High expression of EZH2 is associated with tumor aggressiveness and poor prognosis in patients with esophageal squamous cell carcinoma treated with definitive chemoradiotherapy

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The enhancer of zeste homolog 2 (EZH2), a known repressor of gene transcription, has been reported to be associated with biological malignancy in several cancers. The potential oncogenic role of EZH2 and its clinical/prognostic significance, however, in esophageal squamous cell carcinoma (ESCC) are unclear. In this study, the methods of immunohistochemistry and fluorescence in-situ hybridization were used to examine protein expression and amplification of EZH2 in 98 pretreatment biopsy specimens of ESCC who received definitive chemoradiotherapy (CRT). High expression of EZH2 and amplification of EZH2 was found in 54.1% and 12.0% of ESCCs, respectively. High EZH2 expression was significantly correlated with increased cell proliferation (p = 0.009), high histopathological grade (p = 0.002), regional (p = 0.025) and distant lymph node metastasis (p < 0.001) and lack of clinical complete response to CRT (p = 0.028). Univariate analysis revealed that high expression of EZH2 was associated with poor metastasis-free survival (MFS) (p = 0.003), poor progression-free survival (PFS) (p = 0.001) and poor disease-specific survival (DSS) (p < 0.001). In multivariate analysis, high expression of EZH2, together with lack of clinical complete response, were evaluated as significant independent prognostic factors of MFS, PFS and DSS for patients with ESCC. These findings suggest that high expression of EZH2 correlates with tumor aggressiveness and adverse patient outcome in ESCC treated with definitive CRT. Evaluation of EZH2 expressions might be useful for predicting tumor response to CRT and prognosis for patients with ESCC.

Key words: esophageal squamous cell carcinoma, EZH2, chemoradiotherapy, prognosis, immunohistochemistry

Abbreviations: CR: complete response; CRT: chemoradiotherapy; CT: computed tomography; DSS: disease-specific survival; EGFR: epidermal growth factor receptor; ESCC: esophageal squamous cell carcinoma; EZH2: the enhancer of zeste homolog 2; FISH: fluorescence in-situ hybridization; HCC: hepatocellular carcinoma; HER-2: HER-2/neu; IHC: immunohistochemistry; MFS: metastasis-free survival; NC: no change; PcG: the polycomb group of genes; PD: progressive disease; PFS: progression-free survival; PR: partial response; TNM: tumor-node-metastasis; WHO: World Health Organization

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In general, esophageal squamous cell carcinoma (ESCC) is an aggressive tumor with poor prognosis worldwide, and its overall 5-year survival rate is less than 30%. Most patients present with locally advanced disease. The current standard treatment for advanced localized ESCC is concurrent chemoradiotherapy (CRT) with or without surgery, and whether surgery after neoadjuvant CRT or radical CRT should be performed depends on the tumor response to induction CRT. Therefore, if predictive factors that may identify those patients who would benefit from CRT can be found, a more appropriate therapeutic strategy would be expected. However, the clinical responses of ESCC to CRT are heterogeneous, and the high probability of recurrence and distant metastases is still the main cause of poor quality of life and death. At present, only the stage based on Tumor Node Metastases (TNM) classification and primary complete response to CRT are widely accepted as prognostic factors, which are still far from an accurate predictor. Molecular analyses of ESCC have largely focused on individual candidate genes, such as P53, Bax, Bcl-2, Ki-67, cyclin D1, epidermal growth factor receptor (EGFR) and HER-2/neu (HER-2). However, some of the results investigated by different studies remain conflicting, such reliable markers are still currently lacking.
The enhancer of zeste homolog 2 (EZH2) gene, known as a member of the polycomb group of genes (PcG), has been found to contribute to the maintenance of cell identity, cell cycle regulation and oncogenesis.\textsuperscript{14} It is reported that EZH2 serves as a histone methyl transferase, involves in gene silencing, and disruption of EZH2 expression can lead to cancer.\textsuperscript{15} EZH2 amplification was first reported in hematologic malignancies,\textsuperscript{16,17} and there is an increasing evidence that overexpression of the EZH2 gene occurs in a variety of human malignancies, including breast, prostate, endometrial, gastric, colon, hepatocellular, bladder and oral cancers.\textsuperscript{18–25} The abnormalities of this gene were observed to correlate closely with tumor aggressiveness and/or poor patient prognosis. In our previous study, we observed that knocking down EZH2 expression in hepatocellular carcinoma (HCC) cells was sufficient to significantly reverse tumorigenicity in a nude mice model, and demonstrated the therapeutic value of EZH2 inhibition in vivo for the first time.\textsuperscript{26} Recently, Merola et al.\textsuperscript{27} revealed that Rb2/p130 expression was inversely correlated to that of EZH2 in Barrett's metaplasia with respect to the normal controls, which indicated a role of EZH2 in esophageal malignant progression.

