Effect of SNX-2112 on proliferation of esophageal cancer cells via regulation of excision repair cross-complementing 1, epidermal growth factor receptor, and p53 expression

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ABSTRACT. SNX-2112 is a potential molecular targeted therapeutic drug against esophageal cancer (EC). However, its exact mechanism of action remains to be explained. The aim of this study was to investigate the effect of SNX-2112 on excision repair cross-complementing 1 (ERCC1), epidermal growth factor receptor (EGFR), and p53, to elucidate the mechanism of action of SNX-2112 on EC. Fresh tumor sections were surgically obtained from 65 patients with EC, and the expression of ERCC1, EGFR, and p53 was determined by immunohistochemical staining. Furthermore, the effect of SNX-2112 (0.2 μM) on the proliferation of EC-9706 cells and the expression
of ERCC1, EGFR, and p53 in these cells were analyzed by a cell proliferation assay and western blot, respectively. We observed a significant decrease and increase in ERCC1 (P = 0.001) and p53 (P = 0.043) expression, respectively, and no significant difference in EGFR (P = 0.59) expression, with the TNM stage of EC, which suggested that ERCC1 and p53 could be potential markers for the TNM stage of EC. We also observed a significant increase in ERCC1 expression, and decrease in p53 and EGFR expression, in EC-9706 cells treated with SNX-2112 (P < 0.05), indicating the regulation of EC by SNX-2112. Furthermore, SNX-2112 treatment induced a significant decrease in the proliferation of EC-9706, which confirmed the function of SNX-2112. In summary, SNX-2112 inhibits the proliferation of EC cells by regulating the expression of ERCC1, EGFR, and p53.

Key words: Esophageal cancer; SNX-2112; ERCC1; EGFR; p53; EC-9706 cells

INTRODUCTION

Esophageal cancer (EC) has the second highest mortality rate among gastrointestinal cancers (Mohamed et al., 2014). The “esophageal cancer belt”, comprising Asia, Africa, and Iran, has the highest incidence rate of EC in the world (Kaz and Grady, 2014). Radiotherapy and chemotherapy are the major surgical intervention techniques for EC. However, the traditional chemo-radiotherapeutic approaches are toxic and of limited efficacy, and therefore do not satisfy the clinical requirements for cancer treatment (Chen et al., 2011). Recently, molecular analyses of cancer cells have uncovered key genetic and epigenetic alterations underlying the occurrence and development of tumors, which has in turn paved the way for molecular targeted therapy of cancer (Belkhiri and El-Rifai, 2015). Molecular targeted therapy, considered to be more effective and less harmful to normal cells than traditional forms of treatment, is one of the major modalities of cancer pharmacotherapy. Molecular targeted therapy could block the growth of cancer cells by interfering with specific molecules that induce or affect carcinogenesis and tumor growth (Holohan et al., 2013), as opposed to interfering with all rapidly dividing cells as with traditional chemotherapy.

Heat shock protein 90 (Hsp90) plays a pivotal role in guiding the normal folding and proteolytic turnover of many key regulators of cell growth, differentiation, and survival (Chen et al., 2014). Hsp90 regulates several signal transducing molecules, such as protein kinases and steroid hormone receptors (Taipale et al., 2012). This essential guardian function of Hsp90 is subverted during oncogenesis, allowing for malignant transformation and facilitating rapid somatic evolution. The use of Hsp90 as a target in molecular therapy could be a unique anticancer strategy of considerable promise.

Hsp90 inhibitors are responsible for the destabilization, and consequently, degradation, of Hsp90 client proteins, by interacting specifically with a single molecular target. Additionally, they have shown promising antitumor activity in preclinical model systems (Hall et al., 2014). Recent studies have reported a novel small molecule class of Hsp90 inhibitors that are exemplified by SNX-2112, which competitively binds to the N-terminal adenosine triphosphate binding site of Hsp90. SNX-2112 induces the degradation of multiple Hsp90
SNX-2112 inhibits cancer proliferation by regulating factors

Excision repair cross-complementing 1 (ERCC1) plays a major role in the nucleotide excision repair pathway, and is required for the repair of DNA lesions, such as those induced by UV light or formed by electrophilic compounds like cisplatin (Calderón-Montaño et al., 2014). ERCC1 serves as an important DNA excision repair protein and is reported to be associated with tumor development (Goldstein and Kastan, 2015). Epidermal growth factor receptor (EGFR), which is present on the cell surface, is activated by binding of its specific growth factor ligands. Upon activation, EGFR undergoes transition from an inactive monomeric form to an active homo-dimeric form, which contributes to cell signaling in diverse cellular functions, including cell proliferation, differentiation, motility, and survival (Avraham and Yarden, 2011). Mutations that lead to EGFR overexpression have been associated with a number of cancers. These somatic mutations involving EGFR lead to its constant activation, which in turn is responsible for uncontrolled cell division (Zhen et al., 2014). p53 has been described as “the guardian of the genome” because of its role in conserving stability by preventing genome mutation (Lopez-Serra and Esteller, 2012). The p53 gene plays a crucial role in preventing cancer formation.

