Regulation of Excision Repair Cross-Complementation Group 1 by Snail Contributes to Cisplatin Resistance in Head and Neck Cancer


Abstract

Purpose: We investigated the mechanism and clinical significance of the epithelial-mesenchymal transition (EMT)-induced chemoresistance in head and neck squamous cell carcinoma (HNSCC).

Experimental Design: The correlation between the expression of different EMT regulators and chemoresistance genes, such as excision repair cross-complementation group 1 (ERCC1), was evaluated in cancer cell lines from the NCI-60 database and four human HNSCC cell lines. Ectopic expression of Snail or short-interference RNA-mediated repression of Snail or ERCC1 was done in HNSCC cell lines. Cell viability was examined for cells after cisplatin treatment. A luciferase reporter assay and chromatin immunoprecipitation were used to identify the transcriptional regulation of ERCC1 by Snail. Immunohistochemical analysis of Snail, Twist1, ERCC1, hypoxia inducible factor-1 α (HIF-1α), and NBS1 were done in samples from 72 HNSCC patients receiving cisplatin-based chemotherapy.

Results: The correlation between the expression of Snail and ERCC1 was confirmed in different cell lines, including HNSCC cells. In HNSCC cell lines, overexpression of Snail in the low endogenous Snail/ERCC1 cell lines FaDu or CAL-27 increased ERCC1 expression, and hypoxia or overexpression of NBS1 also upregulated ERCC1. Knockdown of Snail in the high endogenous Snail/ERCC1 cell line OECD-1 downregulated ERCC1 expression and attenuated cisplatin resistance. Furthermore, suppression of ERCC1 in Snail- or NBS1-overexpressing HNSCC cells enhanced sensitivity to cisplatin. Snail directly regulated ERCC1 transcription. In patients with HNSCC, coexpression of Snail and ERCC1 correlated with cisplatin resistance and a poor prognosis.

Conclusions: Activation of ERCC1 by Snail is critical in the generation of cisplatin resistance of HNSCC cells. Clin Cancer Res; 16(18); 4561–71. ©2010 AACR.

The epithelial-mesenchymal transition (EMT), a major mechanism of cancer metastasis, is initiated by repression of the epithelial adhesion molecule E-cadherin by several transcription factors, including Snail (also known as Snail1). Slug (also known as Snail2), Twist1, Zeb1, SIP1, and E47 (1). In most human cancers, metastatic tumors are resistant to chemotherapy; therefore, patients with such tumors typically have poor outcomes. Emerging evidence suggests a correlation between EMT and the resistance to chemotherapy of cancer cells. For example, colorectal cancer cells that are resistant to oxaliplatin undergo phenotypic changes indicative of an EMT (2). Direct regulation of Akt2 by Twist contributes to paclitaxel resistance in breast cancer cells (3). Induction of EMT in breast cancer cells leads to an enrichment of cells with stem-like properties and chemoresistance (4). A recent report found that Zeb1 and other EMT regulators allow pancreatic cancer cells to maintain drug resistance (5). Taken together, these studies suggest that diverse types of cancer cells acquire drug-resistant phenotypes during EMT. Although there is an evident association among EMT, metastasis, and chemoresistance, the underlying mechanisms remain elusive.

Head and neck squamous cell carcinoma (HNSCC) includes cancers that originate in the oral cavity, oropharynx, hypopharynx, and larynx. It is one of the leading causes of
Translational Relevance

Head and neck cancer is one of the major cancers worldwide (6) and has become a major public health problem in Taiwan (7). Multimodal therapies, including surgery, radiotherapy, and chemotherapy, are typically employed to treat HNSCC. The major obstacle of HNSCC treatment is the development of resistance, disease recurrence, and metastasis. Patients with treatment-naïve HNSCC can have a response rate to chemotherapy of >50% (8, 9), but patients with recurrent or metastatic disease have a response rate of <30% (10). It is important to elucidate the mechanisms that mediate the recurrence, metastasis, and treatment resistance of HNSCC and to develop strategies that prevent metastasis so that patients can overcome treatment resistance.

