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Diagnostic Value of SOX2 as A Biomarker for Early-Stage Esophageal Squamous Cell Carcinoma

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Abstract **Original Research Article**

Background: Esophageal squamous cell carcinoma (ESCC), the most common subtype of esophageal cancer, has a complicated and progressive multistep process, from precancerous lesions, hyperplasia, low-grade intraepithelial neoplasia (LGIEN), to high-grade intraepithelial neoplasia (HGIEN), then to ESCC *in situ*, and eventually to invasive ESCC. Early detection and timely intervention are important for a good outcome. Traditional pathology is difficult to identify precancerous lesions and early ESCC, so it is necessary to search for other IHC specific molecules for early clinical diagnosis.This study aimed to determine if immunohistochemistry (IHC) for SRY-related HMG box 2 (SOX2) protein coupled with H&E staining could improve the accuracy of the early pathological diagnosis of ESCC. ***Methods:*** Esophageal tissue samples from 123 patients who underwent endoscopic submucosal dissection (ESD) and were pathologically diagnosed as early ESCC or precancerous lesions were used to determine SOX2 protein expression using EnVision, a standard two-step IHC technique. ***Results:*** IHC staining for SOX2 protein displayed distinct expression features in esophageal epithelial cells of the basal layer in different stages of premalignant lesions and early ESCC. In addition, we compared the value of the pathological diagnosis between H&E staining alone and in combination with IHC staining for SOX2 protein, and found that the detection rates increased from 15.4% to 22.7% for early ESCC, and 12.2% to 17.1% for micro-invasive ESCC. ***Conclusion:*** Our results suggest that the conventional pathological technique of H&E staining in combination with IHC assessment of SOX2 protein expression in basal layer esophageal epithelial cells could be a better diagnostic modality for the pathological diagnosis of precancerous lesions and early ESCC.

Keywords: Esophageal cancer; esophageal squamous cell carcinoma; H&E staining; SOX2.

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the most common form of esophageal cancer, accounting for approximately 90% of diagnosed esophageal cancer cases worldwide [1]. The morbidity and mortality rates of ESCC are particularly high in some regions of China and other countries. Although progress in the diagnostic and therapeutic modalities, including endoscopic submucosal dissection (ESD), have been made over recent years, the 5-year survival rate of patients with ESCC remains less than 15%, mainly due to challenges in the early detection and diagnosis of ESCC as a large proportion of the cancers in the early stage is found to be asymptomatic [1, 2]. Of note, in patients who were diagnosed and intervened early, the poor prognoses of ESCC patients were markedly improved, with a 5-year survival rate as high as 90% [3]. Therefore, early detection, diagnosis, and timely surgical intervention are critical for a good prognosis. Malignant transformation of ESCC is a complicated and progressive process, usually from hyperplasia, low-grade intraepithelial neoplasia (LGIEN), to high-grade intraepithelial neoplasia (HGIEN), then to ESCC *in situ*, and eventually to invasive ESCC. However, histological examination with H&E alone has turned out to be difficult to differentially diagnose HGIEN and early ESCC in clinical practice. Thus, there is an urgent need to explore a more accurate and reliable modality for differentiating early ESCC from HGIEN. While some authors [4,5] have also discussed the immunohistochemical and gene detection of SOX2 protein in normal tissues, tissues with simple hyperplasia, dysplastic tissues (IEN), and ESCC tissues , has also explored the expression of the protein in the tissues of esophageal squamous cell carcinoma.

The esophagus itself does not contain the basal cell layer in the myocortex of the breast cancer or the carcinoma of the prostate. Because of the anatomical position of the esophagus, the special structure of the embryo and histology, the lymphatic drainage is more complicated and early infiltration appears earlier. Therefore, the pathological diagnosis of ESD is not only necessary to determine the nature of the lesion, the degree of differentiation and the state of the cutting edge, but also to determine the exact depth of infiltration to determine whether the lesion is a cure. Accurate measurement of the depth of submucosal infiltration of cancer is an important factor in determining whether patients need additional surgical treatment. However, small nesting cell masses are often seen in the propria and deeper parts of the esophageal specimens after ESD, and it is difficult to judge whether there is an early infiltration of esophageal cancer.

The clinical diagnosis is mainly based on conventional HE staining. It is difficult to judge the infiltration of qualitative primary foci and the depth of submucosal infiltration, and it is lack of objective criteria for identifying HGIEN and early invasive carcinoma of the esophagus. At present, there is a lack of specific markers to identify those minor malignant lesions, but early lesions with more active treatment are needed.