To date, the molecular status of the EZH2 gene in ESCC and the clinicopathological/prognostic significance of expression of EZH2 have not been elucidated. In this study, immunohistochemistry (IHC) and fluorescence in-situ hybridization (FISH) were used to examine the distribution and frequency of protein expression and amplification of EZH2 in a cohort of patients with ESCC treated with definitive CRT, so as to determine whether or not EZH2 expression has predictive value of CRT response and clinical outcome in patients with ESCC.

**Material and Methods**

**Patients and tissue specimens**

In this study, for EZH2 IHC studies, 98 ESCC patients treated with definitive CRT were consecutively selected from the Department of Radiotherapy, Cancer Center, Sun Yat-Sen University between January 2002 and December 2008. The cases selected were based on the following criteria: (i) histologically proven primary ESCC with available biopsy specimens; (ii) no previous malignant disease or a second primary tumor; (c) no previous treatment or severe complications; (d) Karnofsky≥70; (e) no distant metastases except for supraclavicular or celiac lymph nodes; (f) received the same CRT regimen and follow-up regularly. The tumor biopsy specimens were recruited from paraffin blocks of the 98 primary ESCCs from the Department of Pathology of our institutes, and 30 samples of normal esophageal mucosa were used for controls. In addition, to assess the representativeness of using biopsy specimens to evaluate IHC staining of EZH2 in ESCC tissues, additional 20 pairs of ESCC biopsy specimens (before esophagectomy) and surgical resected specimens of the same patients staged pT3 were also examined. The study was approved by the medical ethics committee of our institute.

**Chemoradiotherapy**

All the 98 patients received the same concurrent chemoradiotherapy with PF (Cisplatin/5-fluorouracil) regimen. Cisplatin was administered as i.v. drip at a dose of 80 mg/m\textsuperscript{2} on day 1; 5-fluorouracil 3 g/m\textsuperscript{2} was administered as a continuous i.v. infusion for 48 hr on days 1–2. Two cycles of chemotherapy were done during radiotherapy at 4-week intervals. Radiotherapy was performed by an 8 MV linear accelerator. Two-dimensional or three-dimensional treatment plans using computed tomography (CT) scans were done. The initial treatment volume included the primary tumor with a radial margin of 1.5–2 cm and a proximal and distal margin of 3–4 cm and enlarged lymph nodes. A total radiation dose of 60–70 Gy (1.8–2 Gy/fraction, 5 days a week) was delivered with 3-field technique, and the treatment field was reduced after 40–46 Gy.

**Evaluation and follow-up**

The effect of CRT was evaluated clinically for primary lesions based on esophagography and CT 4 weeks after CRT according to the following criteria. Complete response (CR) was defined as the complete resolution of all assessable lesions. Partial response (PR) was defined as a reduction by 50% or more of the sum of the lesions and no progression of assessable lesions. No change (NC) was indicated by a reduction <50% or increase <25% in tumor size. All these conditions had to last for at least 4 weeks and no appearance of new lesions. Progressive disease (PD) was defined as an increase ≥25% in tumor size or the appearance of new lesions.

