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Original Article

Comparison of Snail1, ZEB1, E-Cadherin Expression Levels in HPV-Induced Cervical Cancer

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Abstract

Background: Molecular profiling techniques are the rapid detection of biomarkers in the human papillomavirus (HPV) infected cells. We aimed to measure the expression level of three cell factors including Snail1, ZEB-1, and E-cadherin in cervical cancer (CC), precancerous and healthy samples, simultaneously, to find potential biomarkers.

Methods: The expression level of the mentioned cell factors were investigated in 72 CC patients, precancerous patients, and healthy controls by using Real-Time PCR.

Results: The results demonstrated a significant reduction in the expression level of E-cadherin in cancer and precancerous cases than that in healthy cases; whereas the expression level of ZEB-1 and Snail1 were upregulated in cancer and precancerous samples. The receiver operating characteristic (ROC) analyses shows the highest AUC value emerged for Snail1: 1(95% CI: 1-1) in comparing CC and healthy groups with a sensitivity of 100.0 % and specificity of 100.0%.

Conclusion: The molecular biomarker Snail1 may be helpful to early diagnosis and prognosis of CC in the HPV-infected human populations. Considering the increased expression level of Snail1 in cancer and precancerous tissue compared to healthy tissue as well as the area under the ROC curve, Snail1 can be used for early detection of CC.

Keywords: Cervical cancer; Human papillomavirus; E-cadherin; Snail1

Introduction

Cervical cancer (CC) is the third widespread cancer, which has the fourth rank among cancer mortality causes of women worldwide (1, 2). The anticipated outbreak is estimated at 21.7 million until 2030, which occasions the death of 13 million of the infected cases due to the population aging (3). CC is 12th leading cause of female disease in Iran (4).

The viral infections are the causative agents of almost 15% of all cancers (5, 6). Human papillo-

mavirus (HPV) performs its life cycle in either mucosal or cutaneous stratified squamous epithelia (7, 8). The persistent high-risk human papillomaviruses (HR-HPVs) are the principal etiologic agent in the CC pathogenesis (9-11). Conversely, HR-HPVs, and especially HPV-16 as the most prevalent virus infecting the cervix, are accompanied by the entire spectrum of cervical intraepithelial neoplasia (CIN) lesions as well as, invasive squamous carcinomas (12, 13).

After HR-HPV infection, several cellular changes associated with the epithelial-mesenchymal transition (EMT) were observed (14, 15). EMT turns epithelial cells into the mesenchymal cells that can invade and migrate. In addition, it contributes to the metastatic progression in human cancer cells (16). One of the significant characteristics of EMT is the functional loss of E-cadherin (17, 18).

E-cadherin is a transmembrane glycoprotein encoded by the *CDH1* gene that its low expression is associated with increased invasiveness and metastasis in several cancers (19, 20). It is inactivated by multiple mechanisms, probably because of the genetic alteration, reduced gene expression, changes of another cadherin–catenin complexes or posttranslational modification of the protein leading to cytoplasmic delocalization (21-23).

The principal mechanism for E-cadherin loss during EMT is transcriptional repression. Several repressors comprise zinc-finger E-box-binding homeobox 1 (ZEB-1), ZEB2/SIP1, Twist, Snail1, and Snail2 can bind to E-box motifs and repress E-cadherin transcription (24-26).

EMT is dynamically regulated during CC progression and the expression level of EMT markers such as E-cadherin and vimentin, change in this dynamic regulation. Transcription factors have a key role in EMT induction. The Snail has been demonstrated to regulate E-cadherin expression and is associated with CC development (27). Studies into the ZEB family in CC are relatively few, and the role of the ZEB family in CC is currently unknown (28).

Among these, Snail1 has a critical role, since its expression is widely observed in EMT processes preceding the remaining EMT-TFs; moreover, ectopic Snail1 induces other EMT-TFs such as Zeb1/2 and Snail2 (29). Also, Snai1 depletion severely impacts mesoderm formation during embryogenesis (30,31).

Snail1 acts as a crucial factor for cellular motion through the progression and metastasis of cancer cells. Moreover, it was found that epidermal growth factor (EGF) stimulation causes the up-regulation and accumulation of Snail1 protein in CC cells (32, 33).

We investigate the biomarker potential of the three cell factors, including E-cadherin, ZEB-1, and Snail1 in CC subjects. For this purpose, the expressions of E-cadherin, ZEB-1 and Snail1 were evaluated in the cervical, precancerous, and healthy tissue samples. Also, the receiver operating characteristic curve analysis was employed to compare selected groups for the diagnosis of CC.

