Expression of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and skp2 increases during esophageal squamous cell cancer progression

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Abstract. Esophageal carcinoma is the sixth most common cause of cancer-related mortality in the world. Senescence and apoptosis are assumed to be two main mechanisms that inhibit age-related carcinogenesis. $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$, which are known to induce senescence by regulating G_1 cell cycle arrest, have been identified as senescence markers. However, the mechanism by which senescence and apoptosis causes neoplasia in esophageal squamous cell carcinoma (ESCC) has not been identified. In this study, 20 cases of normal esophageal tissues, 11 cases of esophageal intraepithelial dysplasia (EID) and 60 cases of ESCC were obtained and pathologically diagnosed. Immunohistochemical staining was performed to assess the expression of $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, skp2, bcl-2 and ki-67. The senescence markers $p14^{ARF}$ and $p16^{INK4a}$ were found to be expressed in 15 and 10% of the normal tissues, 82 and 73% of the EID cases and 100 and 88% of the ESCC cases, respectively. The expression of $p15^{INK4b}$ was low in normal tissues, while 92% of the ESCC specimens were diffusely and markedly stained, involving the basal, middle and upper portion of the epithelium. The nuclear expression markers ki-67 and skp2 were highly expressed in ESCC tissues (100 and 72%, respectively). bcl-2 was expressed weakly in normal tissues (10%) and demonstrated various staining patterns in carcinoma specimens (strong in 60%, negative in 40%). MI was 0.09% in normal tissues and 0.95% in the ESCC specimens. Apart from the increased proliferation in esophageal carcinogenesis, as indicated in the ki-67 and skp2 indices, there was an increased expression of senescence-associated molecular markers in the ESCC specimens, which indicates that the senescence pathway may be activated and become a part of cancer development. Of greatest interest to us was that, when compared with clinical information, the expression of the senescence markers was markedly high in the poorly differentiated specimens with lymph node metastasis, indicating that senescence markers may have diagnostic potential in clinical settings.

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Introduction

Esophageal carcinoma is an age-related neoplasm with a 5-year overall survival rate of less than 35% (1,2). Males more than 40 years of age are at the highest risk of esophageal carcinoma (3). Results of studies have revealed that age-related changes affect the molecular crosstalk between the stromal and epithelial cells, generating a more permissive environment for tumor growth and metastasis (4). Senescence may be one of the mechanism which is responsible for this age-related change in the microenvironment. The definition of senescence is a process that keeps the stable form of cell cycle arrest at the G_1 phase (5), as first reported by Hayflick (6). At the earlier stage of the process, senescence acts as an antiproliferative factor, which prevents tumorigenesis and leads to cell cycle arrest. However, if oncogenic mutations are present, these senescent cells may become immortal and initiate the development of additional genetic 'hits' in tumorigenesis (7-9). Cell cycle regulators within the retinoblastoma (Rb) and p53 pathways, including the cyclin-dependent kinase (CDK) and CDK inhibitor (CDKI) proteins, are the gatekeepers which

maintain the senescence program. $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ have been identified as CDKIs and act as senescence maintenance factors.

The INK4a/ARF locus (9p21) is crucial in the pathogenesis of several types of malignant disorders and encodes two unrelated cell cycle regulators, $p14^{ARF}$ and $p16^{INK4a}$, which have been regarded as significant senescence markers in clinical diagnosis (10). The $p16^{INK4a}$ gene encodes a p16 protein that binds competitively to CDK4 and, during G_1 phase, inhibits the interaction of CDK4 and cyclin D_1 to stimulate passage through the cell cycle (11-13). The p16 protein is often highly expressed in senescent cells in culture and is inactivated in a variety of human cancers. $p15^{INK4b}$ is located centromeric to the p16/p14 gene locus $p14^{ARF}$, which is a major tumor suppressor and causes cell cycle arrest through transforming growth factor β (14).