SNX-2112 is a potential therapeutic agent for EC; moreover, Hsp90 inhibitors are confirmed to be associated with ERCC1 (Tsai et al., 2012), EGFR (Johnson et al., 2015), and p53 (Li et al., 2013). However, to the best of our knowledge, the exact mechanisms of SNX-2112 have not been elucidated, and the efficacy of SNX-2112 in EC has not been assessed based on its expression.

The aim of this study was to confirm the association between the clinicopathological characteristics of EC and the expression of ERCC1, EGFR, and p53, and determine the prognostic implications of these factors in EC. ERCC1, EGFR, and p53 expression in EC-9706 cells was analyzed before and after the SNX-2112 treatment, in order to explain the possible effect of SNX-2112 on EC.

MATERIAL AND METHODS

Patients

This study was approved by the Scientific Ethics Committee of Yantai Liu Huangding Affiliated Hospital at Qingdao University; informed consent was obtained from all patients prior to the study. Sixty-five subjects (average age: 62.46 ± 2.5 years) from 23 Chinese cities diagnosed with EC at our hospital between January 2012 and June 2014 were recruited in this study. The included patients were not subjected to preoperative radiotherapy and/or chemotherapy. EC was confirmed in all patients via a histopathological analysis and a gastroscopy. The cancer stage was determined according to the amount of tumor invasion (T), involvement of lymph nodes (N), and distant metastasis (M) (TNM staging system) (Pennathur et al., 2013). Integrated clinical data, including the age, gender, tumor length, and invasion depth, were collected and have been summarized in Table 1; additionally, data regarding other demographic characteristics not included in this study were obtained from all patients.

Chemicals and reagents

The Hsp90 inhibitor SNX-2112, provided by Serenex (Durham, NC, USA), was
dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 50 mg/mL and stored at -20°C for in vitro analyses. EC-9706 cells cultured in fresh medium were treated with 0.2 µM SNX-2112 for 2 days.

Cell culture

EC-9706 cells were incubated in RPMI 1640 supplemented with 100 mL/L fetal bovine serum, 100 kU/L penicillin, 100 mg/L streptomycin, and 2 mM L-glutamine in a 50-mL/L CO₂ humidified incubator at 37°C.

Immunohistochemical staining

Fresh tumor samples were obtained from the patients via surgery and were fixed immediately in 4% neutral buffered formalin. Fixed tissues were dehydrated in ethanol and embedded in paraffin blocks. Sections (4 mm) of methacarn-fixed tissues were cut using a microtome (Leica, Oberkochen, Germany) and air-dried on poly-L-lysine-coated slides; these were dewaxed with xylene, dehydrated with ethanol, and treated with 3% H₂O₂ at 37°C for 10 min. The sections were then thoroughly rinsed with phosphate-buffered saline (PBS) three times. Subsequently, the sections were placed in Coplin jars containing citric acid buffer, pH = 6.0, and heated in a microwave for 15 min (three 5-min cycles with an interval of 1 min between cycles). The jars were then allowed to cool for 20 min at room temperature. The sections were then rinsed three times in PBS for 5 min each. The sections were blocked with goat serum for 10 min at 37°C, excess serum was removed, and the sections were incubated overnight with the primary antibody at 4°C. In each case, a corresponding section was incubated in PBS without the primary antibody for nonspecific staining (control). The sections were then incubated with a biotinylated secondary antibody at 37°C for 30 min, and subsequently with a peroxidase substrate streptavidin-biotin complex at 37°C for 30 min. The samples were thoroughly rinsed with PBS and immunostained with DAB/H₂O₂. Finally, the sections were counterstained with hematoxylin and mounted with an aqueous mounting medium onto a coverslip; the sections were then observed using a microscope (Olympus, Tokyo, Japan). The control sections were incubated with an equal volume of saline instead of the primary antibody. Ten parallel experiments were conducted for each tumor sample and control group, and each independent group was analyzed in triplicate.

Positive criteria for immunohistochemical staining

The samples were classified into four different stages: non-staining (-), light yellow (+), brownish-yellow (++), and dark brown (+++) staining, based on the degree of immunohistochemical staining. The samples were then observed under a microscope (400X), and labeled according to the proportion of stained cells as <10% (-), 11-50% (+), 51-80% (++), and >80% (+++). The scores (-), (+), (++), (+++) were designated as 1, 2, 3, and 4, respectively. The sum of the two scores was set as the positive criterion; that is, groups with a total score of >4 were considered to be positive (Figure 1).