Materials and Methods

Tissue sample collection

Seventy-two fresh uterine cervix biopsies were collected and kept in RNAlater (Qiagen) at – 80 °C to stabilize RNA. Patients that received any chemo/radiation therapy were excluded. The routine hematoxylin-eosin stain on 5 µm paraffin sections was utilized to make the biopsies at colposcopy and surgery and assessed in participating hospitals and classified as healthy, precancer (CIN1, CIN2, CIN3), or invasive cancer according to international criteria (34). All subjects filled consent before operations at Imam Khomeini Complex Hospital (Tehran, Iran) from 2016 to 2018. This study was approved by the Ethics Committee of Tehran University of Medical Sciences (IR.TUMS.SPH.REC.1395.838).

Total RNA extraction

Total RNA was extracted from tissues with TRIzol (Invitrogen, Carlsbad, CA, USA). The extracted RNAs were stored at – 80 °C until cDNA synthesis. Extraction quality was evaluated by 28s:18s rRNA evaluation using agarose gel electrophoresis stained with SYBR Safe dye (Invitrogen, Carlsbad, CA, The USA) (35).

cDNA synthesis

The reverse transcription (Fermentas, Vilnius, Lithuania) was performed at 42 ˚C for 60 min, followed by 70 ˚C for 5 min according to the manufacturer's instructions (36).

qRT-PCR

The expression of ZEB-1 (37), Snail1 (38), and E-cadherin (39) were analyzed by quantitative Real-Time PCR using the SYBR Green (TAKARA Bio INC., Otsu, Japan) with Applied Biosystems®StepOnePlus™ (Applied Biosystems, Foster City, CA, USA). The human Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was applied to normalize the relative quantity (40- 43).

Statistical analysis

The Mann-Witney non-parametric test and one-way ANOVA were performed to analyze the statistical discrepancy between groups using GraphPad Prism (7.0.1). A *P*-value of < 0.05 was considered remarkable. The area under receiver operating characteristic (ROC) curves were calculated using R software (version 3.4.4).

Results

Patient and control data

Overall 72 fresh specimens (36 normal, 18 CC and 18 precancerous) were prepared for Snail1, ZEB-1, and E-cadherin expression analysis. The mean age of CC, precancerous, and healthy groups were 61 yr (45-81 yr), 47 yr (27–57 yr), and 36 yr (23–49 yr), respectively with no significant discrepancy.

The expression levels of Snail1, ZEB-1, and E-cadherin

The expression levels of Snail1, ZEB-1 and E-cadherin were determined in cervical samples (Fig. 1).

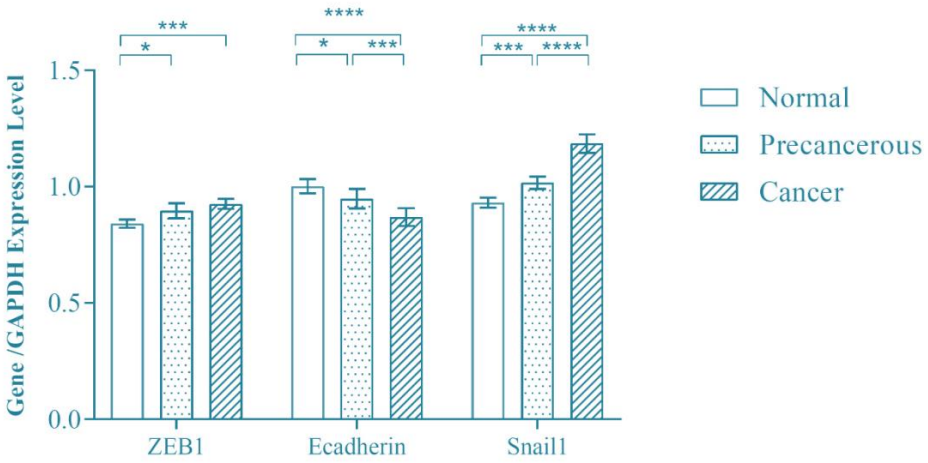

Fig. 1: The relative expression level of Snail1, ZEB-1, and E-cadherin between cervical cancer samples, Precancerous samples, and healthy samples. Each sample was analyzed in triplicate and normalized to GAPDH

The expression of E-cadherin was significantly decreased in cancer and precancer groups in comparison to the healthy group (P <0.0001, P = 0.026), also, in cancer group in comparison to the precancer group (P = 0.02). The expression of ZEB-1 and Snail1 in cervical tissue were remarkably higher in the CC group compared with the healthy group (P =0.0003, P < 0.0001, respectively). In addition, the expression of ZEB-1 and Snail1 in the cancer group, compared to the precancer group, was not significant for ZEB-1 and was significant for Snail1 (P <0.0001). Finally, the expression of ZEB-1 and Snail1 in the cancer group was significantly increased in the precancer

group compared with the healthy group (P = 0.0003, P = 0.026, respectively).