Previous data revealed, through staining, that $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ are abundantly expressed in premalignant lung cancers and have essentially no expression in malignant lung cancers, which indicates that senescence is inactivated in the proliferated lung epithelial cells of lung cancers (15). The same results have been observed in pancreatic, hepatic and breast cancers. Tumorigenesis is defined as an outcome of the accumulation of abnormal stroma facilitated by peripheral senescent fibroblasts and the inactivation of the senescence of proliferating epithelial cells. However, more recent evidence has revealed the high expression of the senescence markers $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and DCR2 in prostate cancer epithelial cells, indicating that senescence continued to be activated in these proliferating epithelial cells.

A balance between proliferation and senescence, instead of the inactivity of senescence only, appears to decide the fate of epithelial cells, converging on the probability of epithelial tumorigenesis.

Since the true mechanism involved in the process from senescence to tumor formation in epithelial cells remains elusive (16-19), in the present study we compared the expression of the senescence markers $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ and the proliferation markers bcl-2, ki-67 and skp2 in tissue blocks from normal esophageal epithelium, esophageal intraepithelial dysplasia (EID) and esophageal squamous cell carcinoma (ESCC). The purpose of the present study was to describe the predictors of biological activity in esophageal carcinoma and to provide evidence that may assist in clinical diagnosis.

Materials and methods

Sample selection. The preparation of the tissue slides was performed as previously described (20). Tissue blocks (n=91) were created from samples obtained from 80 patients from the People's Hospital of Sichuan (Sichuan, China). The samples included 20 cases of normal esophageal tissue, 11 cases of EID, 49 cases of low-grade ESCC and 11 cases of high-grade ESCC Specimens were obtained from patients (aged 42-74 years, mean age 57) who had not received either chemotherapy or irradiation prior to surgery. The basic information of the ESCC patients is summarized in Table I. All samples were diagnosed in duplicate by pathologists from Sichuan University (Sichuan, China) who observed the samples under the microscope and individually scored each slide. The use of human tissue in this study was approved by the Institutional Review Board.

Table I. Clinicopathological features of the ESCC patients.

Feature	n (%)
Gender	
Male	46 (77)
Female	14 (23)
Degree of differentiation	
Well	17 (28)
Poor	43 (72)
Lymph node metastasis	
Negative	28 (47)
Positive	32 (53)
TNM stage	
I	6 (10)
IIA	22 (37)
IIB	14 (23)
III	18 (30)

ESCC, esophageal squamous cell carcinoma.

Immunohistochemical staining. The streptavidin-peroxidase immunohistostaining method was performed as described previously (20). Briefly, samples were fixed in 10% formalin buffer and embedded in paraffin. Tissue sections (4 μm thick) were steamed in universal decloaker (Biocare Medical, Walnut Creek, CA, USA) for antigen retrieval, followed by 19 min protein-blocking (Biocare Medical). All slides were first incubated against $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ (1:300, for 1 h at room temperature; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and then treated with anti-rabbit secondary antibody (Biocare Medical) and horseradish peroxidase for 15 min each. The tissues were stained for 3 min with high sensitivity 3,3'-diaminobenzidine tetrahydrochloride, counterstained with hematoxylin, dehydrated and then mounted (21,22).

On the basis of the expression patterns in the esophageal epithelial cells, the expression of the $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, bcl-2, ki-67 and skp2 proteins was considered positive when any positive staining was observed in the epithelial cells. Immunostained tissue slides were semi-quantitatively scored by two independent pathologists (Z.J. and Y.F.) blinded to the clinical information. Quantification was performed using a four-score grading system.

Statistical analysis. All statistical analyses were performed using the SPSS 10.0 statistical software program (SPSS, Chicago, IL, USA). The χ^2 and Fisher's exact tests were used to assess the correlation between the expression of these biological markers and the clinical parameters of the patients. $P<0.05$ was considered to indicate a statistically significant result.

