Predictive biomarkers for combined chemotherapy with 5-fluorouracil and cisplatin in oro- and hypopharyngeal cancers

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Abstract. The present study aimed to identify significant correlations between gene expression and chemotherapy response to 5-fluorouracil (5-FU)/cisplatin in head and neck squamous cell carcinoma (HNSCC), and to identify patients who would benefit from induction chemotherapy for both organ preservation and survival. A total of 64 patients who underwent radical treatment for HNSCC were enrolled. All patients received induction chemotherapy with 5-FU/cisplatin and tumor responses were evaluated. Pretreatment biopsy specimens from all patients were assayed for mRNA expression of thymidylate synthase, dihydropyrimidine dehydrogenase (DPD), orotate phosphoribosyltransferase, tymidine phosphorylase, glutathione S-transferase-pi, p53, RB Transcriptional Corepressor 1, B-cell lymphoma 2 (Bcl-2), Bcl-xL, E2F Transcription Factor 1, epidermal growth factor receptor, human epidermal growth factor receptor 2, phosphoinositide 3-kinase, phosphatase and tensin homolog, vascular endothelial growth factor (VEGF), cyclooxygenase-2, XPA, DNA Damage Recognition And Repair Factor, excision repair cross-complementing 1 (ERCC1), multidrug resistance gene 1 (MDR1), multidrug resistance-associated protein 1, equilibrative nucleoside transporter 1 and β-tubulin by reverse transcription-quantitative polymerase chain reaction, and the association between the expression levels of these genes and patient response to chemotherapy was determined. The complete response (CR) group and non-CR group for induction chemotherapy comprised 32.8 and 67.2% of patients, respectively. The 5-year overall survival rate was significantly higher for the CR group (95%) compared with the non-CR group (57%). According to univariate analysis, chemotherapy response was associated with T-class and mRNA expressions of DPD, ERCC1, XPA, p53, Bcl-2, VEGF and MDR1. Multivariate analysis identified ERCC1 expression and T-class as significant predictors of response to chemotherapy, indicating that a DNA-repair pathway and apoptosis pathway are pivotal mechanisms governing response to chemotherapy. The findings suggest that ERCC1 expression could be a predictive biomarker for chemotherapy response to 5-FU/cisplatin in HNSCC. Assessing mRNA expression is a standard method for these studies, however further investigations examining polymorphisms and mutations in addition to apoptotic responses are required to determine target gene activation in HNSCC.

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Abbreviations: 5FU, 5-fluorouracil; Bcl-2, b-cell lymphoma 2; Bcl-xL, b-cell lymphoma-extra large; CI, confidence interval; COX2, cyclooxygenase-2; CR, complete response; CT, computed tomography; CT, threshold cycle; DPD, dihydropyrimidine dehydrogenase; EGFR, epidermal growth factor receptor; ENT1, equilibrative nucleoside transporter 1; ERCC1, excision repair cross-complementing 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase, GST-pi, glutathione S-transferase-pi; HDRA, histoculture drug response assay; HER2, human epidermal growth factor receptor 2; HNSCC, head and neck squamous cell carcinoma; MDR1, multidrug resistance gene 1; MRP1, multidrug resistance-associated protein 1; NER, nucleotide excision repair; OPRT, orotate phosphoribosyltransferase; OR, odds ratios; PI3K, phosphoinositide 3-kinase; PR, partial response; PTEN, phosphatase and tensin homolog; RECIST, response evaluation criteria In solid tumor guidelines; RT-PCR, reverse Transcription-PCR; TP, tymidine phosphorylase; TS, thymidylate synthase; UICC, union for international cancer control; VEGF, vascular endothelial growth factor