The patients were followed every 3 month for the first year and then every 6 months for the next 2 years, and finally annually. The diagnostic examinations consisted of esophagography, CT, chest X-ray, abdominal ultrasonography and bone scan when necessary to detect recurrence and/or metastasis. Disease progression was defined as cases in which the tumor evaluated as PD after CRT or recurrence after CR (local progression) and/or cases in which new distant metastasis occurred (distant progression).

**Immunohistochemistry**

Immunohistochemistry (IHC) staining was performed on 5-μm tissue sections rehydrated through graded alcohols. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 15 min. For antigen retrieval, tissue slides were boiled in tris (hydroxymethyl) aminomethane-EDTA buffer (pH 8.0) in a pressure cooker for 12 min (EZH2) or microwave-treated for 10 min (Ki-67). Nonspecific binding was blocked with 10% normal rabbit serum for 20 min. The tissue slides were incubated with anti-EZH2 (BD Transduction Laboratories, Franklin Lakes, NJ, 1:100 dilution) or anti-Ki-67 (Dako, Glostrup, Denmark, 1:100 dilution) for 60 min at 37°C in a moist chamber. Subsequently, the slides were sequentially incubated with biotinylated rabbit antimouse immunoglobulin at a concentration of 1:100 for 30 min at 37°C.
and then reacted with a streptavidin-peroxidase conjugate for 30 min at 37°C and 3′,3′-diaminobenzidine as a chromogen substrate. The nucleus was counterstained using Meyer’s hematoxylin. A negative control was obtained by replacing the primary antibody with a normal murine IgG. Positive expression of EZH2 in ESCC and normal esophageal mucosa cells was primarily a nuclear pattern (Fig. 1). Known immunostaining positive slides were used as positive controls.

Two independent observers blinded to the clinicopathologic information performed scoring using a previously validated scoring system for EZH2 expression. This system scores nuclear EZH2 expression by recording the percentage of nuclei staining positive for the EZH2 protein, irrespective of staining intensity, in which EZH2 immunoreactivity was classified into 2 groups: low expression, when positive cells were less than 50% (Fig. 1a); and high expression, when at

Figure 1. Immunohistochemical stainings of EZH2 and Ki-67 and FISH of EZH2 in human esophageal tissues. (a) The adjacent normal esophageal mucosa of ESCC (case 19) showed low expression of EZH2 protein, in which less than 50% of normal esophageal squamous cells showed positive staining of EZH2 in nuclei. (b) High expression of EZH2 was detected in the same ESCC case 19, in which more than 70% squamous cell carcinoma cells showed positive staining of EZH2 protein in nuclei. (c) High expression of EZH2 was observed in another ESCC (case 37), where more than 90% carcinoma cells demonstrated positive staining of EZH2. (d) Amplification of EZH2 gene was observed by FISH in the same ESCC case 37, in which EZH2 gene signals (red) was detected at least 3 times more than centromere signals of chromosome 7 (green). (e) Another primary ESCC (case 55) was observed high expression of EZH2, where all carcinoma cells showed positive staining of EZH2 protein in nuclei. (f) More than 60% of carcinoma cells were observed positive expression of Ki-67 in the same ESCC case 55. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]
least 50% of the cells showed positive immunoreactivity in the nuclei (Figs. 1b and 1c left and 1d left).18,23,28 In this study, a minimum of 500 epithelial cells was counted for each normal or tumor case.

### Fluorescence in-situ hybridization

Two-color fluorescence in-situ hybridization (FISH) was undertaken using a Spectrum Orange-labeled BAC clone (RP11-731B4) at chromosome 7q35-q36 containing the EZH2 gene and a Spectrum Green-labeled reference centromeric probe on chromosome 7 (Vysis, Downers Grove, IL). The FISH reaction was performed as described previously with slight modification.29 In brief, the deparaffinized tissue section was treated with proteinase K (400 μg/ml) at 37°C for 45 min, followed by denaturing in 70% formamide, 2× SSC at 75°C for 6 min. Fifty nanograms of each probe were mixed in a 10 μl hybridization mixture (containing 55% formamide, 2× SSC, and 2 g human Cot1 DNA), denatured at 75°C for 6 min and then hybridized to the denatured TMA section at 37°C for 24 hr. After washing, the section was counterstained with 1 μg/ml DAPI in an anti-fade solution and examined with a Zeiss Axio phot microscope equipped with a triple-band pass filter. FISH signals from 300 cells in each sample were counted. The criteria for EZH2 gene amplification were defined as the presence of either 6 (or more) gene signals or more than 2.5 times as many gene signals than centromere signals of chromosome 7 (Fig. 1c right).