The correlation analysis between Snail1, ZEB-1, and E-cadherin expression

To comprehend the associations between Snail1, ZEB-1, and E-cadherin expressions, the correlation values were explored. A significant and inverse correlation was found between ZEB-1 and E-cadherin in healthy group (r = - 0.432, P-value = 0.05). The ZEB 1 and Snail1 had an inverse and significant relationship in cancer group (r = - 0.94, P-value<0.00001). Finally, a meaningful and inverse correlation was observed between ZEB-1 and E-cadherin in cancer group (r = - 0.703, P-value = 0.005). Any considerable correlation was not observed.

Receiver operating characteristic (ROC) curve analysis

The ROC curves were generated and they are under area analyses were performed to evaluate the diagnostic value of Snail1, ZEB-1, and E-cadherin expression levels in the samples of CC, precancerous and healthy (Table. 1).

ROC curves showed that the area under the curve (AUC) values in CC and healthy groups were Snail1: 1(95% CI: 1-1), ZEB-1: 0.99 (95% CI: 0.97-1) and E-cadherin: 0.90 (95% CI: 0.81-1) (Fig. 2). The AUC value in CC and precancerous groups were Snail1: 0.94 (95% CI: 0.88-1), ZEB-1: 0.67(95% CI: 0.48-0.85) and E-cadherin: 0.71 (95% CI: 0.54-0.89). Finally, the AUC in the precancerous and healthy groups were Snail1: 0.88 (95% CI: 0.78-0.98), ZEB-1: 0.74 (95% CI: 0.58-0.90) and E-cadherin: 0.71 (95% CI: 0.55-0.87). So, the highest AUC value was obtained for Snail1: 1(95% CI: 1-1) in comparing CC and healthy groups. It indicated that Snail1 has a strong potential diagnosis value for differentiating of CC from precancerous and healthy groups. Moreover, the sensitivity of Snail1 was higher than two other proteins in comparing CC and healthy groups (100%), precancerous and healthy groups (73.7), and CC and precancerous groups (78.9) (Table 2).

Table 1: ROC curve analysis. Area under the curve (AUC) value of Snail1, ZEB-1, and E-cadherin

Cell Factors	*Cervical cancer and Normal groups*	*Precancerous cervical and Normal groups*	*Cervical cancer and Precancerous cervical groups*
E-cadherin	AUC	AUC	AUC
	95% CI :(0.81-1)	95% CI : (0.55-0.87)	95% CI : (0.54-0.89)
ZEB-1	AUC	AUC	AUC
	95% CI : (0.97-1)	95% CI : (0.58-0.90)	95% CI : (0.48-0.85)
Snail1	AUC	AUC	AUC
	95% CI : (1-1)	95% CI : (0.78-0.98)	95% CI : (0.88-1)

Table 2: The Snail1, ZEB-1, and E-cadherin cellular factors and their sensitivity and specificity estimation according to the ROC Curve results

Cell Factors	*Sensitivity and Specificity*	*Cervical cancer and Normal groups*	*Precancerous cervical and Normal groups*	*Cervical cancer and Precancerous cervical groups*
E-cadherin	Sensitivity	75	30	65
	Specificity	90.4	90	80
ZEB-1	Sensitivity	100	45	20
	Specificity	95	95	95
Snail1	Sensitivity	100	73.7	78.9
	Specificity	100	84.2	94.7