SRY-related HMG box 2 (SOX2) is a transcription factor with a key role primarily in the pluripotency of stem cells, early development, and self-renewal [6]. In 2009, a novel oncogenic role for SOX2 was initially unveiled [7]; thereafter, the overexpression of SOX2 has been reported in many forms of malignant tumors, including lung and esophageal cancers [7-9]. Most recently, Yokota and colleagues have reported that all types of ESCC cultured cells express SOX2, and more than 87.5% of ESCC tissues from human subjects express SOX2 protein [10].

In the present study, we aim to examine the diagnostic value of SOX2 as a biomarker for ESCC in a Chinese population. The findings obtained from this study may provide a scientific basis by which a better diagnostic modality for ESCC could be developed and, therefore, may ultimately improve the clinical outcomes of ESCC patients.

SUBJECTS AND METHODS

Study subjects

A total of 123 patients, including 72 males and 51 females with a mean age of 63 years old, who underwent ESD and were diagnosed as having early ESCC or precursor lesions by two expert pathologists at the Diagnostic Center during the period between May 2012 and December 2017 in the First Affiliated Hospital of Jilin University (Changchun, Jilin, China) were examined in this study. The study protocol was reviewed and approved by the Ethics Committee of the First Affiliated Hospital of Jilin University, with the need for a written informed consent was waived due to the nature of the present study.

Criteria for pathological interpretation

According to the WHO classification of tumours of the digestive system (2010 edition), these lesions were divided into LGIEN, HGIEN, and infiltrative squamous cell carcinoma.

Histology and IHC

The esophageal sections were obtained during the ESD. Standard H&E staining was conducted for histological assessment, while IHC was performed to examine the SOX2 protein in the cells of the esophageal sections using EnVision, a standard two-step IHC technique developed by Dako Cooperation (Carpinteria, CA, USA). In brief, paraffin sections of esophageal tissues were dewaxed, hydrated, and washed. The slides were initially incubated with rabbit monoclonal SOX2 (Dako) primary antibody for 1 h at room temperature. A working solution with a rabbit anti-mouse polyclonal secondary antibody was added, and the reaction proceeded for another 1 h at room temperature. After washing with phosphate-buffered saline (0.01 M, pH 7.5) for a minimum of three times, the resulting slides were visualized with diaminobenzidine, counterstained with H&E, and mounted in a neutral resin. SOX2 protein expression was analyzed under a microscope, according to the manufacturer's instructions.

RESULTS

SOX2 protein expression in epithelial cells of the basal layer in normal esophageal tissues (Fig. 1)

The squamous epithelial of the normal epithelial mucosa is flat, with its lamina propria papillae projecting into the epithelium. We initially examined whether SOX2 protein was expressed in normal esophageal squamous epithelial cells in a total of 64 normal esophageal tissues. As shown in Fig. 1, SOX2 protein was observed predominantly in epithelial cells of the lamina propria and occasionally in corneal epithelial cells in the normal esophageal squamous epithelium, which is the location of epithelial stem cells. However, SOX protein expression was undetectable in the epithelial cells located above the basal layer in the normal esophageal squamous epithelium. This result supported the hypothesis that epithelial stem cell-associated factors are activated in cancer cells.

SOX2 protein expression in esophageal epithelial cells of the basal layer in different stages of premalignant lesions (Table 2)

The majority of the premalignant changes of ESCC or precancerous lesions of the esophageal squamous epithelium were originated from the transformation of the basal layer of the epithelium, accompanied by the appearance of alterations in the flat basal layer. After detecting the aberrant expression of

SOX2 protein in the basal layer of the esophageal squamous epithelium, we sought to determine if SOX2 could be a diagnostically useful marker to distinguish premalignant lesions from ESCC. To test this hypothesis, we examined the SOX protein expression levels in the ESD specimens in different stages from premalignant lesions to early ESCC.

As shown in (Fig.2), we detected SOX2 protein expression in the basal layer of the ESD specimens from patients with LGIEN (n = 86), which were mainly characterized by an enhanced proliferation of squamous epithelial cells in the basal layer that became crowded with a clear lamina propria. In addition, the squamous epithelial cells in the basal layer were arranged in an orderly manner, and the aberrant cellular changes were seen beneath the surface. The observations from IHC analysis of SOX2 protein indicated that the aberrant epithelial cells were dominantly presented in the basal layer in the study subjects, who were morphologically diagnosed as having LGIEN.

