Expression and clinical implications of enhancer of Zeste homolog 2 and p53 protein in squamous cell carcinoma and precancerous lesions in the cervix

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ABSTRACT. We investigated the expression and clinical implications of enhancer of Zeste homolog 2 (EZH2) and p53 protein in cervical squamous cell carcinoma (SCC) and precancerous lesions. EZH2 and p53 expressions in SCC (168), cervical intraepithelial neoplasia (CIN)-I (19), CIN-II (35), and normal tissues (30) were detected by streptavidin-peroxidase-conjugation. The correlation between co-expression of EZH2 and p53 protein and the clinic pathological features and prognosis of SCC were discussed. The positive expression rates of EZH2 and p53 were 6.7, 37.0, and 75.6%, and 3.3, 21.1, and 39.3% in normal cervical tissues, CIN, and SCC, respectively, which were significantly different (P < 0.05). The positive expression rate of EZH2 and p53 protein in SCC patients with and without lymph node metastasis was 82.9 and 70.4% (EZH2) and 45.7 and 34.7% (p53), respectively, which was also a significant difference (P < 0.05). The progression-free survival (PFS) rates in followed-up patients (N = 143) who were EZH2- and p53-negative, EZH2- or p53-positive, and EZH2-

and p53-positive were 71.3 ± 1.9, 66.1 ± 2.0, and 51.3 ± 3.8 months, respectively, which was a significant difference (P < 0.001); the overall survival among these groups was 72.9 ± 1.1, 68.6 ± 1.8, and 57.4 ± 3.4 months, respectively (P < 0.001). Multivariate analyses revealed that EZH2 expression, lymph node metastasis, and tumor staging were independent prognostic factors of SCC. EZH2 and p53, which affect lymph node metastasis and prognosis of SCC, may play a key role in the occurrence and development of SCC.

Key words: EZH2; p53 protein; Squamous cell carcinoma; Cervical intraepithelial neoplasia; Immunohistochemistry; Survival analysis

INTRODUCTION

Cervical carcinoma, a high incidence cancer type, affects the female genitalia; squamous cervical carcinoma (SCC) is the most common tumor affecting the cervix. There has been a growing incidence of cervical carcinoma over the past few years, with a large percentage of cases affecting young women (Parkin et al., 2001). Cervical intraepithelial neoplasia (CIN) is a precancerous cervical lesion, and must be diagnosed and treated early to avoid cancer progression. Enhancer of zeste homolog 2 (EZH2) inhibits cell differentiation and target gene expression. EZH2, in conjunction with the p53 protein, induces tumor cell proliferation, metastasis, and immortalization. Highly expressed in various malignant tumors, EZH2 and p53 protein are closely related to the degree of malignancy and prognosis of tumors (Bracken et al., 2003; Tang et al., 2004; Dong et al., 2007). In this study, we attempted to detect the expression of EZH2 and p53 in different types of cervical lesions by immune histochemical analysis, and discuss their clinical implications.

MATERIAL AND METHODS

Patients and specimen collection

Cervical lesions were collected from 253 patients receiving surgical treatment for cervical cancer from January 2005 to December 2006 at the First Affiliated Hospital of Sun Yat-sen University. Among these, 30 patients underwent a hysterectomy for hysteromyoma (normal control), and 19 and 35 patients were at stage II and III of CIN, respectively. One hundred and sixty-eight patient shad SCC. The cancer stages were determined according to the 2009 International Federation of Gynecology and Obstetrics (FIGO) staging system: 13, 71, and 84 cases were at stage IA, IB, and IIA of cancer. Among the 168 SCC patients, 98 showed lymph node metastasis, while the others did not. All cases were followed up for 60-84 months; all 25 patients whose follow-up was discontinued were women with SCC (lost follow-up rate: 9.9%).

Immunohistochemistry

The expression of EZH2 and p53 was detected by the SP method, using citrate buffer (pH = 6.0) for repair. The staining intensity was determined by comparing the sample with the

positive and negative controls. The proportion of positive cells in each slide was calculated; samples with <30 and ≥30% positive cells were considered as negative and positive samples, respectively.

## Statistical analysis

All statistical analyses were performed using SPSS v.18.0 (IBM, Armonk, NY, USA). The count data was analyzed by the χ^2 test. The data was also subjected to Kaplan-Meier survival analysis and log-rank test. Multivariate analysis was performed using Cox's proportional hazard model, with significance set at $\alpha = 0.05$.