Key words: biomarkers, chemotherapy, head and neck cancer, ERCC1, p53

Introduction

Head and neck squamous cell carcinoma (HNSCC) is highly sensitivity to anticancer agents. Various types of agents have been studied for the treatment of HNSCC. Recently, new agents designed to target specific molecular defects unique to the cancer have been developed. However, combination chemotherapy with 5-fluorouracil (5-FU) and cisplatin is still the most

common regimen for HNSCC, showing major response rates of 60-90% and complete response (CR) rates of 20-50% (1,2). Patients with CR or partial response (PR) show improved survival, while those with no response show no improvements in either organ preservation or survival. However, the optimal chemotherapy regimen and the role of induction chemotherapy and adjuvant chemotherapy remain unclear. Moreover, there is currently no promising way to assess a patient's response to chemotherapy or to identify patients who are sensitive to individual anticancer agents. Drug resistance, especially in patients with recurrent and metastatic disease, may be a major factor in preventing favorable outcomes in HNSCC patients. Therefore, identification of patients who respond to chemotherapy can help to develop specifically tailored therapies to enhance survival rates and quality of life. One clinically sound method would be to assess molecular markers for chemoresistance using conventional methods to aid in establishing a more effective regimen for patients. While many biomarkers have been implicated as potential candidates for resistance to anticancer agents, the research on these remains minimal, especially with regard to HNSCC.

We previously reported the mRNA expression for several candidate markers of chemoresistance in HNSCC patients, and showed an association between EGFR and HER-2 expression and *in vitro* chemosensitivity using a histoculture drug response assay (HDRA) (3). Here, we aimed to identify biomarkers that significantly predict response to chemotherapy in HNSCC patients by examining multigene mRNA expression in pretreatment biopsy specimens from HNSCC patients who were scheduled to undergo induction chemotherapy with 5-FU and cisplatin.

Patients and methods

Patient selection. Sixty-four patients who underwent radical treatment for HNSCC at the Department of Head and Neck Surgery, Aichi Cancer Center, Japan were enrolled in this study. The study protocol was approved by the Ethics Committees of the Aichi Cancer Center. Informed consent for participation in this study was obtained from all patients.

Treatment plan. All enrolled patients received induction chemotherapy with 5-FU and cisplatin before definitive therapy to select patients for organ preservation. For the recently treated arm, 35 patients (54.7%) received continuous infusion of 5-FU 800 mg/m^2/day for 5 days followed by cisplatin 80 mg/m^2 on day 6, repeated every 3 weeks. For the previously treated arm, 27 patients (42.2%) received continuous infusion of 5-FU 600 mg/m^2/day for 6 days followed by cisplatin 80 mg/m^2 on day 7, repeated every 3 weeks. Two patients (3.1%) with poor renal function received cisplatin 25 mg/m^2 on day 1 followed by continuous infusion of 5-FU 1,000 mg/m^2/day for 2 days (day 1-2), repeated every week. Tumor responses were evaluated in accordance with the Response Evaluation Criteria In Solid Tumor Guidelines (RECIST) (4) after induction chemotherapy using head and neck computed tomography (CT) followed by biopsy with histopathological diagnosis. Responders of induction therapy then received definitive radiotherapy or concurrent chemoradiotherapy for organ preservation, while non-responders were treated with surgery.

Molecular assessment. Tumor biopsy samples were obtained before administration of induction chemotherapy. The samples were assessed for the mRNA expressions of 22 candidate predictive biomarkers (Table I). We subsequently investigated the association between the mRNA expression levels of the biomarkers and response to induction chemotherapy with 5FU/cisplatin.

RNA extraction and cDNA synthesis. Tumor tissue samples, obtained immediately after biopsy, were submerged in liquid nitrogen and stored at -80˚C for subsequent quantification of mRNA expression. Total RNA was isolated by a single-step guanidinium isothiocyanate-phenol-chloroform-based method using the ISOGEN RNA extraction kit (Nippon Gene, Inc., Tokyo, Japan) according to the manufacturer's instructions. After RNA isolation, cDNA was synthesized using the ThermoScript™ real-time polymerase chain reaction (RT-PCR) System (Invitrogen, Carlsbad, CA, USA) and 1 μg of total RNA primed with oligo dT primer (Invitrogen), as described previously (3).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) quantification. mRNA levels of each gene were measured by RT-PCR based on TaqMan chemistry and quantified using an ABI PRISM 7900-HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA), as described previously (3). PCR reactions (25 μl) were conducted in a 96-well plate. Reaction mixtures consisted of TaqMan Universal PCR Master mix (Applied Biosystems), forward and reverse primers (900 nM each), probe (250 nM), and cDNA template (equal to 1 ng total RNA). Reactions were performed at 50˚C for 2 min and 95˚C for 10 min, then 55 cycles at 95˚C for 15 sec and 60˚C for 1 min. Gene expression analysis was performed in duplicate in the same PCR experiment. The primer and probe sequences used are summarized in Table I. To examine gene expression levels across the different tumor samples, we compared the relative expression level to that of a calibrator using the comparative C_T method. The threshold cycle (C_T) was defined as the fractional cycle number at which the fluorescence generated by cleavage of the probe exceeded a fixed threshold level above baseline. We normalized the amount of total RNA present in each reaction by amplifying the housekeeping gene GAPDH. The mRNA amount in tissue normalized to GAPDH mRNA was expressed as follows: $-\Delta C_T = -[C_{T\ target} - C_{T\ GAPDH}]$. The ratio of the amount of target mRNA to the amount of GAPDH mRNA was determined from $2^{-\Delta CT}$ x K, where K is a constant.