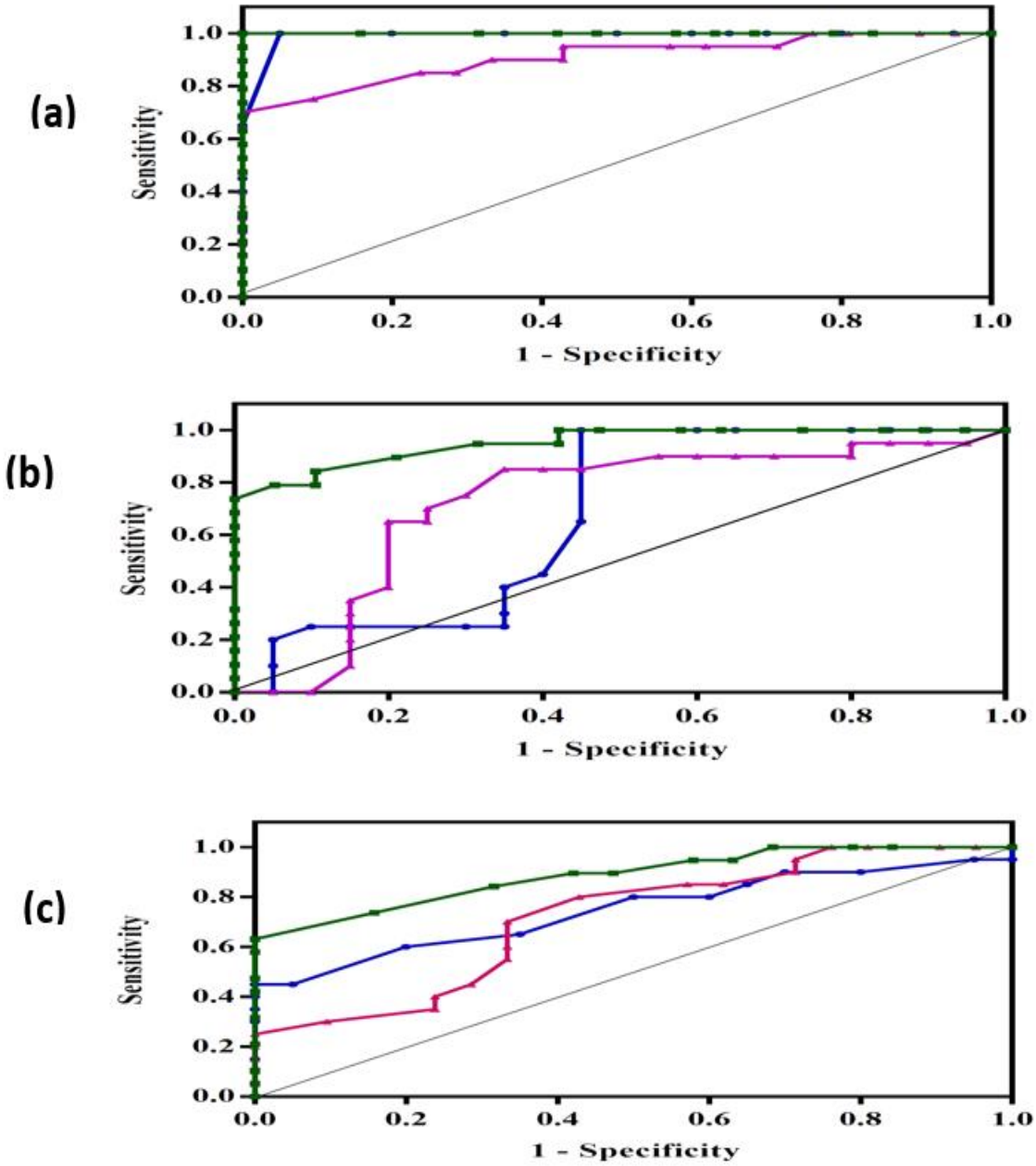

Fig. 2: (a) Receiver-operating characteristics (ROC) curve analysis using Snail1, ZEB-1, and E-cadherin for discriminating cervical cancer and healthy groups in tissue. The green, blue and violet lines represent Snail1, ZEB-1, and E-cadherin, respectively.
(b) Receiver-operating characteristics (ROC) curve analysis using Snail1, ZEB-1, and E-cadherin for discriminating cervical cancer and precancerous groups in tissue. The green, blue, and violet lines represent Snail1, ZEB-1, and E-cadherin, respectively.
(c) Receiver-operating characteristics (ROC) curve analysis using Snail1, ZEB-1, and E-cadherin for discriminating precancerous and healthy groups in tissue. The green, blue, and violet lines represent Snail1, ZEB-1, and E-cadherin, respectively

Discussion

Finding new biomarkers for CC has a great importance to diagnose disease before the tumor becomes invasive. The HPV infection of cervical cells leads to many cellular alterations. In this study, we surveyed potential value of three candidate genes as CC tissue biomarkers. The outcomes revealed that the E-cadherin down-regulation and up-regulation of ZEB-1 and Snail1 in cancerous/precancerous samples in comparison to healthy tissue.

Epithelial to mesenchymal transition (EMT) take places through physiological states e.g. development and tissue damage repair, also in pathological situations like cancer initiation and progression. In this phenomenon, epithelial cells trans-differentiate to mesenchymal cells.