Platinum-based drugs, especially cisplatin and carboplatin, are among the most active anticancer agents available and are used widely for HNSCC treatment (11, 12). Previous studies have identified the main mechanisms involved in the development of platinum resistance, including reduction of cellular accumulation, intracellular inactivation, enhancement of DNA repair, and blocking the induction of apoptosis (11, 12). Among these four mechanisms, induction of the DNA repair protein excision repair cross-complementation group 1 (ERCC1) has been regarded as one of the most important factors controlling cisplatin resistance. ERCC1 plays a key role in nucleotide excision repair and in removing platinum-induced DNA adducts. Previous studies have found that increased ERCC1 expression correlates with failure of cisplatin treatment and is associated with a poor prognosis for patients with ovarian cancer, lung cancer, and HNSCC (13–15). The mechanisms that regulate ERCC1 expression are not yet clearly established.

We previously showed that NBS1 (a DNA repair protein) regulates Snail and that Snail contributes to the EMT associated with HNSCC metastasis (16). In the present study, we investigated the role of Snail in cisplatin resistance and regulation of ERCC1 expression in two cultured cell lines of HNSCC and in the cancerous cells of patients with HNSCC.

Materials and Methods

Analysis of microarray expression datasets

Microarray gene expression data of tumor cell lines of diverse origin (NCI-60 panel) were downloaded from the website of the Stanford NCI-60 Cancer Microarray Project (17). Data were generated by Gene Logic Inc. using Affymetrix U133. The correlation between transcripts was analyzed statistically, and the heat maps were created by dChip.

Cell lines, plasmids, stable transfection, and oxygen deprivation

Four human HNSCC cell lines, namely, FaDu, SAS, OECM-1, and CAL-27, and the human embryonic kidney cell line HEK-293T were obtained from the Bioresource Collection and Research Center of Taiwan in 2005. These cell lines have been tested and authenticated according to the published guideline before the initiation of the study (18). The pcDNA3-Snail was generated by insertion of a 795 bp fragment of the full-length human Snail cDNA into the HindIII/EcoRI sites of the pcDNA3.1 vector. pcDNA3-SnailΔSNAG, pcDNA3-SnailΔ60, and pcDNA3-SnailΔ129 were generated by truncating the SNAG domain (a.a. 1-10), the first 60 a.a and the first 129 a.a of Snail and inserting the fragment into the HindIII/EcoRI sites of the pcDNA3.1 vector. pFLAG-Twist1 and pcDNA3-Slug have previously been described (19, 20). The plasmids for short-interference RNA (siRNA) experiments were generated by inserting an oligonucleotide that contained a specific siRNA target sequence against Snail, Slug, Twist1, or ERCC1, or a scrambled sequence into the pSUPER vector (Supplementary Table S1). All clones presented in this report were generated by stable transfection. Stable clones were generated by transfection of expression vectors and/or siRNA plasmids and were selected by appropriate antibiotics. Hypoxic conditions were generated in an Astec ACM-165 multigas incubator (ASTEC Co., Ltd.) with 1% O2, 5% CO2, and 94% N2 for 18 hours.

Protein extraction, Western blot analysis, RNA extraction, and quantitative real-time reverse-transcription PCR analysis

Cells were harvested and lysed. The protein content was determined by the Bradford method (Bio-Rad Laboratories). Protein extracts were loaded onto SDS-polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were probed with the primary and secondary
antibodies, and signals were developed using an ECL chemiluminescence kit (Amersham Biosciences). Supplementary Table S2 lists all antibodies used.

For analyzing the amount of different transcripts, total RNAs from cultured cells were extracted using the Trizol reagent (Invitrogen Life Technologies), and 1 µg of RNA was used for cDNA synthesis. Quantitative real-time PCR using the StepOnePlus real-time PCR system (Applied Biosystems Inc.) was applied to quantify mRNA expression, and GAPDH was selected as an internal control. Supplementary Table S1 shows the sequences of primers used for real-time PCR experiments.

**Chromatin immunoprecipitation assay**

The regulatory region of the ERCC1 gene (n.t. −843 to +349, surrounding the transcription start site) was cloned by PCR amplification of genomic DNA and then inserted into the HindIII/BglII sites of the pXP2 vector to generate the ERCC1-LucA parental construct. ERCC1-LucB and ERCC1-LucC constructs were generated from ERCC1-LucA to identify the major regulatory regions of ERCC1. The ERCC1-LucA(me1), ERCC1-LucA (me234), and ERCC1-LucA(me1234) constructs were made by site-directed mutagenesis of the ERCC1-LucA vector. A reporter construct that contained the proximal promoter of E-cadherin (Ecad-Luc486) was used as a control to confirm the suppressive effect of Snail on the E-cadherin promoter (20). For the luciferase reporter assay, the reporter constructs were cotransfected into HEK-293T cells with a different expression vector or an empty vector pcDNA3.1. A plasmid expressing the bacterial β-galactosidase gene (pCMV-βgal) was cotransfected in each experiment as an internal control for transfection efficiency. Cells were harvested after 48 hours of transfection, and luciferase activities were assayed (19). The promoter activities were assessed by normalization of the luciferase activity by β-galactosidase activity, i.e., luciferase activity/β-galactosidase activity. All experiments were done in triplicate, and the results are presented as the change in the relative promoter activity in cells transfected with the expression vector versus the empty vector.