Statistical analysis. The covariates of interest were gender (male vs. female), age, T-class, N-class, and mRNA expression level (continuous variables). While mRNA levels were not normally distributed, the log-transformed levels fit a normal distribution (5). The outcome of interest was the response to induction chemotherapy. Student's t test was used to analyze correlations between the variables and response to induction chemotherapy. Variable selection with the entry and removal criterion $P<0.05$ was used to construct a multivariate logistic regression analysis based on factors identified using univariate analysis. The odds ratios (OR) and 95% confidence interval (CI) were calculated from the logistic regression models for predictors of response.

Kaplan-Meier curves were used to compare differences in survival calculated using the log-rank test. Survival end points included overall survival and disease-free survival; overall survival considered all deaths as events while disease-free survival was defined as the time to recurrence. All statistical analyses were performed using EZR version 1.34 (6). A P-value ≤0.05 was considered statistically significant.

Results

Patient population. The characteristics of this study population are summarized in Table II. Fifty patients (78.1%) were male and 14 (21.9%) were female with an age range of 31 to 79 years. The primary sites of cancer were the oropharynx (n=30; 46.9%) and hypopharynx (n=34; 53.1%). According to the Union for International Cancer Control (UICC) TNM classification, tumors were classified as T1/T2, T3/T4, N0 and N1-3 in 37 (57.8%), 27 (42.2%), 14 (21.9%), and 50 patients (78.1%), respectively. There were 8 (12.5%), 11 (17.2%) and 45 patients (70.3%) with Stage II, III, and IV cancer, respectively. Twenty-one patients (32.8%) showed CR after induction chemotherapy.

Association between survival and chemotherapy response. Survival curves according to response to induction chemotherapy are shown in Figs. 1 and 2. The CR group included 21 patients (32.8%), while the non-CR group for induction chemotherapy comprised 43 patients (67.2%). CR rates of three cisplatin/5-FU arms were 37% (13/35) in recently treated arm, 30% (18/27) in previously treated arm and 0% (0/2) in weekly arm, respectively. There was no significance among three arms in chi-square test.

The CR group received definitive radiotherapy or concurrent chemoradiotherapy after induction chemotherapy for organ preservation. The non-CR group was treated with radical surgery. The 5-year overall survival rate was 95 and 57%, while the 5-year disease-free survival rate was 90 and 53% for the CR group non-CR group, respectively. The CR group for induction chemotherapy demonstrated significantly better prognoses than the non-CR group for overall (P=0.0009) and disease-free (P=0.0141) survival.

Chemotherapy response and gene expression. Patient response to induction chemotherapy for primary tumors was compared to tumor characteristics and gene expression (Tables III and IV). Univariate analysis using Student's t test demonstrated that a patient's clinical T-class was the only significant variable among the clinical factors (P=0.0002), indicating that T1/T2 tumors were associated with increased chemotherapy response. Response to induction chemotherapy was significantly correlated with the log-transformed mRNA expression levels of ERCC1 (P=0.0054), XPA (P=0.0154), p53 (P=0.0092), DPD (P=0.0274), Bcl-2 (P=0.0135), VEGF (P=0.0213), and MDR1 (P=0.0389). Interestingly, high expression levels of these genes were significantly associated with increased sensitivity to chemotherapy. Multivariate logistic regression analysis was conducted using significant variables identified in the univariate model. ERCC1 expression (OR, 36; 95% CI, 1-1100; P=0.040) and clinical T-class (OR, 0.119; 95% CI, 0.022-0.637; P=0.013) were identified as independent predictors of response to combination chemotherapy with 5-FU/cisplatin.