Cell proliferation assay

A standard CCK-8 assay kit (Beyotime Biotechnology, Beijing, China) was used to
analyze the proliferation of EC-9706 cells. Cells (1 x 10^5) were seeded into each well of a 96-well plate and cultured for 24 h. The cells were then treated with SNX-2112 or DMSO (control) and incubated for 0, 12, 24, 36, and 48 h. Subsequently, 10 µL CCK-8 was added to each well and the plate was re-incubated for 1 h. The absorbance at 450 nm of each well was detected. Cell proliferation in each group was measured in five wells, and each independent group was analyzed in triplicate.

![Figure 1](image.png)

**Figure 1.** Representative expression of (A) ERCC1, (B) EGFR, and (C) p53.

**Western blot assay**

Total protein was isolated from cells using the cell extraction buffer (Biosource, Camarillo, CA, USA) supplemented with protease and phosphatase inhibitors. Total protein was pre-cleared by centrifugation and the protein concentration was measured using a standard bicinchoninic acid protein assay kit (Pierce, Rockford, IL, USA). The primary antibodies used in this study (including those against β-actin, ERCC1, EGFR, and p53) were purchased from Abcam (Cambridge, UK). Equal protein loading was confirmed by probing for β-actin expression. The cells were then incubated with anti-mouse or anti-rabbit immunoglobulin G (IgG; GE Healthcare, Little Chalfont, UK) secondary antibodies conjugated to horseradish peroxidase. The proteins were then visualized using an Amersham Enhanced Chemiluminescence detector (GE Healthcare).

**Statistical analysis**

All data were estimated according to the results of ten independent experiments. Statistical analysis was performed using the SPSS software platform (version 20.0; SPSS Inc., Chicago, IL, USA). Data were compared using the chi-square test; associations between the factors were determined by Spearman correlation. P < 0.05 was considered to be statistically significant.

**RESULTS**

**Association between ERCC1, EGFR, and p53 expression and clinicopathological characteristics of EC**

The effect of ERCC1, EGFR, and p53 on EC was confirmed by recording the clinico-
pathological characteristics of patients; among these, the age, gender, tumor length, invasion depth, G staging, and TNM stage of all patients are summarized in Table 1. The expression of ERCC1, EGFR, and p53 was detected by immunohistochemical staining. We observed no significant association of ERCC1, EGFR, and p53 expression with patient age and gender or with tumor length and invasion depth. Additionally, we observed a significant decrease and increase in the expression of ERCC1 and p53, respectively, with the TNM stage of EC, which suggested ERCC1 and p53 as potential markers for the TNM stage of EC. EGFR overexpression was suggested to be associated with a number of cancers (Bastid, 2012); however, in this study, the expression of EGFR did not increase significantly with the TNM stage of EC, which suggested that the mechanisms of EC were unique, and that EGFR might be a key factor affecting this variation (Table 1).

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<th>p53</th>
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<td>16 (57.14)</td>
<td>12 (42.86)</td>
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SNX-2112 inhibits the proliferation of EC cells by regulating the expression of ERCC1, EGFR, and p53

The mechanism of SNX-2112 on the effect of ERCC1, EGFR, and p53 in EC was investigated by treating the EC-9706 cells with 0.2 µM SNX-2112. The cell proliferation assay showed that the proliferation of EC-9706 cells was inhibited significantly by SNX-2112 (Figure 2). Furthermore, western blot (Figure 3) showed that the application of SNX-2112 resulted in increased ERCC1 expression, and significantly decreased p53 and EGFR expression (P < 0.05), which indicated that SNX-2112 was responsible for the regulation of ERCC1, EGFR, and p53 in EC.
SNX-2112 inhibits cancer proliferation by regulating factors

**Figure 2.** SNX-2112 inhibits the proliferation of EC-9706 cells. Proliferation of EC-9706 cells was evaluated after the application of 1) no solution (control group); 2) DMSO (DMSO group); and 3) SNX-2112 (SNX-2112 group). *P < 0.05 compared to the wild-type group. #P < 0.05 compared to the DMSO group.

**Figure 3.** SNX-2112 regulates the expression of ERCC1, EGFR, and p53 in EC-9706 cells. The black columns (before group) comprise of EC-9706 cells before the application of SNX-2112 and gray columns (after group) comprise cells analyzed after the application of SNX-2112. *P < 0.05.

**DISCUSSION**

Recent years have seen advances in research on tumor pathology at a molecular level. These analyses have indicated that tumor development is a result of the interaction between proto-oncogenes and anti-oncogenes; moreover, multiple genes are reported to be involved in the occurrence and development of tumors (Rivlin et al., 2011). EC is one of the most common types of malignant tumors, whose exact mechanism, however, remains unexplained. Therefore,
research on EC at the molecular level could help identify the key genes or markers for accurate and early diagnosis and treatment of EC, in turn providing novel methods for clinical practice.