RESULTS

EZH2 and p53 expression in cervical lesions

The EZH2 and p53 protein expression in different cervical lesions were analyzed by immunohistochemistry (Figure 1). The positive expression rate of EZH2 in normal cervical tissue, and CIN, and SCC tissues was 6.7% (2/32), 37.0% (20/54), and 75.6% (127/168), respectively, while that of p53 in these tissues was 3.3% (1/30), 21.1% (11/54), and 39.3% (66/168), respectively; all differences were statistically significant ($P < 0.05$). The positive expression rates of EZH2 and p53 in SCC patients (N = 168) with (N = 70) and without (N = 98) lymph node metastasis were 82.9% (58/70) and 70.4% (69/98) and 45.7% (32/70) and 34.7% (34/98), respectively; these differences were found to be statistically significant ($P < 0.05$). The patients were divided into 3 groups based on the expression of EZH2 and p53: EZH2- and p53-positive, EZH2- or p53-positive, and EZH2- and p53-negative. EZH2 and p53 expressions in different types of cervical lesions are summarized in Table 1.

A   B   C
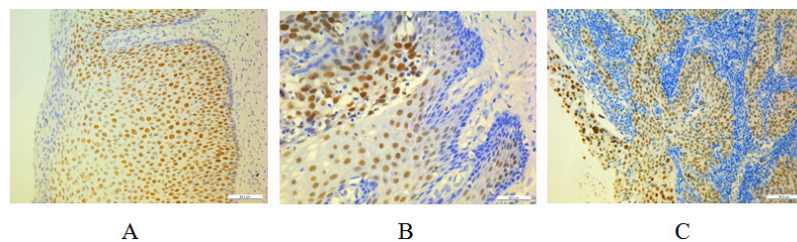
Figure 1. EZH2 and p53 protein expression in CIN and SCC tissues. **A.** Positive expression of p53 in CINI (immunohistochemistry; 200X magnification). **B.** Positive expression of EZH2 at the boundary between CIN and SCC (immunohistochemistry; 400X magnification). **C.** Positive expression of EZH2 in SCC (immunohistochemistry; 200X magnification). All data expressed as percentages. CIN = cervical intraepithelial neoplasia; EZH2 = enhancer of Zeste homolog 2.

Table 1. Expression of the enhancer of Zeste homolog 2 (EZH2) and p53 protein in different types of cervical lesions.

Group	Cases	Positive for EZH2 and p53	Positive for EZH2 or p53	Negative for EZH2 and p53	P value
Normal control	30	1 (3.3)	1 (3.3)	28 (93.3)	0.001
CIN II	19	2 (10.5)	6 (31.6)	11 (57.9)	0.009
CIN III	35	4 (11.4)	14 (40.0)	17 (48.6)	0.039
SCC	168	51 (30.4)	92 (54.8)	25 (14.9)	0.027

Correlation between EZH2 and p53 expression and clinic pathological features of SCC

The correlation between EZH2 and p53 expression and the clinic pathological features of SCC are summarized in Table 2. EZH2 and p53 expression differed significantly between patients with difference stages of SCC (IB and IIA; $P < 0.05$), and between patients with and without lymph node metastasis ($P < 0.05$).

Table 2. Correlations between enhancer of Zeste homolog 2 (EZH2) and p53 expression and clinicopathological features of squamous cell carcinoma (SCC).

Clinicopathological features	Cases	Positive for EZH2 and p53 (N = 51)	Positive for EZH2 or p53 (N = 92)	Negative for EZH2 and p53 (N = 25)	P value
Clinical staging					
Stage IA	13	4	4	5	0.377
Stage IB	71	16	42	13	0.021
Stage IIA	84	31	46	7	0.019
Lymph node metastasis					
No	98	23	59	16	0.014
Yes	70	28	33	9	0.027
Size of local foci (cm)					
<4	137	41	78	18	0.077
≥4	31	10	14	7	0.311
Microinfiltration of vessels					
No	139	42	79	18	0.097
Yes	29	9	13	7	0.379