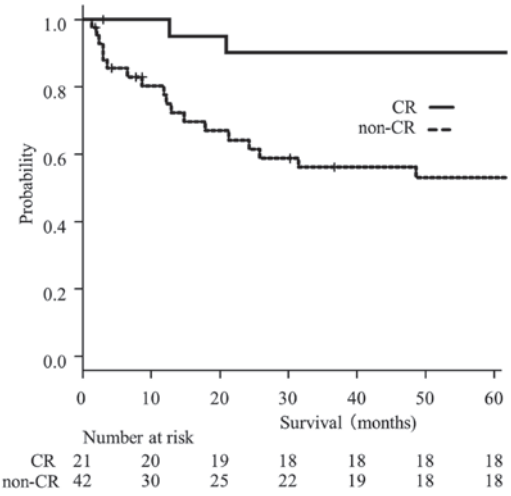

Figure 1. Kaplan-Meier curves indicating disease-free survival. The CR group includes patients with complete response or good partial response for induction chemotherapy. The non-CR group includes all other patients. CR, complete response.

Discussion

Platinum-based induction chemotherapy has been integral to comprehensive therapies for patients with HNSCC. Recently, many studies have focused on induction chemotherapy to assess both organ preservation and survival, demonstrating its encouraging potential as a novel therapeutic approach in HNSCC. Response to induction chemotherapy also allows for the predictive identification of patients who may respond to subsequent radiotherapy, and can help select patients for organ preservation. In the current study, 32.8% of patients showed CR after induction chemotherapy and were selected to undergo definitive radiotherapy or concurrent chemoradiotherapy for organ preservation. Patients who had good response to induction chemotherapy also showed significantly longer survival for both overall and disease-free survival than non-responders.

Intrinsic drug resistance and acquired resistance are critical factors for the efficacy of chemotherapeutic agents. Identifying molecular predictors of chemotherapy efficacy can provide important tools for designing individualized treatment regimens. We therefore investigated the association between gene expression and response to induction chemotherapy with 5-FU and cisplatin. Although newer regimens, such as paclitaxel or docetaxel plus cisplatin or carboplatin, have been extensively studied (7,8), the combination chemotherapy of 5-FU and cisplatin remains the gold standard regimen in HNSCC.

5-FU is converted to the three main active metabolites fluorodeoxyuridine monophosphate, fluorodeoxyuridine triphosphate, and fluorouridine triphosphate via the enzymes thymidylate synthase (TS), orotate phosphoribosyltransferase (OPRT), tymidine phosphorylase (TP), and dihydropyrimidine dehydrogenase (DPD) (9,10). Therefore, a large number of studies have focused on assessing the expressions of TS, OPRT,

Table I Primers and probe sequences of investigated genes.