Subsequently, we examined the expression of SOX2 protein in the basal cell layer of the ESD specimens from patients with HGIEN (n = 161). As shown in (Fig.3), the following main features of epithelial cells in the basal layer were identified: enlargement of the nucleus, increase in chromatin, disappearance of nuclear polarity, and completely positive expression of SOX2 protein. Moreover, the squamous epithelial cells became nearly cancerous, with the abnormal changes expanded into the surface of the epithelium. The basal layer appeared as a wave-shape structure, and the expanded basal cells grew to the propria, whereas the basement membrane was usually integrated in HGIEN.

Different from the precancerous lesions, the early invasive ESCC tissues (n = 62) exhibited the following characteristics as observed by IHC analysis of SOX2 protein expression. The cancerous cells in the tumor cell nests penetrated the membrane to enter into the lamina propria, and the basement membrane was no longer integrated in early invasive ESCC. In combination with IHC analysis of SOX2 protein expression, the detection rate of early invasive ESCC was increased from 15.4% to 22.7% (Table 2).

SOX2 protein expression in esophageal epithelial cells of the basal layer in invasive ESCC (Fig.4)

Of 69 invasive ESCC cases diagnosed by histological analysis with H&E staining, the basal cell layer completed disappeared histologically, and cells aberrantly expressed SOX2 protein. Furthermore, esophageal squamous epithelial cancer cell nests were observed in a proportion of invasive ESCC specimens (6/69), in which the basal cell layer was completely undetectable.

SOX2 protein expression in esophageal epithelial cells of the basal layer in micro-invasive ESCC (Fig.5)

A total of 18 micro-invasive ESCC cases were diagnosed following histological analysis with H&E staining, of which 8 exhibited invasions into the muscle layer with cancer cells diffusely scattered, and 10 showed small cancer cell clusters without invasion into the vertical margin. After combining with IHC staining for SOX2 protein expression, the detection rate of micro-invasive ESCC was increased from 12.2% to 17.1% (Table 2). The majority of micro-invasive ESCC occurred in poorly differentiated ESCC subjects.

Figure 1 Immunohistochemical (IHC) analysis of SOX2 protein expression in the squamous epithelium of normal esophageal tissues. IHC was conducted to determine SOX2 protein expression in the squamous epithelium of normal esophageal specimens using EnVision, according to the instructions provided by the manufacturer (Dako Cooperation, Carpinteria, CA, USA). SOX2 protein was predominantly expressed in epithelial cells of the lamina propria and occasionally in corneal epithelial cells in the normal esophageal squamous epithelium, whereas SOX protein expression was undetectable in the epithelial cells located above the basal layer in the normal esophageal squamous epithelium. (A) IHC staining for SOX2 at a lower magnification; (B) IHC staining for SOX2 at a higher magnification.

Figure 2 Immunohistochemical (IHC) analysis of SOX2 protein expression in the squamous epithelium of esophageal specimens with low-grade intraepithelial neoplasia (LGIEN). IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens with LGIEN using EnVision, according to the instructions provided by the manufacturer (Dako Cooperation, Carpinteria, CA, USA). SOX2 protein expression was detected in the basal layer of the ESD specimens from patients who were morphologically diagnosed with LGIEN. The proliferation of the squamous epithelial cells in the basal layer was enhanced, resulting in visible crowding of cells. (A) IHC staining for SOX2 at a lower magnification; (B) IHC staining for SOX2 at a higher magnification.

Figure 3 Immunohistochemical (IHC) analysis of SOX2 protein expression in the squamous epithelium of esophageal specimens with high-grade intraepithelial neoplasia (HGIEN) and early invasive ESCC. IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens with precancerous HGIEN lesions (A–D) and early invasive ESCC (E–H) using EnVision, according to the manufacturer's protocol (Dako Cooperation, Carpinteria, CA, USA). (A) & (C) IHC staining for SOX2 in specimens with HGIEN at a lower magnification; (B) & (D) IHC staining for SOX2 in specimens with HGIEN at a higher magnification; (E) & (G) IHC staining for SOX2 in early invasive ESCC

specimens at a lower magnification; (F) & (H) IHC staining for SOX2 in early invasive ESCC specimens at a higher magnification. In HGIEN, an enlarged nucleus, an increase in chromatin, the disappearance of nuclear polarity, and completely positive expression of SOX2 protein were visualized. In early invasive ESCC, the cancerous cells in the tumor cell nests penetrated the membrane to enter into the lamina propria. In addition, the basement membrane was no longer integrated as visualized under a microscope.