Survival rate of SCC patients with varying EZH2 and p53 expression

Progression-free survival (PFS) in followed-up SCC patients (N = 143) who were EZH2- and p53-negative, EZH2- or p53-positive, and EZH2- and p53-positive was 71.3 ± 1.9, 66.1 ± 2.0, and 51.3 ± 3.8 months, respectively; the differences between the cases were statistically significant ($P < 0.001$; Figure 2). EZH2- and p53-protein negative, EZH2- or p53-positive, and EZH2- and p53-positive was 72.9 ± 1.1, 68.6 ± 1.8, and 57.4 ± 3.4 months, the differences between the cases were statistically significant ($P < 0.001$; Figure 3).
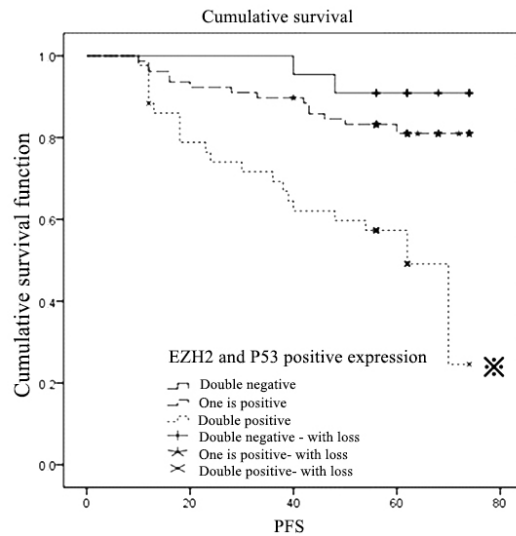
Figure 2. Progression-free survival (PFS) curve of SCC with varying EZH2 and p53 expression. SCC = squamous cell carcinoma; EZH2 = enhancer of Zeste homolog 2.
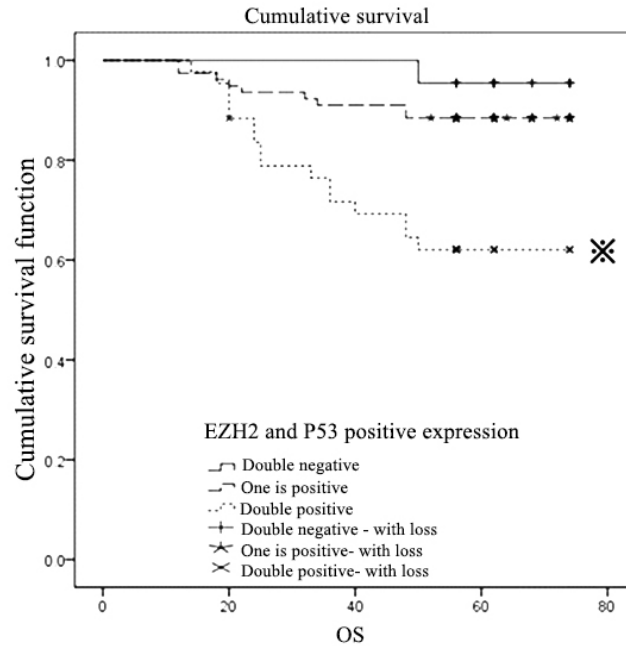
Figure 3. Survival curve of SCC cases with differential EZH2 and p53 expression. SCC: squamous cell carcinoma; EZH2: enhancer of Zeste homolog 2.

Univariate and multivariate analysis of factors influencing the prognosis of SCC

Univariate analysis revealed a correlation between the age and body mass index (BMI) of patients, and the EZH2 and p53 expression and SCC prognosis (Table 3). The multivariate analysis indicated that the EZH2 expression, lymph node metastasis, and tumor staging were independent factors influencing the prognosis of SCC (Table 4).

Table 3. Univariate analysis of factors influencing the prognosis of 143 squamous cell carcinoma (SCC) cases.

Factor		Cases	Survival time (months, means ± SD)	P value
Age (years)	<50	91	68.9 ± 1.2	0.032
	≥50	52	63.7 ± 0.9	
Occupation	Farmer	35	65.7 ± 1.2	0.721
	Others	108	65.9 ± 1.7	
Familial history	No	126	66.7 ± 1.8	0.498
	Yes	17	64.2 ± 0.7	
Childbearing history	Having borne no children	2	61.3 ± 0.9	0.278
	Having borne children	141	65.9 ± 1.5	
Menopause	No	87	68.3 ± 1.7	0.375
	Yes	56	64.1 ± 2.0	
BMI (kg/m^2)	<24	96	70.1 ± 0.9	0.023
	≥24	47	64.9 ± 1.2	
EZH2	-	35	73.3 ± 0.7	<0.001
	+	108	63.5 ± 1.9	
p53	-	87	68.9 ± 1.6	0.018
	+	56	61.3 ± 2.8	

EZH2: enhancer of Zeste homolog 2; BMI: body mass index.

Table 4. Multivariate analysis of prognostic factors of squamous cell carcinoma (SCC; N = 143).

Factor	B value	Standard error	Wald value	RR (95%CI)	P value
EZH2 expression	2.152	1.025	4.409	1.735 (1.056-2.379)	0.036
p53 expression	0.574	0.421	1.859	1.147 (0.403-1.585)	0.173
Lymph node metastasis	1.999	0.629	10.110	2.773 (1.364-3.631)	0.001
Tumor stage	1.414	0.505	7.833	2.127 (1.351- 2.794)	0.005
Age	-0.015	0.019	0.561	0.993 (0.477-1.021)	0.454
BMI	-0.082	0.061	1.825	0.985 (0.955-1.005)	0.177

SCC = squamous cell carcinoma; EZH2 = enhancer of Zeste homolog 2; BMI = body mass index.