**Cell viability assay**

Cell viability was determined by alamarBlue (AbD Serotec), which uses the natural reducing power of living cells to convert resazurin to resorufin, a fluorescent molecule. Different HNSCC clones were seeded on 96-well plates at a density of 1 × 10⁴ cells/well in culture medium. After treatment with different concentrations of cisplatin for 24 hours, cells were incubated with alamarBlue for 4 hours at 37°C. The product was quantified with a microplate reader that measured absorbance at 570 nm and 600 nm. All experiments were done in triplicate, and mean values are presented.

**Patients and treatment**

From January 2000 to March 2005, 72 patients diagnosed with locally advanced squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx, and larynx (T3-4, N0-M0) at the Taipei Veterans General Hospital (Taiwan) were enrolled. All patients gave written informed consent prior to enrollment, and the study was approved by the Institutional Review Board of the Taipei Veterans General Hospital. All patients were considered poor candidates for resection because surgery causes significant functional impairment. Study samples, including cancerous and normal tissues, were obtained during diagnostic biopsy. Normal tissues were derived from sites that were neighboring, but outside, the tumor. Pathologists confirmed the identity of cancerous and normal tissues.

An organ preservation protocol, with two courses of cisplatin-based induction therapy followed by concurrent chemoradiotherapy (cisplatin-based chemotherapy and radiotherapy for primary tumor and neck cancer, up to 70 Gy in 35 fractions over 7 weeks), was given to all patients. An evaluation of the treatment response was done after induction chemotherapy and 2 months after chemoradiotherapy. The response criteria were based on Response Evaluation Criteria for Solid Tumors guidelines (21). Chemoresistance was defined as "less than partial response" (i.e., stable disease or progressive disease) to induction chemotherapy. The medium follow-up period was 27 months (range, 18-56 months).

Supplementary Table S3 summarizes the clinical characteristics of the HNSCC patients.

**Immunohistochemistry and scoring**

Sections (6 µm thick) of tumor and normal tissue were cut from frozen specimens of 72 HNSCC samples for immunohistochemical analysis. We used the previously described procedure to stain the serial-sectioned samples with anti-hypoxia inducible factor-1 α (HIF-1α), NBS1, Snail, and Twist1 antibodies (19, 22). For ERCC1 immunohistochemistry of these 72 HNSCC samples, we used the anti-ERCC1 antibody from FL-297 clone (Santa Cruz Biotechnology, Inc.) to prevent nonspecific staining results (23). Supplementary Table S2 lists the characteristics of all antibodies. The histology and immunohistochemistry images were captured by
Olympus BX51 High Class System Microscope (Olympus Corporation) equipped with an Olympus DP71 microscope digital camera, Olympus U Plan FL objectives, and Olympus WHB 10× eyepieces. The acquisition software was Olympus DP controller (Olympus Corporation). The depth of captured image was 10 bit. Two independent specialists used previously described criteria to interpret ERCC1, HIF-1α, NBS1, Snail, and Twist1 images (15, 19, 22).

Statistical analysis

Pearson’s χ² or Fisher’s exact test was used to compare dichotomous variables. An independent Student’s t-test or an ANOVA was used to compare continuous variables. Pearson’s correlation coefficient test was used to assess the correlation between two continuous variables. The Kaplan-Meier estimation method was used for overall survival analysis, and a log-rank test was used to compare differences. The level of significant statistical difference was set at 0.05.