Figure 4 Hematoxylin-eosin (H&E) staining and immunohistochemical (IHC) analysis for SOX2 protein expression in the squamous epithelium of esophageal specimens of invasive ESCC. H&E and IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens of invasive ESCC using EnVision, according to the manufacturer's instructions (Dako Cooperation, Carpinteria, CA, USA). **(A)** H&E staining at a lower magnification; **(B)** IHC staining for SOX2 protein at a lower magnification; **(C)** H&E staining at a higher magnification; **(D)** IHC staining for SOX2 protein at a higher magnification.

Figure 5 Hematoxylin-eosin (H&E) staining and immunohistochemical (IHC) analysis for SOX2 protein expression in the squamous epithelium of esophageal specimens of micro-invasive ESCC. H&E and IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens of micro-invasive ESCC using EnVision, according to the manufacturer's instructions (Dako Cooperation, Carpinteria, CA, USA). (A) H&E staining at a lower magnification; (B) IHC staining for SOX2 protein at a lower magnification; (C) H&E staining at a higher magnification; (D) IHC staining for SOX2 protein at a higher magnification.

Figure 6 Schematic diagram of the diagnosis based on alterations in SOX2 protein expression and the basal layer. The pathological diagnosis of precancerous lesions and ESCC was made according to hematoxylin-eosin staining alone and in combination with the immunohistochemical (IHC) staining for SOX2 protein in the squamous epithelium of the esophageal specimens, suggesting that the conventional pathological method in combination with IHC staining of SOX2 protein could be a better modality for the pathological diagnosis of precancerous lesions and early ESCC.

Table-1: Changes of podoplanin and the basal layer in esophageal squamous cell carcinoma And precancerous lesions

Basal layer	Normal	LGIEN	HGIEN	Early invasive ESCC	Invasive ESCC	Micro-invasive ESCC
Non-complete	64	86	161	62	69	18
Compelte	64	81	99	53	5	2
		5	62	9	64	16

NOTE: LGIEN, low-grade intraepithelial neoplasia; HGIEN, high-grade intraepithelial neoplasia

Table-2: Hematoxylin-eosin and immunohistochemical staining for SOX2 in the 123 ESD specimens

Staining	LGIEN	HGIEN	Early invasive ESCC	Invasive ESCC	Micro-invasive ESCC
H&E(+)	33	60	19	11	15
H&E(-)	0	9	9	0	6
SOX2(+)	33 (27%)	51 (42%)	28 (22.7%)	11 (9%)	2 (17.1%)

NOTE: ESD, endoscopic submucosal dissection; LGIEN, low-grade intraepithelial neoplasia; HGIEN, high-grade intraepithelial neoplasia; ESCC, esophageal squamous cell carcinoma

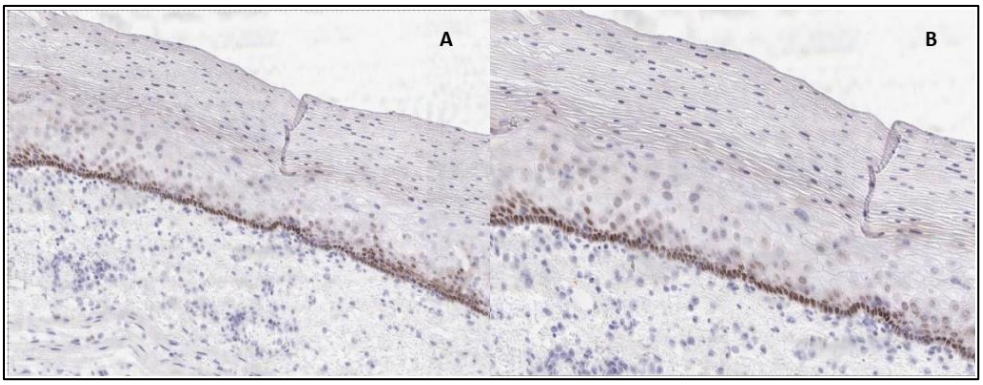


Fig-1: Immunohistochemical (IHC) analysis of SOX2 protein expression in the squamous epithelium of normal esophageal tissues.

IHC was conducted to determine SOX2 protein expression in the squamous epithelium of normal esophageal specimens using EnVision, according to the instructions provided by the

manufacturer (Dako Cooperation, Carpinteria, CA, USA). SOX2 protein was predominantly expressed in epithelial cells of the lamina propria and occasionally in corneal epithelial cells in the normal esophageal squamous epithelium, whereas SOX protein expression was undetectable in the epithelial cells located above the basal layer in the normal esophageal squamous epithelium. **(A)** IHC staining for SOX2 at a lower magnification; **(B)** IHC staining for SOX2 at a higher magnification.