### Statistical analysis

Statistical analysis was performed with the SPSS software (SPSS Standard version 13.0, SPSS). The associations of EZH2 expression with ESCC patient's clinicopathological features were assessed by the χ² test. An independent sample t-test was used to assess the expression of EZH2 and Ki-67 between different groups. Metastasis-free survival (MFS), progression-free survival (PFS) and disease-specific survival (DSS) were analyzed with the Kaplan–Meier method and compared by the log rank test. MFS, PFS and DDS were defined as the time from diagnosis to tumor metastasis, tumor progression and cancer-relative death, respectively. Multivariate survival analysis was performed on all parameters that were found to be significant on univariate analysis using the Cox regression model. p values of <0.05 were considered significant.

### Results

#### Patient characteristics

The clinicopathological characteristics of the 98 patients studied were summarized in Table 1. According to the 6th edition of the TNM classification of the International Union Against Cancer (UICC, 2002), 8 patients were classified into Stage II, 51 cases were Stage III and 39 cases were Stage IV. All the patients received the same regimen of concurrent CRT described earlier. CR, PR, NC and PD were achieved in 19 patients, 42 patients, 36 patients and 1 patient, respectively. Of the 79 patients who did not get CR, 22 cases received adjuvant chemotherapy, 2 cases received radical esophagectomy. The other patients did not receive any anti-tumor treatments until tumor progression.

### Table 1. Clinicopathologic correlation of EZH2 expression in ESCC

| Variables                      | EZH2 expression (%) | p    |
|--------------------------------|--------------------|------|
| Age (years)                    |                    |      |
| ≤55                            | 28 (51.9)          | 26 (48.1) | 0.192 |
| >55                            | 17 (38.6)          | 27 (61.4) |
| Gender                         |                    |      |
| Male                           | 36 (43.9)          | 46 (56.1) | 0.365 |
| Female                         | 9 (56.3)           | 7 (43.8) |
| Location                       |                    |      |
| Cervical/Upper Thoracic        | 29 (60.4)          | 19 (39.6) | 0.005 |
| Middle/Lower Thoracic          | 16 (32.0)          | 34 (68.0) |
| WHO grade                      |                    |      |
| G1                             | 17 (70.8)          | 7 (29.2) |
| G2                             | 23 (46.0)          | 27 (54.0) |
| G3/4                           | 5 (20.8)           | 19 (79.2) |
| Tumor size (cm)                |                    |      |
| ≤6                             | 28 (50.0)          | 28 (50.0) | 0.349 |
| >6                             | 17 (40.5)          | 25 (59.5) |
| T status                       |                    |      |
| T2/3                           | 24 (51.1)          | 23 (48.9) | 0.326 |
| T4                             | 21 (41.2)          | 30 (58.8) |
| N status                       |                    |      |
| N0                             | 12 (70.6)          | 5 (29.4) | 0.025 |
| N1                             | 33 (40.7)          | 48 (59.3) |
| M status                       |                    |      |
| M0                             | 36 (61.0)          | 23 (39.0) | <0.001 |
| M1-lym                         | 9 (23.1)           | 30 (76.9) |
| Lymph node metastasis          |                    |      |
| NOM0                           | 12 (70.6)          | 5 (29.4) | 0.001 |
| N1M0                           | 24 (57.1)          | 18 (42.9) |
| M1-lym                         | 9 (23.1)           | 30 (76.9) |
| CRT response                   |                    |      |
| CR                             | 13 (68.4)          | 6 (31.6) | 0.028 |
| Not CR                         | 32 (60.5)          | 47 (59.5) |
| Locoregional progression       |                    |      |
| Present                        | 21 (44.7)          | 26 (55.3) | 0.813 |
| Absent                         | 24 (47.1)          | 27 (52.9) |
| Distant progression            |                    |      |
| Present                        | 8 (22.2)           | 28 (77.8) | <0.001 |
| Absent                         | 37 (59.7)          | 25 (40.3) |