EMT also plays a key role in the tumor progression and metastasis with stem cell properties that render resisting to cancer treatment (18, 31, 44-48).

The EMT procedure involves cellular and cell adhesion abnormalities. It is accompanied by betterment in attacking and dynamic exclusivities (49). There are currently other complementary ways with EMT not yet known. EMT is becoming a chosen aim for anti-cancer treatment (15). Although, some include well-known genes and markers such as E-cadherin and Vimentin that can be helpful in identifying EMT in tumors (50, 51).

The loss of E-Cadherin is a sign of completing EMT in epithelial tumors. E-cadherin, as the prevailing cell-cell adhesion molecule is found in the epithelial cell types (52). Some studies disclosed the important role of E-cadherin during tumor progression and invasion (53, 54). Moreover, they are critical factors in the designation final E-cadherin level via binding to its inhibitory factors such as transcription factors zinc finger E-box-binding proteins 1 and 2 (ZEB-1 and ZEB2) and Snail1 levels (55-57). Snail1 is a zinc finger protein known as the transcription factor, which is expressed by some epithelial tumor cells and fibroblasts (58). Snail1 straightly binds to the E-boxes present in the proximal E-cadherin promoter and suppresses E-cadherin gene expression (23, 59). Moreover, Snail blocks the progression of cell cycle and participates in cell movement and survival, and transcriptional regulation of cytokines to mediate invasion and inflammation (60). The expression levels of Snail1 and ZEB-1 up-regulate due to abrupt changes in the tumor microenvironment. In cancer, it has been reported that promoting ZEB1 expression plays a vital role in progression and metastasis in the renal cell carcinoma (61), endometrial cancer (62), invasive breast cancer (63) and lung adenocarcinoma (50). Down-regulation of ZEB1 the expression can prevent invasive tumors from converting to mesenchymal phenotype by reducing the proliferation and mobility of CC cells, which suggests that ZEB1 may be a potential therapeutic target for cervical squamous cell carcinoma (64).

The function of E-cadherin in counteracting with the invasive property of cancer cells can lead to design treatment methods, which reduce the suppressor genes and induce the increase of the E-cadherin expression (25).

Overexpression of Snail was reported in association with metastasis and poor prognosis in gastric cancer (65). In addition, the correlation of high expression level of ZEB-1 with loss of E-cadherin expression was found in various cancer cells (66-70). Herein, the similar results as above were obtained, so that the up-regulation of Snail1 and ZEB-1 were along with the down-regulation of E-cadherin in the cervical cancerous cells.

We used the ROC curves to investigate the predictive power of Snail1 as a diagnostic biomarker for CC. The uttermost AUC value emerged for Snail1: 1(95% CI: 1-1) in comparing CC and the healthy groups with a sensitivity of 100.0% and specificity of 100.0%. Moreover, the highest AUC value and sensitivity were obtained in comparing precancerous cancer and the healthy groups and precancerous cancer compared with the cancer groups. Therefore, the expression level of Snail1 may be tracked as a tissue marker for CC to its prognosis and diagnosis. The overexpression of ZEB1 and Snail1 and the regulation of E-cadherin expression are closely related to the differentiation

status and the invasive capacity of cervical carcinoma. In cervical carcinoma tissue, ZEB1 and Snail1 are highly expressed, which may further enhance the regulation of the expression of E-cadherin. This may cause an increase in malignancy and invasiveness of CC cells. This study also demonstrated that the critical role of ZEB1, Snail1, and E-cadherin in cervical carcinoma, which provides a theoretical basis for the purpose of gene therapy in the metastasis of cervical carcinoma.

Ideal tumor markers should have high sensitivity and specificity to differentiate cancer patients from healthy subjects. They should be secreted into the circulation and activities at a concentration proportional to tumor burden.

Conclusion

The expression level of several cell factors changes in CC cells. The identification of these factors is beneficial to cancer prognosis and early treatment. We found that up-regulation of the tissue cell factors including Snail1, ZEB-1, and down-regulation of E-cadherin are considerable biomarkers for the diagnosis of the HPV associated CC. Finally, we found out a correlation between expression levels of ZEB-1 and E-cadherin in healthy and cancer groups, which were in agreement with other previous reports. The molecular biomarker Snail1 may be helpful to early diagnosis and prognosis of CC in the HPV-infected human populations. Then Snail1 can be used for early detection of CC.

Ethical considerations

Ethical issues (Including plagiarism, informed consent, misconduct, data fabrication and/or falsification, double publication and/or submission, redundancy, etc.) have been completely observed by the authors.

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Conflict of interest

The authors declare that there is no conflict of interest.

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