Results

Correlation of Snail and ERCC1 expression in cancer cell lines

We initially investigated the correlation in expression of EMT regulators (Snail, Slug, Twist1, SIP1, Zeb1, and E47) and genes related to chemoresistance, including genes of the ATP-binding cassette transporter family (ABCB5, ABCC1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC10, ABCC11, and ABCG2) and genes known to contribute to cisplatin resistance (ERCC1, GST-π1, and γ-GT), taxane resistance (βIIb-tubulin, βIII tubulin), and 5-fluorouracil resistance (thymidylate synthase) in microarray datasets of the NCI-60 panel (Supplementary Fig. S1A). To elucidate the significance of the induction of EMT and chemoresistance, we selected cell lines from the NCI-60 panel in which the expression of the EMT regulator was inversely correlated with that of E-cadherin (correlation coefficient r < −0.6; Fig. 1A, Fig. 1B, Fig. 1C, Fig. 1D).

![Graphs](https://example.com/graphs.png)

Fig. 1. Correlation between the expression of ERCC1 and Snail in different cancer cell lines. A, correlation between the relative expression levels of Snail and E-cadherin in cancer lines selected from the NCI-60 panel. B, a heat map generated from 21 cancer cell lines showing the expression levels of Snail, E-cadherin, and ERCC1. C, correlation between the relative expression levels of Snail and ERCC1 in cancer cell lines from an NCI-60 panel. D, relative mRNA expression of ERCC1 and EMT regulators (Snail, Slug, Twist1, SIP1, Zeb1, and E47) in four HNSCC cell lines that were not included in the NCI-60 panel.
Supplementary Table S4), indicating the functional significance of the EMT regulator in these cell lines. Next, we observed the correlation between the EMT regulator and chemoresistant genes. Among the genes related to chemoresistance, the expression of \textit{ERCC1} was significantly correlated with that of \textit{Snail} (\( r = 0.775 \); Fig. 1B and C, Supplementary Tables S4 and S5). The correlation between \textit{ERCC1} and other EMT regulators was not significant (Supplementary Fig. S1B, Supplementary Table S4). To confirm this finding in HNSCC, we analyzed the relative mRNA levels of \textit{ERCC1} and EMT regulators in four HNSCC cell lines that were not included in the NCI-60 panel (FaDu, SAS, CAL-27, and OECM-1). The most significant correlation was shown between the expression level of \textit{Snail} and \textit{ERCC1} (Fig. 1D). Taken together, these findings suggest a correlation between the expression of \textit{Snail} and \textit{ERCC1} in different kinds of cancer cells, including HNSCC.

**Snail upregulates \textit{ERCC1} and contributes to cisplatin resistance in HNSCC cells**

We hypothesized that \textit{ERCC1} is regulated by \textit{Snail} rather than other EMT regulators because only the expression level of \textit{Snail} was correlated with that of \textit{ERCC1}. To further confirm whether \textit{ERCC1} is only regulated by \textit{Snail} and not by the other EMT regulators, we analyzed the regulatory region of the \textit{ERCC1} gene. There are putative binding sites for different EMT regulators, including E-boxes for \textit{Snail}, \textit{Slug}, \textit{Twist1}, and \textit{E47}; \textit{Z-boxes} for \textit{Zeb1}; and \textit{SIP1} binding sites (Supplementary Fig. S2A). To examine the regulation of \textit{ERCC1} by EMT regulators, we manipulated the expression of \textit{Snail} and two other EMT regulators (\textit{Slug} and \textit{Twist1}) by overexpression or...
a siRNA strategy in HNSCC cells and observed a change
in ERCC1 expression. The results showed that overexpres-
sion of Snail in FaDu and CAL-27 upregulated the ex-
pression of ERCC1 (Fig. 2A and B), and knockdown of
Snail in OECM-1 downregulated ERCC1 (Fig. 2C). We
further examined whether the upstream effectors of Snail,
hypoxia, and NBS1 (16, 24) augmented ERCC1 expres-
sion. These results show that both hypoxia and NBS1
overexpression increased ERCC1 expression, and repres-
sion of Snail by siRNA or LY294002, a phosphatidylinos-
itol 3-kinase inhibitor that represses Snail expression
(16), in a FaDu-NBS clone blocked the upregulation of
ERCC1 (Supplementary Fig. S3). Regarding the correla-
tion of ERCC1 and other EMT regulators, there was no
change in ERCC1 expression after manipulation of
Twist1 or Slug expression (Supplementary Fig. S2B and
C). These results suggest that ERCC1 is regulated by Snail
rather than the other EMT regulators.