1Chi-square test. 2Mean age. 3Mean tumor size. Abbreviation: T, tumor; N, node; M, metastases; M1-lym, distant lymph node metastasis.
Expression of EZH2 in ESCC

In this study, protein expression of EZH2 was examined by IHC in 98 cases of primary ESCC, 30 cases of normal esophageal mucosa and additional 20 pairs of ESCC biopsy specimens and surgical resected specimens of the same patients staged pT3. As no heterogeneous expression of EZH2 between biopsy specimens and its paired surgical materials, and no obvious difference of EZH2 expression among ESCCs in different layers of the esophageal wall was observed (Supp. Info. Table SI), we think that the results of this study performed using biopsy specimens are reliable for the evaluation of IHC staining of EZH2 in ESCC tissues. Using the criteria described earlier, high expression of EZH2 was observed in 53/98 (54.1%) of the ESCCs but in none of the normal esophageal mucosa. The association between clinicopathological features and EZH2 expression levels of the 98 ESCCs were summarized in Table 1. High EZH2 expression correlated closely with tumor location \( (p = 0.005) \), World Health Organization (WHO) grade \( (p = 0.002) \), Node (N) status \( (p = 0.025) \) and distant lymph node metastasis (M-lym) status \( (p < 0.001) \). Further analysis we found that the expression levels of EZH2 increased steadily from no lymph node metastasis to regional lymph node metastasis, and distant lymph node metastasis \( (p = 0.001) \) (Table 1).

Correlation between clinicopathological variables, EZH2 expression and CRT response

Primary CR was achieved in 19.4% \( (19/98) \) of the patients with ESCC. EZH2 expression was the only factor that showed a significant correlation with CRT response \( (p = 0.028) \) (Table 1) No significant association was found between CRT response and clinicopathologic variables, such as patient’s age, gender and tumors histopathological grade, location, size, T status and radiotherapy dose \( (p > 0.05) \).

Correlation between clinicopathological variables, EZH2 expression and ESCC patient survival

Of the 98 patients with ESCC, none was lost to follow-up. The median observation period was 23.9 months \( (2.3–80.7 \text{ months}) \), with 73 tumor progressions and 68 cancer-related deaths. The median survival time was 21.9 months. The 3-year PFS and DSS for the entire cohort of patients were 25.5% and 30.0%, respectively. For the 59 patients without distant metastasis at the time of diagnosis, 12 distant metastases were observed, and the 3-year MFS was 40.7%.

A significant association between high expression of EZH2 and the present of distant progression but not regional progression was demonstrated by our \( \chi^2 \) test (Table 1). In univariate analysis, high EZH2 expression was evaluated to correlate closely with poor PFS \( (p = 0.001) \) and poor DSS \( (p < 0.001) \) for the whole cohort, and poor MFS \( (p = 0.003) \) for 59 M0 cases (Fig. 2, Table 2). Further, our multivariate analysis showed that EZH2 expression and CRT response were independent predictors of PFS, DSS and MFS (Table 3).