Genes	Sequences (5'-3')		
	Forward primer	Reverse primer	Probe
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	CAAGCTTCCCGTTCTCAGCC
TS	GAATCACATCGAGCCACTGAAA	CAGCCCAACCCCTAAAGACTG	TTCAGCTTCAGCCAGAACCCAGA
DPD	AATGATTCGAAGAGCTTTTGAAGC	GTTCCCCGGATGATTCTG	TGCCCTCACCAAAACTTTCTCTCTTGATAAGGA
OPRT	GCCTCCTTATTGCGGAAATG	CATTCTAACCGCTGCTCTAGTGTAGT	CTCCACCGGCTCCCTGGCC
TP	CCTGCGGACGGAATCCT	TCCACGAGTTTCTTACTGAGAATGG	CAGCCAGAGATGTGACAGCCACCG
MDR1	AGAAAGCGAAGCAGTGGTTCAG	CGAACTGTAGACAAACGATGAGCT	CGACCTTTTCTGGCCTTATCCAGAGCC
MRP1	CAATGCTGTGATGGCGATG	GATCCGATTGTCTTTGCTCTTCA	AGACCAAGACGTATCAGGTGGCCCAC
COX2	GCTCAAACATGATGTTTGCATTC	GCTGGCCCTCGCTTATGA	TGCCCAGCACTTCACGCATCAGTT
EGFR	TGCGTCTCTTGCCGGAAT	GGCTCACCCTCCAGAAGCTT	ACGCATTCCCTGCCTCGGCTG
HER2	CTGAACTGGTGTATGCAGATTGC	TTCCGAGCGGCCAAGTC	TGTGTACGAGCCGCACATCCTCCA
VEGF	GCACCCATGGCAGAAGG	CTCGATTGGATGGCAGTAGCT	ACGAAGTGGTGAAGTTCATGGATGTCTATCAC
Bcl-2	CGCCCTGTGGATGACTGAGT	GGGCCGTACAGTTCCACAA	CTGAACCGGCACCTGCACACCTG
Rb1	TCTATAACTCGGTCTTCATGCAGAGA	GAATCCGTAAGGGTGAACTAGGAA	CAGGCCCCCTACCTTGTCACCAATACCT
E2F1	CAGCGCCTGGCCTACGT	GGGCTTTGATCACCATAACCA	CGTAGCATTGCAGACCCTGCAGAGC
GST-pi	TCACTCAAAGCCTCCTGCCTA	TTGGACTGGTACAGGGTGAGGT	CAGCTCCCCAAGTTCCAGGACGGA
ERCC1	GACTGTCCGTTTTGTTGACTGACT	ATGGAGAAGCTAGAGCAGGACTTC	TGGTCAGACATTCAGTCACCCGGG
XPA	TTCTTCTGGTCCATACTCATGTTGA	AGAATTGCGGCGAGCAGTAA	ATCGTCTCCCTTTTCCACACGCTGCT
ENT1	ACTGTGGTCTTCGAGCACGAT	GCAGAGGCTGGCGAGGTA	TCATGGCTGCCTTTGCCTTCTCCA
β-tubulin	TGATCAGTGATGAACATGGCATC	ATGGTCCCAGGTTCTAGATCCA	AGGTGGCAAATATGTTCCTCGTGCCATC
p53	GCGAGCACTGCCCAACA	CAGCTCTCGGAACATCTCGAA	AGAATATTTCACCCTTCAGATCCGTGG
Bcl-xL	AGCGGTTGAAGCGTTCCT	GCGGCTGGGATACTTTTGTG	CTCTCGGCTGCTGCATTGTTCCCAT
PIK3CA	CAATTGGTCTGTATCCCGAGAAG	TTGAGCTGTTCTTTGCTATTTTCC	TCCCACGCAGGACTGAGTAACAGACTAGC
PTEN	CAGTGGCGGAACTTGCAAT	GCTGAGGGAACTCAAAGTACATGA	ATATTCCTCCAATTCAGGACCCACACGAC

Table II. Patient characteristics.

	No. of patients n=64 (%)
Sex	
Male	50 (78.1)
Female	14 (21.9)
Age, years	
Range	31-79
Median	61
Site	
Oropharynx	30 (46.9)
Hypopharynx	34 (53.1)
Tumor status	
T1	3 (4.7)
T2	34 (53.1)
T3	19 (29.7)
T4	8 (12.5)
Node status	
N0	14 (21.9)
N1	6 (9.4)
N2	37 (57.8)
N3	7 (10.9)
Stage	
I	0 (0)
II	8 (12.5)
III	11(17.2)
IV	45 (70.3)
Chemo-respnse	
CR	21 (32.8)
Non-CR	43 (67.2)

CR, complete response.