Next, we investigated the correlation between the ex-
pression of Snail/ERCC1 and cisplatin resistance by ex-
amining the IC50 for cisplatin in the HNSCC cell lines.
We found that OECM-1, which has the highest levels of
both Snail and ERCC1, was the most resistant to cisplat-
in. Conversely, FaDu and CAL-27 were relatively sensi-
tive to cisplatin treatment (Supplementary Fig. S4A). In
these cell lines, however, the expression of Snail/ERCC1

Fig. 3. Direct regulation of ERCC1 by Snail. A, left, schematic of the genomic organization of the regulatory region of ERCC1 and representation of the
reporter constructs used in transient transfection assays. TSS, transcription start site. Middle, transcriptional activation of ERCC1-LucA, ERCC1-LucB,
and ERCC1-LucC by Snail. Luciferase activity/β-galactosidase of HEK-293T cells cotransfected with ERCC1-LucA and pDNA3.1 was applied as the
baseline control. Right, transcriptional repression of the E-cadherin promoter construct Ecad-Luc486 by Snail. B, left, representation of the reporter
constructs with mutated E-box(s) used in transient transfection assays. Right, promoter activity assays of wild-type and mutated ERCC1 promoter
constructs transfected with Snail versus the control vector. C, left, schematic of the design of ChIP and control primers. The ChIP-1 and ChIP-2
fragments contain the Snail-binding sequence, whereas the control fragment does not contain any Snail-binding sequence. Middle, PCR amplification
of the ChIP-1, ChIP-2, and control fragments of ERCC1 promoter in CAL27-cDNA3 versus CAL27-Snail-1. Right, PCR amplification of the fragment of
E-cadherin promoter containing the Snail-binding sequence was used as a positive control. Input, 2% of total input lysate. Bars, mean of three independent
experiments ± SE. *, P < 0.05 between experimental and control groups.
was not completely correlated with cisplatin resistance. Therefore, we examined the relative expression levels of phosphorylated p38, phosphorylated Akt, phosphorylated Erk1/2, and the mRNA levels of GST-π1, γ-GT, and bcl-2 to evaluate if there was any other molecule involved in cisplatin resistance. The results showed a complicated expression profile of these molecules (Supplementary Fig. S4B and C), suggesting the complexity of developing cisplatin resistance in HNSCC cells. We focused on the role of Snail-ERCC1 in cisplatin resistance due to the significant correlation of these proteins in HNSCC cells. We did a cell viability test after cisplatin treatment of the HNSCC clones described above. The results showed that overexpression of Snail in FaDu and CAL-27 cells enhanced resistance to cisplatin (Fig. 2A and B) and that suppression of Snail in OECM-1 attenuated cisplatin resistance (Fig. 2C). Collectively, these results suggest that increased Snail expression promotes expression of ERCC1 and contributes to cisplatin resistance in HNSCC cells.

Snail directly regulates ERCC1 transcription

Next, we sought to determine whether ERCC1 is required for Snail-mediated cisplatin resistance. The cell

![Fig. 4. SNAG domain of Snail is responsible for transactivation of ERCC1 by Snail.](Image)

Snail directly regulates ERCC1 transcription

Next, we searched the regulatory region of the ERCC1 gene for possible Snail binding sites. There are four putative Snail-binding sites (E-boxes, consensus sequence: CANNTG) in the regulatory region of the ERCC1 gene (Fig. 3A). We first mapped the major regulatory region of the ERCC1 promoter that was responsible for Snail-induced overexpression by generating ERCC1 promoter constructs of different lengths (ERCC1-LucA, ERCC1-LucB, and ERCC1-LucC) and then carried out promoter activity assays. The results showed that Snail overexpression activated both regulatory regions of ERCC1 genes (~843 to +1 in ERCC1-LucB and ~18 to +349 in ERCC1-LucC) and that the full-length promoter (ERCC1-LucA) had the highest activity. The suppressive effect of Snail on E-cadherin promoter was confirmed as a control (Fig. 3A).

To identify the sites most critical for Snail binding, we did site-directed mutagenesis to generate reporter constructs with different E-box mutations. We then carried out reporter assays on these constructs. The results indicated that mutation of various E-boxes partially suppressed Snail-induced promoter activation and that mutation of all four E-boxes led to the greatest suppression (Fig. 3B). A ChIP assay confirmed that Snail binds directly to the E-boxes of the ERCC1 regulatory region (Fig. 3C).