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**Figure 2.** Kaplan–Meier survival analysis in patients with ESCC. Progression-free survival (a) and disease-specific survival (b) curves for the whole cohort of patients with ESCC \( (n = 98) \) and metastasis-free survival (c) curve for M0 cases \( (n = 59) \) according to EZH2 expression status.
Correlation between EZH2 expression and cell proliferation in ESCCs

The expression level of Ki-67 was assessed as a labeling index (LI), i.e., as the percentage of Ki-67 positive cells in each tumor.23,28,29 As shown in Table 4, both the LIs of EZH2 and Ki-67 were significantly higher in ESCCs than in esophageal normal mucosas (\( p < 0.001 \)). In addition, EZH2 LIs also correlated with CRT response, being highest for the NC/PD group and lowest for the CR group (\( p = 0.013 \)). However, no significant association of the LIs of Ki-67 and CRT response

| Variables                        | PFS (months) | DSS (months) | MFS (months) |
|----------------------------------|--------------|--------------|--------------|
|                                  | Case Median  | Case Median  | Case Median  |
| Age (years)                      |              |              |              |
| ≤552                             | 54 10.9      | 21.9         | 35 22.6      |
| >55                              | 44 12.4      | 21.3         | 24 21.3      |
| Gender                           |              |              |              |
| Male                             | 82 10.4      | 18.5         | 47 20.2      |
| Female                           | 16 60.9      | 31.7         | 12 63.8      |
| Location                         |              |              |              |
| Cervical/upper thoracic          | 48 10.7      | 22.7         | 37 30.4      |
| Middle/lower thoracic            | 50 11.3      | 16.7         | 22 20.2      |
| WHO grade                        |              |              |              |
| G1                               | 24 14.8      | 22.7         | 16 52.8      |
| G2                               | 50 10.9      | 22.1         | 31 21.3      |
| G3/4                             | 24 7.4       | 13.5         | 12 6.9       |
| Tumor size (cm)                  |              |              |              |
| ≤63                              | 56 12.4      | 22.6         | 38 22.7      |
| >6                               | 42 10.8      | 14.1         | 21 10.8      |
| T status                         |              |              |              |
| T2/3                             | 47 14.7      | 22.7         | 26 52.1      |
| T4                               | 51 9.0       | 17.6         | 33 18.5      |
| N status                         |              |              |              |
| N0                               | 17 29.5      | NR           | 17 NR        |
| N1                               | 81 10.7      | 18.0         | 42 16.0      |
| M status                         |              |              |              |
| M0                               | 59 12.8      | 23.2         | – –          |
| M1-lym                           | 39 10.7      | 14.7         | –        |
| RT dose (GY)                     |              |              |              |
| ≤602                             | 68 10.4      | 20.0         | 36 20.2      |
| >60                              | 30 16.0      | 27.1         | 23 52.1      |
| Adjuvant Chemotherapy            |              |              |              |
| Yes                              | 22 10.9      | 21.9         | 12 27.2      |
| No                               | 76 12.4      | 22.6         | 47 20.2      |
| CRT response                     |              |              |              |
| CR                               | 19 60.9      | 63.3         | 12 NR        |
| Not CR                           | 79 9.3       | 16.6         | 47 16.0      |
| EZH2 expression                  |              |              |              |
| Low                              | 45 24.5      | 52.1         | 36 52.1      |
| High                             | 53 8.6       | 14.0         | 23 9.3       |

1Log-rank test. 2Mean age. 3Mean tumor size. 4No cases observed. 5Median radiotherapy dose.
was observed ($p = 0.174$). Furthermore, when dividing the ESCCs into high and low EZH2 expression groups, high expression of EZH2 was found to be associated with a high LI of Ki-67 ($p = 0.009$) (Table 4).

**Discussion**

In this study, we first examined the expression of EZH2 and its clinicopathological/prognostic significance in 98 primary patients with ESCC treated with definitive CRT. Our results showed that the expression of EZH2 correlated with cell proliferation, histopathological grade, lymph node metastasis status at diagnosis, CRT response and prognosis in ESCC.