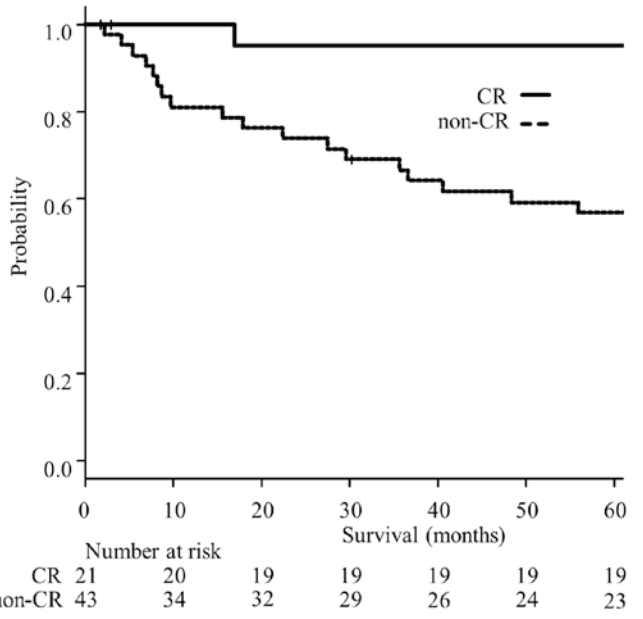

Figure 2. Kaplan-Meier curves indicating overall survival. CR, complete response.

TP, and DPD as key enzymes for the regulation 5-FU resistance. We showed that only DPD expression was significantly associated with response to chemotherapy, with higher DPD expression correlating with enhanced response to 5-FU/cisplatin. A previous study showed that DPD was the first key enzyme linked to 5-FU catabolism, and that it was predictive of 5-FU responsiveness in HNSCC (11). Overexpression of DPD is also associated with resistance to 5-FU in head and neck and colorectal cancer (11,12). In contrast, however, another study demonstrated that heterogeneity, rather than intensity, of DPD expression regulated 5-FU sensitivity in oral SCC (13). While many studies have reported a correlation between high DPD expression levels and chemoresistance or poor survival rate, the prognostic value of DPD expression is still controversial in induction chemotherapy with 5-FU/cisplatin. Further studies that assess DPD levels using an enzyme assay or that examine the modification of molecular structures caused by mutations, as a previous study indicated (14), may clarify a potential role for DPD as a marker for response to induction chemotherapy with 5-FU/cisplatin.

The cytotoxic mode of action of cisplatin is mediated by its interaction with DNA to form DNA adducts, primarily intrastrand crosslink adducts. After binding to DNA, cisplatin inhibits replication and leads to arrest in the G2 phase of the cell cycle, resulting in apoptosis. Cisplatin resistance appears to be associated with several molecular alterations, including drug detoxification, upregulation of DNA repair enzymes, overexpression of anti-apoptotic proteins, and detoxifying enzymes (15-17). Based on their mechanisms for effecting cancer cells, 5-FU and cisplatin appear to be associated with distinct factors of drug resistance. However, reports indicate that tumors that fail to respond to cisplatin are cross-resistant to diverse unrelated anticancer agents, suggesting that cisplatin likely shares common mechanisms of resistance with other agents (18).

Nucleotide excision repair (NER) is a highly conserved mechanism that repairs DNA-damaged lesions caused by platinum compounds (19). The basic steps in this pathway include DNA damage recognition and demarcation of the affected area, followed by formation of a complex to unwind the damaged DNA, excision of the DNA at the lesion site, removal of the damaged strand, and synthesis of new DNA that is complimentary to the remaining strand and ligation (15). Excision repair cross-complementing 1 (ERCC1) plays a key role in NER. ERCC1 dimerizes with xeroderma pigmentosum complementation group F to form a complex that is required for excising the damaged DNA (15). XPA is a protein involved in the initial damage recognition and recruitment stage of the NER pathway (20). A previous study investigating the contribution of the XPA-binding region of ERCC1 on NER activity showed that the interaction between ERCC1 and XPA was essential for NER (21). Evaluations of ERCC1 mRNA expression from various types of cancers have shown an inverse correlation between response to platinum-based chemotherapy and survival in testicular, ovarian, colorectal, and non-small cell lung cancers (22-25). However,

Table III. Univariate analysis of predictive factors for response to chemotherapy using Student's t test.