Next, we sought to identify the region in Snail responsible for ERCC1 activation. Therefore, we generated mutated constructs of Snail with different lengths (pcDNA3-SnailΔSNAG, pcDNA3-SnailΔ60, or pcDNA3-SnailΔ129). SNAG is a 10-amino-acid domain located at the NH2-terminal region of Snail (Fig. 4A) and has been reported to be critical in Snail-induced transcriptional suppression (25, 26). Interestingly, Snail-induced activation of ERCC1 was diminished after transfection of pcDNA3-SnailΔSNAG, pcDNA3-SnailΔ60, or pcDNA3-SnailΔ129 compared with wild-type Snail (Fig. 4B). As expected, truncation of SNAG abolished the Snail-induced repression of the E-cadherin promoter (Fig. 4C). Collectively, these results suggest that Snail directly regulates ERCC1 and that the four E-boxes in the regulatory region of ERCC1 contribute to this activation. The SNAG domain of Snail contributes to the suppression of E-cadherin and to the activation of ERCC1.

ERCC1 is required for the Snail-induced cisplatin resistance of HNSCC cells

Next, we sought to determine whether ERCC1 is required for Snail-mediated cisplatin resistance. The cell
viability assay showed that knockdown of ERCC1 in
Snail-overexpressing CAL-27 cells increases the sensitivity
to cisplatin (Fig. 5A). We had previously shown that over-
expression of the DNA repair protein NBS1 induces the
EMT of HNSCC cells by activation of Snail (16). To eluci-
date the role of activation of the Snail/ERCC1 axis in the
promotion of cisplatin resistance of NBS1-overexpressing
HNSCC cells, we did a cell viability assay in FaDu cells
transfected with an empty vector (FaDu-CMV), FaDu cells
overexpressing NBS1 (FaDu-NBS1), FaDu-NBS1 cells
receiving siRNA against ERCC1 (FaDu-NBS1-si-ERCC1) and FaDu cells receiving a
scrambled sequence. The results showed that overexpres-
sion of NBS1 in FaDu cells increased resistance to cisplatin
(Fig. 5B), and repression of ERCC1 in NBS1-overexpressing
FaDu cells abrogated the resistance (Fig. 5C). These results
indicate the critical role of activation of the Snail/ERCC1
axis in the mediation of cisplatin resistance of HNSCC cells.

Clinical significance of activation of Snail/ERCC1 axis
in HNSCC patients
Next, we investigated the expression of ERCC1 and Snail
in HNSCC samples and their contribution to cisplatin
resistance and prognosis. Immunohistochemical analyses of
ERCC1 and Snail were done for 72 pairs of normal epithelium
and tumor samples from advanced HNSCC patients
who were undergoing cisplatin-based chemotherapy. A
representative immunohistochemistry result is shown in
Fig. 6A. In tumor cells, nuclear expression of ERCC1
occurred in 42 cases (58.3%) and overexpression of Snail
occurred in 32 cases (44.4%). There was a clear association
between Snail and ERCC1 expression (P = 0.002; Supple-
mentary Table S6). Overexpression of Snail or ERCC1 in-
creased the risk of cisplatin resistance in HNSCC cases,
and coexpression of both markers augmented this effect
(Fig. 6B, Supplementary Table S7). We also evaluated
the importance of Twist1 in HNSCC (Supplementary
Fig. S5). The result showed that Twist1 was not associated
with ERCC1 (P = 0.467; Supplementary Table S6). In-
creased Twist1 expression also increased the probability
of developing cisplatin resistance (Supplementary Table
S7), and 10 of the 17 cases (58.8%) with ERCC1(+)/
Snail(−) were Twist1 positive.

We further confirmed by immunohistochemistry that
the Snail/ERCC1 axis is activated by its upstream sig-
als, HIF-1α or NBS1, in HNSCC samples. We found
that overexpression of HIF-1α was correlated with
increased levels of Snail and ERCC1 ($P = 0.010$ and 0.002, respectively; Supplementary Table S8). Increased NBS1 expression was also correlated with the upregulation of Snail and ERCC1 ($P < 0.001$ for both; Supplementary Table S8).

Finally, we analyzed the prognostic impact of each molecule and the combined effect of Snail/ERCC1 in HNSCC. The results showed that overexpression of HIF-1α, NBS1, Snail, or Twist1 was associated with a worse survival (Supplementary Fig. S6A–D). Increased ERCC1 expression was also associated with a trend toward a worse outcome ($P = 0.081$; Supplementary Fig. S6E), and coexpression of Snail and ERCC1 was associated with a poorer survival rate (Fig. 6C). Collectively, these results suggest that activation of the Snail/ERCC1 axis promotes chemoresistance, and combination of Snail and ERCC1 expression improves the predictive power of chemoresistance and prognosis in HNSCC patients.