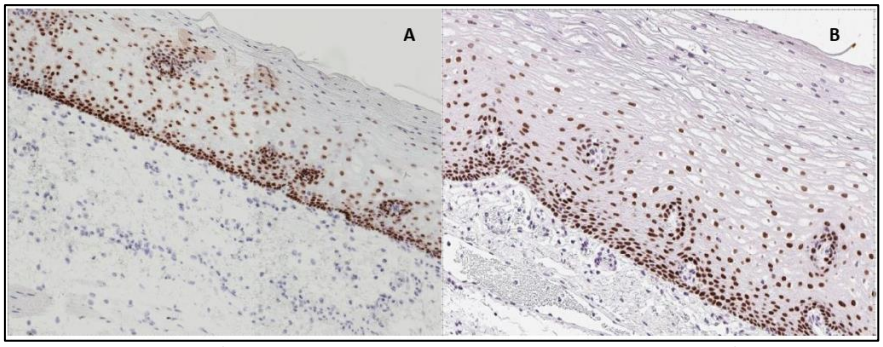
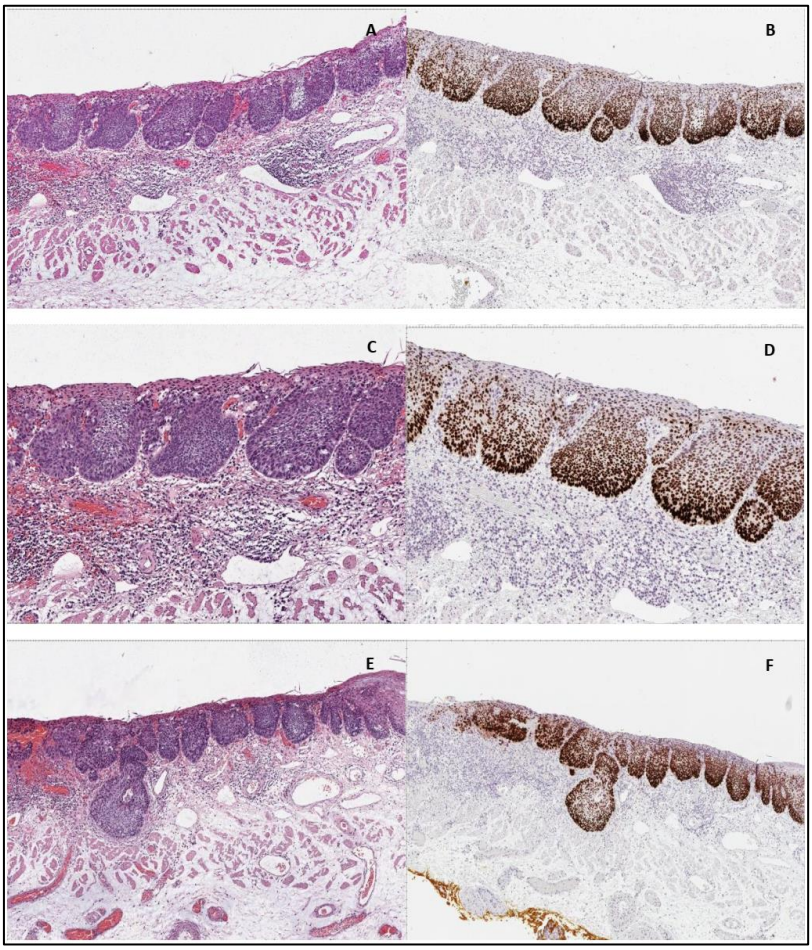


Fig-2: Immunohistochemical (IHC) analysis of SOX2 protein expression in the squamous epithelium of esophageal specimens with low-grade intraepithelial neoplasia (LGIEN).

IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens with LGIEN using EnVision, according to the instructions provided by the manufacturer (Dako Cooperation, Carpinteria, CA, USA). SOX2 protein expression was detected in the basal layer of the ESD specimens from patients who were morphologically diagnosed with LGIEN. The proliferation of the squamous epithelial cells in the basal layer was enhanced, resulting in visible crowding of cells. **(A)** IHC staining for SOX2 at a lower magnification; **(B)** IHC staining for SOX2 at a higher magnification.