DNA repair is important for maintaining normal cellular functions. Genetic variants of DNA repair genes and reduced DNA repair capacity are believed to contribute to increased susceptibility to cancer (Tong et al., 2009). ERCC1 plays a pivotal role in several DNA repair pathways and also forms a structure-specific heterodimeric endonuclease with xeroderma pigmentosum complementation group F, which cleaves damaged 5’-DNA in the lesion (Doll et al., 2010). Previous research has shown a strong association between the decrease in ERCC1 expression and the occurrence of cancer (Fan et al., 2011). The results of our study indicated that low ERCC1 expression was correlated with poor prognosis of EC, and SNX-2112 could be used to treat EC by increasing the expression of ERCC1. Interestingly, some researchers have suggested that patients who had advanced EC and also showed low expression of ERCC1 displayed improved response to preoperative chemo-radiotherapy. Additionally, preoperative chemo-radiotherapy may be more effective than immediate surgery in patients with low ERCC1 expression (Kim et al., 2008). Although the results of our study suggested that SNX-2112 played an important role in DNA repair through regulating the expression of ERCC1, its exact influence on the overall survival and mechanism of EC needs to be investigated further.

Generally, EGFR exerts a signal transducing effect on the growth of cells, thereby regulating the growth, proliferation, and differentiation of cells (Worster et al., 2012). EGFR overexpression, caused by gene mutations, could also result in abnormal signal transduction and cell growth, leading to carcinogenesis (Rahman et al., 2014). Therefore, increased EGFR expression is a strong prognostic feature of multiple tumor types, and EGFR inhibition could have substantial therapeutic benefits. The results of this study suggested that the expression of EGFR could increase with the occurrence and development of EC, suggesting a positive correlation between the expression of EGFR and development of EC; although this association was not significant, it still indicated that EGFR could promote growth and metastasis in EC. Additionally, EGFR is an oncogenic kinase, which is required for proper functioning of Hsp90 (Bachleitner-Hofmann et al., 2011). FW-04-806 (Huang et al., 2015), ganetespib (Patel et al., 2014), and other Hsp90 inhibitors are believed to decrease the expression of EGFR. The results of this study also suggested that SNX-2112 induced a decrease in the expression of EGFR, which indicated the important role played by SNX-2112 in inhibiting Hsp90, via the downregulation of EGFR.

p53 plays a major role in transcription in humans and is responsible for maintaining normal cell growth, regulating apoptosis, and preventing malignant transformation in cells (Timani et al., 2015). Mutations in the p53 gene could lead to decreased anticancer activity and promote malignant transformation (Bywater et al., 2012). Low expression of p53 is also associated with high response to chemotherapy-based treatment in EC (Zhang et al., 2013); for example, phospholipase C epsilon 1 modulates the apoptosis mechanism by suppressing p53 expression in esophageal cancer (Li et al., 2014) cells, while ECRG2 combined with cisplatin has been shown to promote apoptosis in EC cells by upregulating p53 (Song et al., 2014). This indicated that p53 is an important mediator in this complex system. In this study, we confirmed that p53 expression increased significantly with the development of EC; additionally, SNX-2112 treatment was shown to induce a significant decrease in p53 expression, which indicated that SNX-2112 also played an important role in the apoptosis process by interfering with the p53 expression. To the best of our knowledge, the mechanism by which SNX-2112 affects p53 has not been documented; the results of the study suggested that this mechanism exerted a significant effect on the treatment of EC.
SNX-2112 inhibits cancer proliferation by regulating factors

Hsp90 has been shown to stimulate apoptosis and inhibition of proliferation of esophageal squamous cell cancer (ESCC) cells; moreover, SNX-2112 treatment was shown to induce a decrease in the expression of proliferating cell nuclear antigen and a corresponding increase in the number of apoptotic cells in ESCC tissues (Wang et al., 2015). SNX-2112 combined with 5-FU has an antagonistic effect on esophageal cancer cells by affecting the growth inhibition, cell cycle, and apoptosis mechanisms (Liu et al., 2015). We further confirmed that SNX-2112 inhibits the proliferation of EC-9706 cells by regulating ERCC1, EGFR, and p53. However, the exact mechanisms remain to be explained. This could be confirmed by further studies with larger sample sizes.

In summary, ERCC1, EGFR, and p53 play an important role in the complex EC system and are potential prognostic markers for EC. Furthermore, SNX-2112 inhibits the proliferation of esophageal cancer cells by regulating the expression of ERCC1, EGFR, and p53.

Conflicts of interest

The authors declare no conflict of interest.

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