**Discussion**

Previous studies have shown that the EMT, which underlies cancer metastasis, correlates with the presence of malignant phenotypes, including resistance to chemotherapy (2–5, 27–29) and radiation therapy (27), generation of cells that have stem-like properties (30), and angiogenesis (31, 32). The pleiotropic activities induced by EMT regulators beyond the induction of cytoskeletal rearrangement and cellular migration lead to the complex behavior of cancer cells during metastatic evolution and may be responsible for the difficulties encountered in the treatment of metastatic cancers. However, very few reports have examined the molecular mechanism that underlies EMT-induced chemoresistance.

In this report, we showed that ERCC1 plays a crucial role in Snail-induced chemoresistance based on numerous lines of evidence: (a) expression of ERCC1 and Snail
was highly correlated in diverse cancer cell lines; (b) Snail directly regulated ERCC1 transcription; (c) suppression of ERCC1 in Snail-overexpressing HNSCC cells attenuated the cisplatin resistance; and (d) coexpression of Snail and ERCC1 in primary HNSCC samples was associated with cisplatin resistance and a poor prognosis. To our knowledge, the present study is the first to show that Snail directly regulates the target gene related to platinum resistance.

One limitation of our study is that the in vitro drug sensitivity experiments did not always reflect the in vivo situation because tumor vascularization and the effect of tumor stroma are not accounted for in vitro. The mechanism of Twist1, another EMT regulator, in promoting chemoresistance has been examined previously (3). In our study, Twist1 expression was shown in 58.8% of ERCC1 (+)/Snail(−) cases, suggesting the complexity of the regulation of chemoresistance in HNSCC. Due to the limited number of cases included here, further study is necessary to confirm this finding.

The cisplatin-induced DNA damage is repaired by the nucleotide excision repair pathway, in which ERCC1 is the key protein involved (12). Although the significance of ERCC1 in cisplatin resistance is well known, most previous studies have focused on the expression pattern and polymorphism of ERCC1 (13–15, 33) and have not examined its regulatory mechanisms. The results presented here indicate that Snail positively promotes ERCC1 transcription by binding to E-boxes in the ERCC1 promoter. Quintela-Fandino and colleagues previously showed no relationship between ERCC1 genotype and survival rates in HNSCC (34). Together with our findings, this suggests that the level of ERCC1 expression, rather than ERCC1 polymorphism, is clinically relevant in predicting cisplatin resistance and survival in HNSCC.

Snail is known to act as a transcriptional repressor; very few reports, however, have shown that Snail activates target genes, with the exception of the direct activation of myosin Va by Snail (35). Here, we showed that the SNAG domain of Snail is responsible for the promotion of ERCC1 transcription. Previous research has identified a critical role for SNAG in the repression of target genes. The SNAG domain of Snail has been shown to contribute to recruitment of histone deacetylase and assembly of a repressor complex (25), and Snail interacts with the AJUBA/PRMT5 complex via its SNAG domain (26). Taken together with our results, we conclude that the SNAG regulatory domain has different effects on different target genes. We had previously shown that Slug, which is another EMT regulator in the zinc-finger family with the similar sequence to Snail, directly activates membrane type-4 matrix metalloproteinase (20). These findings suggest that future studies should attempt to further dissect the molecular mechanisms of these regulators.

In conclusion, our study indicates that Snail activates the chemoresistance gene ERCC1, which is crucial to the development of cancer cell chemoresistance during the EMT process. The Snail/ERCC1 axis plays a central role in cisplatin resistance of HNSCC regardless of signal inputs. Coexpression of both proteins is associated with cisplatin resistance and poor survival of HNSCC patients who are undergoing cisplatin-based chemotherapy. Our results provide valuable insight for the development of future therapies that attempt to overcome cisplatin resistance in the treatment of HNSCC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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ERCC1 Regulated by Snail Promotes Cisplatin Resistance


Regulation of Excision Repair Cross-Complementation Group 1 by Snail Contributes to Cisplatin Resistance in Head and Neck Cancer

Dennis Shin-Shian Hsu, Hsin-Yi Lan, Chi-Hung Huang, et al.


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