			Response				
			CR		nonCR		
Markers	Variables	Scale	Mean	SD	Mean	SD	P-value
Clinical	Sex	Male/female	-	-	-	-	0.3340
	Age	Year	58.8196	7.2268	60.8125	11.1649	0.4600
	T class	T1-T4	2.0000	0.4472	2.7442	0.7896	**0.0002**
	N class	N0-N3	1.6667	0.7958	1.5349	1.0316	0.6090
Biological	TS	Log-transformed level of mRNA	-2.3916	0.8924	-2.5538	0.5462	0.3720
	ERCC1		-1.2602	0.4269	-1.5298	0.3092	**0.0054**
	XPA		-1.1154	0.5074	-1.4032	0.3943	**0.0154**
	p53		-1.0214	0.7949	-1.5197	0.6446	**0.0092**
	E2F1		-1.4983	0.7463	-1.7150	0.4665	0.1600
	Rb1		-1.7118	0.4796	-1.8984	0.4409	0.1330
	PI3K		-1.5902	0.5023	-1.6718	0.3359	0.4490
	ENT1		-1.3960	0.5023	-1.5197	0.3539	0.2590
	DPD		-1.9756	0.4096	-2.2797	0.5454	**0.0274**
	OPRT		-0.9528	0.3762	-1.1755	0.4763	0.0657
	TP		-0.4993	0.4881	-0.5135	0.4294	0.9060
	Bcl-2		-2.1529	0.6099	-2.6697	0.8028	**0.0135**
	Bcl-xL		-0.8639	1.0317	-1.0097	0.6018	0.4780
	PTEN		-1.2380	0.5045	-1.3281	0.4101	0.4470
	VEGF		-1.1783	0.3474	-1.4444	0.4550	**0.0213**
	COX2		-1.6233	0.7336	-1.8537	0.6593	0.2110
	EGFR		-1.6129	0.6780	-1.7037	0.5523	0.5690
	HER2		-0.8955	0.5601	-1.0512	0.4565	0.2390
	MDR1		-2.6895	0.7712	-3.1987	0.9235	**0.0389**
	MRP1		-1.1319	0.5547	-1.2508	0.5677	0.4310
	GST-pi		0.3794	0.3245	0.2560	0.3598	0.1890
	β-tubulin		-0.3397	0.3344	-0.3311	0.2673	0.9110

Bold print denotes significant values and variables.

Table IV. Multivariate analysis of predictive factors for response to chemotherapy using logistic regression.

				95%CI		
Markers	Variables	Scale	Odds ratio	Lower	Upper	P-value
Clinical	**T class**	T1-T4	0.119	0.022	0.637	**0.013**
Biological	**ERCC1**	Log-transformedl level of mRNA	36	1	1,100	**0.040**
	XPA		0.570	0.047	6.930	0.659
	p53		2.160	0.657	7.070	0.205
	DPD		0.546	0.107	2.770	0.465
	Bcl-2		1.020	0.222	4.660	0.981
	VEGF		0.683	0.094	4.990	0.707
	MDR1		1.800	0.464	6.960	0.397

Bold print denotes significant values and variables.

we found a significant correlation between high expressions of ERCC1 and XPA and increased response to chemotherapy in HNSCC. Increased ERCC1 expression has been shown to be correlated with improved outcomes in gastric cancer patients (26). Overexpression of ERCC1 has also been shown to improve treatment outcomes in lung cancer patients (27). Further, a clinical study demonstrated that reduced DNA repair capacity increases the risk of developing lung cancer (28). Interestingly, a recent study showed that a defect in the interaction between ERCC1 and XPA that disrupts NER has no major effect on cellular sensitivity to cisplatin, suggesting that ERCC1-mediated NER is not a key determinant of cellular sensitivity to cisplatin (21). While a large number of studies have demonstrated that lower ERCC1 expression is associated with increased response to platinum-based chemotherapy and survival (15,29), DNA damage is known to be associated with an increased risk of cancers in which reduced DNA repair capacity may accelerate the alteration and mutation of essential genes, causing carcinogenesis. Therefore, the reduced DNA repair capacity of ERCC1 and XPA may not always result in increased response to chemotherapy, particularly in patients in which cancer progression is caused by low ERCC1 or XPA expression. Because the DNA repair pathway is a markedly complex process involving NER, mismatch repair, base excision repair, and gene-specific repair, previous studies have suggested that regulation of the efficacy of chemotherapeutic agents is not likely to only comprise a simple mechanism in which the NER pathway and apoptotic pathways are linked in a complFex (24). Further studies are needed to clarify the mechanisms of the NER pathway, such as by identifying the associated gene polymorphisms and mutations.