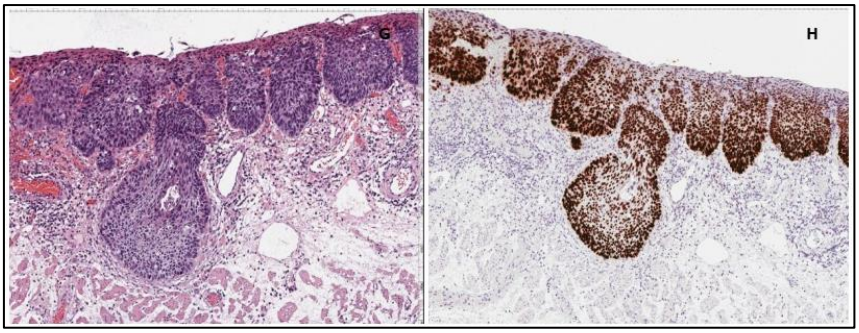


Fig-3: Immunohistochemical (IHC) analysis of SOX2 protein expression in the squamous epithelium of esophageal specimens with high-grade intraepithelial neoplasia (HGIEN) and early invasive ESCC.

IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens with precancerous HGIEN lesions (A–D) and early invasive ESCC (E–H) using EnVision, according to the manufacturer's protocol (Dako Cooperation, Carpinteria, CA, USA). (A) & (C) IHC staining for SOX2 in specimens with HGIEN at a lower magnification; (B) & (D) IHC staining for SOX2 in specimens with HGIEN at a higher magnification; (E) & (G) IHC staining for SOX2 in early invasive ESCC specimens at a lower magnification; (F) & (H) IHC staining for SOX2 in early invasive ESCC specimens at a higher magnification. In HGIEN, an enlarged nucleus, an increase in chromatin, the disappearance of nuclear polarity, and completely positive expression of SOX2 protein were visualized. In early invasive ESCC, the cancerous cells in the tumor cell nests penetrated the membrane to enter into the lamina propria. In addition, the basement membrane was no longer integrated as visualized under a microscope.

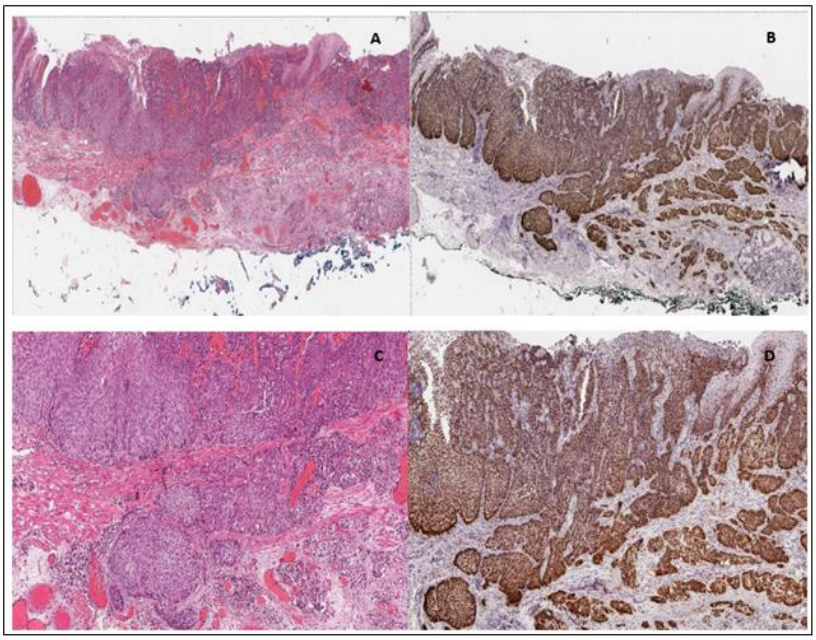


Fig-4: Hematoxylin-eosin (H&E) staining and immunohistochemical (IHC) analysis for SOX2 protein expression in the squamous epithelium of esophageal specimens of invasive ESCC.

H&E and IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens of invasive ESCC using EnVision, according to the manufacturer's instructions (Dako Cooperation, Carpinteria, CA, USA). **(A)** H&E staining at a lower magnification; **(B)** IHC staining for SOX2 protein at a lower magnification; **(C)** H&E staining at a higher magnification; **(D)** IHC staining for SOX2 protein at a higher magnification.