DISCUSSION

Two major types of proteins with conservative structure and functions are responsible for regulating the cell proliferation and differentiation post-embryonic development: thepolycomb (PcG) and trithorax (TrxG) groups (Mills, 2010). EZH2 is a PcG protein that promotes cell proliferation, inhibits cell differentiation and target gene expression, and is closely associated with tumors. The *EZH2* gene was first identified in humans in 1996 (Hobert et al., 1996), and was found to be homologous to the *EZH2* gene in fruit flies; moreover, the resultant protein also possessed similar structure and functions to that seen in fruit flies. *EZH2* is localized to chromosome 7q35, regulates cell growth, and promotes cell proliferation by inhibiting the target genes in the chromatin. *EZH2* inhibits transcription, and thus reduces the activity of various genes, especially the expression of factors inhibiting tumor metastasis, such as TIMP (Shin and Kim, 2012) and RKIPz (Ren et al., 2012). EZH2 generally promotes tumor cell invasion and metastasis and reduces the expression of target genes by inhibiting the binding of transcription factors to DNA. It acts on the pRb2/p130 C-terminal domain that is associated with histone deacetylase (HDAC1). By restoring the activity of the cyclin A promoter, mediated by the pRb2/p130-HDAC1 complex, EZH2 accelerates the cell cycle progression and malignant transformation (Tonini et al., 2004).

EZH2 is expressed in tumors of the urinary system, breast tumors, and lymphoma; the expression of EZH2 in middle- and late-stage tumors and metastatic tumors is much higher than that in early-stage tumors and non-metastatic tumors. This implies that EZH2 expression is related to tumor invasion and metastasis (Visser et al., 2001; Hoffmann et al., 2007; Kunju et al., 2011). Bachmann et al. (2006) and Zhang et al. (2006) also demonstrated that EZH2 promoted the occurrence, development, and invasion of endometrial carcinoma. In this study, normal cervical squamous epithelia, CIN II, CIN III, and SCC tissues all showed increased expression of EZH2; moreover, the latter was also expressed in precancerous lesions. This suggested the role of the activation and expression of *EZH2* in the onset and development of SCC. The prevalence of *EZH2* was greater in patients with lymph node metastasis; therefore, EZH2 expression is an adverse factor that promotes tumor progression.

However, some studies have also shown that EZH2 is not a direct factor inducing tumor occurrence and development. EZH2 alters cell proliferation and differentiation by regulating the expression of other genes or proteins, which in turn leads to malignancy. The factors regulated by *EZH2* include matrix metalloproteinase (*MMP*), heat shock protein (*HSP*), and metastasis-associated gene (*MTA*). p53 protein expression is related to cervical HPV infection and the occurrence of cervical carcinoma (Zhang et al., 2006). However, the interactions between *EZH2* and p53 in cervical carcinoma remainnunclear. Recent reports have suggested a negative correlation between EZH2 and p53 expression; moreover, wild-type

p53 is believed to bind to EZH2 promoter, thereby inhibiting EZH2 expression, and arresting human fibroblasts at the G_2 phase, inhibiting its proliferation, and inducing apoptosis (Tang et al., 2004). Bracken et al. (2003) reported conflicting results in osteosarcoma cells. In these cells, silencing of the *EZH2* gene by RNA interference led to downregulation of the *p53* gene. Dong et al. (2007) demonstrateda positive correlation between EZH2 expression and that of mutant-type p53 protein in breast cancer. It was inferred that the two proteins acted in synergy during the development of breast cancer, although the specific mechanism was not elucidated.

In this study, we observed a correlation between EZH2 and p53 expression and lymph node metastasis and tumor staging in SCC. Simultaneous detection of EZH2 and p53 helped predict the prognosis of SCC, which was consistent with the previous discovery that EZH2 and p53 expression is associated with an increase in tumor metastasis. Kaplan-Meier survival analysis indicated that the PFS and OS of EZH2- and p53-positive SCC patients were significantly lower than those of EZH2- and p53-negative, or EZH2- or p53-positive patients. Multivariate analyses revealed that lymph node metastasis, tumor stage, and the EZH2 expression were independent prognostic factors of SCC. Hence, the study into the mechanism of action of EZH2 and p53 protein in cervical carcinoma contributes to the understanding of the molecular biology of tumor occurrence and development, and helps predict the malignant transformation of cervical lesions. Similar to factors such as infiltration of deep cervical muscles, having multiple sexual partners, and complicating pregnancy (Zhang et al., 2004), EZH2 expression plays a key role in the diagnosis, treatment, and prognostic evaluation of cervical carcinoma and precancerous lesions.

In conclusion, EZH2 and p53 are involved in the development of SCC, independently or in synergy. The detection of EZH2 and p53 expression can benefit the prediction of tumor stage, lymph node metastasis, and prognosis of SCC compared to the detection of EZH2 or p53 alone.

Conflicts of interest

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

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