p53, a tumor suppressor gene, is an essential regulator of apoptosis and a well-known regulatory gene for cisplatin resistance. When DNA damage occurs in cells, increased levels of active p53 induce either G1 cell cycle arrest or apoptosis followed by suppression of tumorigenesis (30). p53 mutations occur in 40-70% of HNSCC patients, leading to extensive research on wild-type p53 as a potential therapeutic target (30). Previous reports have indicated that overexpression of the p53 protein in tumor cells is strongly associated with chemotherapy response and larynx preservation (31,32). High expression of wild-type p53 was also shown to be important for apoptotic cell death in cisplatin-treated cells (33). In agreement with these previous findings, we found that high expression of p53 was correlated with response to chemotherapy.

Expression of Bcl-2 is upregulated in various types of tumors. Many studies have reported a significantly worse treatment outcome in patients with high Bcl-2-expressing tumors (34). An important function of Bcl-2 is to inhibit apoptosis induced by radiation and chemotherapeutic agents (35). While we found that overexpression of Bcl-2 was associated with good chemotherapy response, we could not verify the mechanism of action. One of several studies that identified a correlation between Bcl-2 expression and a favorable outcome also showed that local control probability was significantly improved for patients with Bcl-2-expressing tumors treated by radiotherapy (36). High expression of Bcl-2 has also been reported to be a good prognostic indicator in breast cancer, which supports our findings (37). Expressions of other Bcl-2 family members, which are primary regulators of apoptosis, may affect the function of Bcl-2 (38,39).

The role of Bcl-2 as a proangiogenic signaling molecule for both tumor and vascular endothelial cells is well established (39). Previous studies have shown that stimulation of the VEGF signaling pathway results in increased expression of Bcl-2 in tumor and endothelial cells (40). Moreover, Bcl-2 expression is significantly upregulated in HNSCC-associated endothelial cells compared to endothelial cells in normal oral mucosa, and Bcl-2 induces VEGF expression in neovascular endothelial cells through a STAT3-mediated pathway (41). We observed a significant association between chemotherapy response and VEGF expression, suggesting that the VEGF-Bcl-2 pathway may be important for patient response to anticancer agents in head and neck cancer.

Multidrug resistance gene 1 (MDR1) functions as an ATP-dependent pump that transports foreign substances out of cells, including anticancer drugs (42,43). Previous studies have demonstrated that cisplatin enhanced MDR1 expression and its function, resulting in drug resistance in cancer cells, even though cisplatin is not a substrate for MDR1 (42,44). Interestingly, rather than an association between high MDR1 expression and resistance to chemotherapy, we found a correlation between high MDR1 expression and chemosensitivity. MDR1 expression in tumor cells is increased following each course of chemotherapy, leading to enhanced resistance to anticancer agents (44). Therefore, changes in the expression of MDR1 prior to and following induction chemotherapy should be measured in the present set of patients to verify whether sequential changes in expression are associated with chemotherapy response.

In summary, we showed that ERCC1 expression and T-class were independent predictors of response to induction chemotherapy using 5-FU and cisplatin. In a meta-analysis of 1,288 HNSCC patients receiving platinum-based therapy, Bišof *et al* reported that ERCC1 may be a predictive and prognostic factor for individualized therapies for HNSCC patients (45). Our findings also suggest that ERCC1 may be a predictive biomarker for response to chemotherapy with 5-FU/cisplatin in HNSCC patients. A DNA repair pathway and an apoptosis pathway are pivotal to the mechanism underlying response to chemotherapy. Although efficient DNA repair activity inhibits cancer cell progression and invasion via apoptosis signaling, it might be potentially disadvantageous for response to anticancer agents. While assessing mRNA expression is a standard method for these studies, the mechanisms underlying drug resistance are complex and require additional investigation. Further studies examining ERCC1 polymorphisms and mutations and assessing apoptotic response associated with p53 activation in HNSCC are needed to clarify genetic associations with response to chemotherapy in HNSCC patients.

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