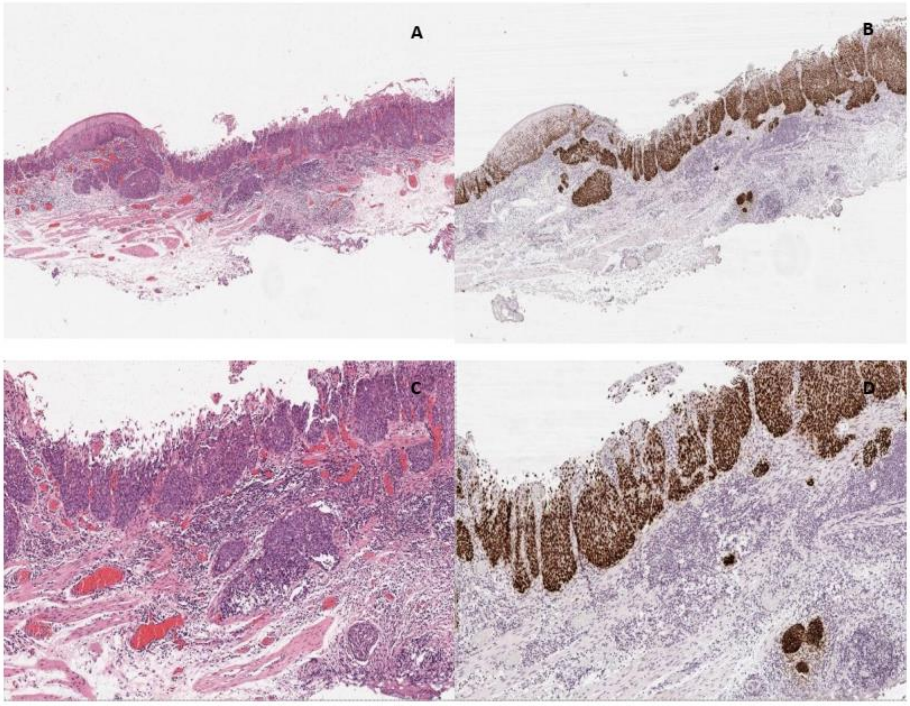


Fig-5: Hematoxylin-eosin (H&E) staining and immunohistochemical (IHC) analysis for SOX2 protein expression in the squamous epithelium of esophageal specimens of micro-invasive ESCC.

H&E and IHC staining for SOX2 protein was performed in the squamous epithelium of esophageal specimens of micro-invasive ESCC using EnVision, according to the manufacturer's instructions (Dako Cooperation, Carpinteria, CA, USA). **(A)** H&E staining at a lower magnification; **(B)** IHC staining for SOX2 protein at a lower magnification; **(C)** H&E staining at a higher magnification; **(D)** IHC staining for SOX2 protein at a higher magnification.

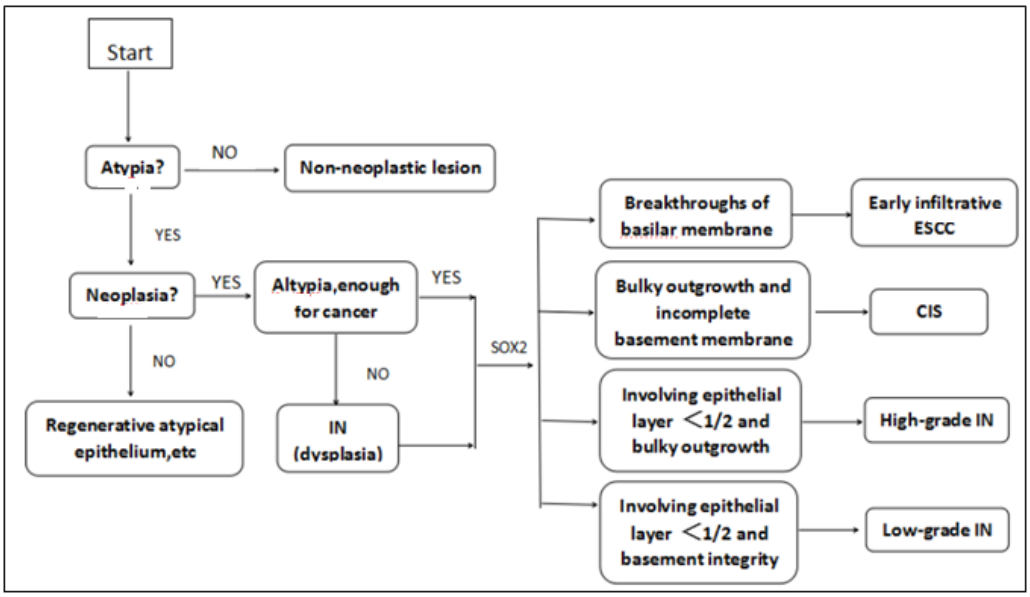


Fig-6: Schematic diagram of the diagnosis based on alterations in SOX2 protein expression and the basal layer.