**Amplification of EZH2 in ESCCs**

In our FISH study, the FISH analysis was informative in 14/30 (46.7%) of the normal esophageal tissues and 50/98 (51.0%) of the ESCCs. Samples without FISH signal and samples with weak target signals or those with a strong signal background were the main reasons for the noninformative cases. FISH results demonstrated that the amplification of EZH2 was not detected in any of the normal esophageal tissues but was detected in 6/50 (12.0%) of the informative ESCCs. Four of the 6 cases belong to the NC group; the other 2 cases have PR and PD, respectively. In each of the 6 cases with EZH2 amplification, high level of EZH2 expression was observed. In the remaining 44 informative cancers without amplification of EZH2, 23 (52.3%) cases showed low expression of EZH2, while 21 (47.7%) cases were observed high EZH2 expression.

**Table 3. Multivariate Cox regression analysis for MFS, PFS and DSS in patients with ESCC**

| Variable       | MFS (n = 59) |         | PFS (n = 98) |         | DSS (n = 98) |         |
|----------------|--------------|---------|--------------|---------|--------------|---------|
|                | HR (95% CI)  | p       | HR (95% CI)  | p       | HR (95% CI)  | p       |
| EZH2 expression| 0.031        |         | 0.010        |         | 0.006        |         |
| Low            | 1.000        |         | 1.000        |         | 1.000        |         |
| High           | 2.089 (1.070–4.076) | 1.906 (1.170–3.107) | 2.192 (1.247–3.855) |         |         |
| CRT response   | 0.014        |         | 0.005        |         | 0.010        |         |
| CR             | 1.000        |         | 1.000        |         | 1.000        |         |
| Not CR         | 4.919 (1.385–7.472) | 2.979 (1.388–6.394) | 2.997 (1.306–6.877) |         |         |

**Table 4. Association of EZH2 expression and cell proliferation in esophageal tissues**

| Variables       | Cases | EZH2 expression (%) | Ki-67 labeling index (%) |
|-----------------|-------|----------------------|--------------------------|
|                 |       | Mean ± SE            | Mean ± SE                |
| Pathology       |       |                      | p<0.001                  | <0.001                   |
| Normal mucosa   | 30    | 21.7 ± 2.4           | 7.3 ± 1.6                |
| Tumor           | 98    | 50.0 ± 3.1           | 25.2 ± 2.1               |
| CRT response    |       |                      | p<0.013                  | 0.174                    |
| CR              | 19    | 41.2 ± 7.3           | 30.5 ± 4.2               |
| PR              | 42    | 49.0 ± 4.7           | 24.7 ± 2.9               |
| NC/PD           | 37    | 59.1 ± 5.1           | 22.4 ± 3.3               |
| EZH2 expression |       |                      | p<0.009                  | 0.009                    |
| Low             | 45    | –                    | 20.7 ± 1.9               |
| High            | 53    | –                    | 29.1 ± 2.3               |

1Independent sample t-test. Abbreviation: SE, standard error.

Abbreviations: HR, hazard ratio; CI, confidence interval.

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contrast, a steady increasing of EZH2 expression was noted in ESCCs from no lymph node metastasis to regional lymph node metastasis, and distant lymph node metastasis. Taken together, these data suggest that EZH2 may play an important role in tumorigenic process and provide a selective advantage in lymph node metastasis of ESCC.

It has been widely accepted that clinical CR to CRT is the most important predictor of outcome for patients with ESCC treated with concurrent CRT with or without surgery. However, numerous clinical trials have shown that preoperative CRT benefits only the 25% of patients who get pathologic CR, whereas the remaining 75% present CRT-resistant and highly aggressive cancers. Thus, it will be of great value identifying a molecular signature that would suggest a highly CRT-sensitive cancer for patients with ESCC at the time of diagnosis. In this study, we observed that EZH2 expression was the only factor that showed a significant correlation with ESCC CRT response. We have previously found that in HCC Bel7404 cells, the cell sensitivity to chemotherapeutic doxorubicin could be enhanced by suppressing EZH2 expression. Similar results were also observed by Ougolkov and colleagues that EZH2 depletion sensitized pancreatic cancer cells to doxorubicin and gemcitabine, leading to a significant induction of apoptosis. These findings suggest a potential impact of EZH2 on human tumor cellular responses to ionizing radiation and cytotoxic drugs.