The pathological diagnosis of precancerous lesions and ESCC was made according to hematoxylin-eosin staining alone and in combination with the immunohistochemical (IHC) staining for SOX2 protein in the squamous epithelium of the esophageal specimens, suggesting that the conventional pathological method in combination with IHC staining of SOX2 protein could be a better modality for the pathological diagnosis of precancerous lesions and early ESCC.

DISCUSSION

Histologically, ESCC is the most prevalent form of esophageal cancer, and timely intervention largely relies on detection and diagnosis in the early stage. However, the current pathological diagnosis with H&E staining of ESD specimens has posed challenges to distinguish early ESCC from precancerous lesions [11]. To the best of our knowledge, this is the less study to evaluate the diagnostic value for conventional H&E staining coupled with IHC for SOX2 protein expression in the differential diagnosis and early detection of ESCC in esophageal tissue samples from a Chinese population. The present study of esophageal tissue samples of early ESCC and precancerous lesions had the following main novel findings: (1) SOX2 protein expression of esophageal epithelial cells in the basal layer exhibited distinct features in different stages of premalignant lesions and early ESCC; (2) The diagnostic accuracy and reliability of H&E staining in combination with IHC for SOX protein were improved; (3) Detection rates using the combination of H&E and IHC staining for SOX2 for early ESCC and micro-invasive ESCC were 22.7% and 17.1%, respectively, which were higher than 15.4% and 12.2% using H&E staining alone. These findings suggested that the conventional pathological technique of H&E staining in combination with IHC assessment of SOX2 expression in esophageal epithelial cells of the basal layer could be a better diagnostic modality for the pathological diagnosis of precancerous lesions and early ESCC.

The effective treatment of early ESCC has progressed in recent years. In fact, the 5-year survival rate of early ESCC patients who underwent the ESD procedure is as high as 100%. A successful resection by ESD will largely depend on an accurate and reliable evaluation of the depth of invasion. However, histological examination with H&E staining alone is challenging for pathologists to differentially diagnose HGIEN and early ESCC in clinical practice. In our study, SOX2 protein expression exhibited distinct features in the multistep process of ESCC malignant transformation: hyperplasia, LGIEN, HGIEN, early ESCC *in situ*, and invasive ESCC.

SOX2, a member of the SOX family located at chromosome 3q26, is an essential transcription factor for the maintenance of stem cells and the early development of adult mammalian tissues, as reported in a number of previous studies [12-15]. As a transcription factor, SOX2 exerts its multiple roles in mediating the development of embryos and tissues, self-renewal and cell differentiation, as well as determining the fate of embryonic stem cells mainly via the HMG domain binding to its target genes [16-20]. Recently, SOX2 has emerged as a potential oncogene, which is activated in ESCC and lung squamous cell carcinoma by recurrent 3q26.3 amplification [21]. Interestingly, the amplification of chromosome 3q26 is among the most common genetic alterations related to ESCC and lung squamous cell carcinoma [20, 21]. Chen and colleagues have investigated the mRNA and protein expression of the SOX2 gene in ESCC in comparison with normal esophageal mucosal tissues and have demonstrated that its aberrant overexpression is associated with invasion and malignant transformation of ESCC [22]. These findings provide scientific evidence supporting the potentially novel role of SOX2 as a new biomarker for the detection and early diagnosis of ESCC.

This study also provided additional evidence that SOX2 protein expression and its features were altered in the multistep process of ESCC malignant transformation. In particular, in the LGIEN specimens, we observed that the aberrant cellular changes were restricted to beneath the surface with an intact lamina propria, while the HGIEN specimens showed different features with cancerous cells predominantly in the epithelium and aberrant cells entered into the propria. In early invasive ESCC, we observed that the cancerous cells in the tumor cell nests entered into the lamina propria, and the basement membrane was no longer integrated. Thus, the different features in the IHC analysis of SOX2 protein expression in esophageal epithelial cells of the basal layer were able to provide important information for a more accurate assessment of invasion.

Taken together, our results demonstrated that the results of the traditional pathological method of H&E staining in combination with IHC analysis of SOX2 protein expression in esophageal epithelial cells of the basal layer could be a better diagnostic modality for the pathological diagnosis of precancerous lesions and early ESCC (Fig.6).

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