With regards to the biologic function of EZH2, a recent cDNA microarray study demonstrated that disruption of EZH2 expression can retard cell proliferation, while overexpression of EZH2 was found to shorten the G1 phase of the cell cycle and lead to accumulation of cells in the S phase. In this study, we observed an overall significant positive association of upregulated expression of EZH2 and increased ESCC cellular proliferation. These results suggest a potential important role of EZH2 in the control of cell proliferation, an activity that might be responsible, at least in part, for tumorigenesis and/or progression of ESCC, which might also contribute to the tumor resistance to radiotherapy. It is known, as an essential downstream target of pRB/E2F pathway, EZH2 is a critical mediator of E2F function, in which E2F1 has been shown to be associated with radiosensitivity and/or chemosensitivity in certain types of tumors. Moreover, increased E2F-1 expression via tumor cell proliferation and decreased apoptosis were found to correlate with adverse prognosis in patients with ESCC. Thus, we suppose that the pRB/E2F/EZH2 pathway is likely to be one of the mechanisms involved in tumor progression and response to CRT. Clearly, further studies should be carried out to precisely understand the potential oncogenic function of EZH2 in human ESCC pathogenesis and which signaling pathway is involved in the sensitivity of ESCC to CRT.

The prognostic significance of EZH2 expression in ESCC, especially its clinical implication of distant metastasis, is the most important finding of this study. We found a strong correlation between high expression of EZH2 and the present of distant metastasis in patients with ESCC. Similar results were also observed in prostate and breast cancers. One of the earliest reports was a gene profiling study where EZH2 was scored as the most significant gene upregulated in metastatic prostate cancer compared to clinically localized prostate. Significantly, EZH2 overexpression in prostate cell lines led to silencing of a discrete set of >100 target genes. The implication of EZH2 in tumor metastasis may be explained by the existence of several metastasis-suppressing genes that EZH2 works to suppress, such as Rho GTP-ase-acting protein 1. Furthermore, in our univariate and multivariate survival analysis, we demonstrated that high expression of EZH2 was a significant predictor of poor MFS for M0 cases, as well as an independent prognostic factor of PFS and DSS for the whole cohort of patients with ESCC. These findings underscore a potentially important role of EZH2 as an underlying biological mechanism in the progression of ESCC. Therefore, EZH2 expression might be used as an additional tool in identifying patients with ESCC who will be at high risk of metastasis and/or progression, which may be useful in optimizing individual therapy management at the time of diagnosis.

To determine whether high expression of EZH2 in ESCCs was caused by gene amplification, we examined the amplification status of EZH2 by FISH. In our 50 informative cases of ESCC by both IHC and FISH simultaneously, high expression of EZH2 was detected in all (6/6) ESCCs that had EZH2 amplification. These findings were in good agreement with the results of studies in breast, gastric and bladder cancers, in which a strong correlation between DNA copy number and levels of transcription was reported. However, in our study, amplification of EZH2 was not observed in 21 other ESCCs with high expression of EZH2. These data suggest that, in some subsets of tumors, high expression of EZH2 is likely to be due to a specific amplification of EZH2 gene, however, molecular mechanisms other than gene amplification, might also play a role in the regulation of EZH2 expression in ESCC. It has been reported that EZH2 promoter can respond to ectopic expression of E2F1, E2F2, E2F3 and E2F4. Furthermore, the longest EZH2 promoter construct, which was efficiently transactivated by the E2Fs, has been found to be consistent kinetically with the cell growth-regulated expression of the EZH2 mRNA. These results collectively, suggest that the regulation of protein expression of EZH2 is quite complicated and it might be regulated not only by gene amplification, but also by other molecular mechanisms including transcriptional regulation.

In summary, in this study, we describe, for the first time, protein expression and amplification patterns of EZH2 in normal human esophageal tissues and in ESCCs. Our results provide a basis for the concept that high expression of EZH2, as detected by IHC, may be an ideal predictor of aggressive ESCC with CRT resistance and an independent prognostic factor of patients with ESCC treated with definitive CRT.
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