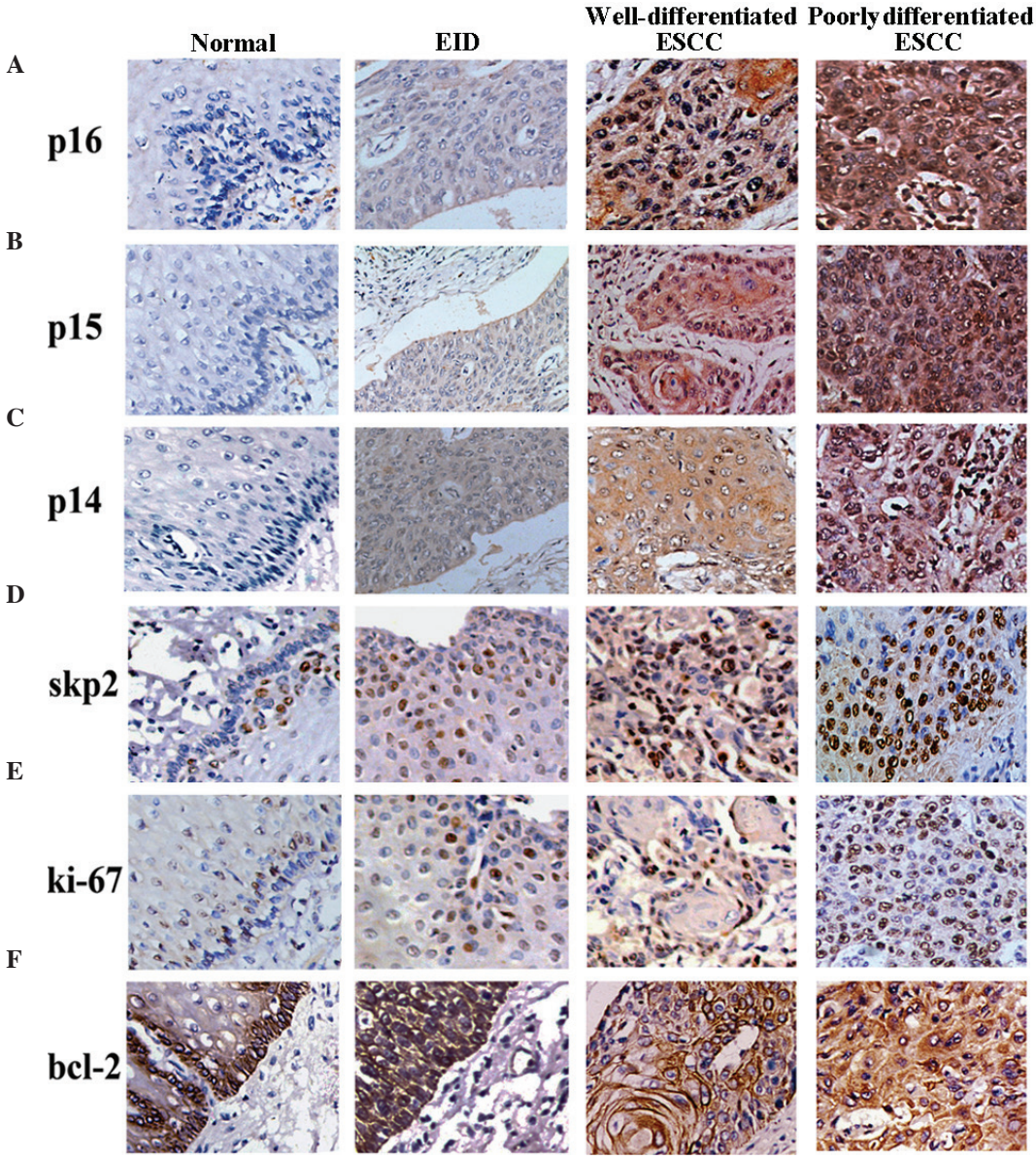

Figure 1. Expression of different markers in esophageal tissues (x40): Normal tissue, from top to bottom: (A) p16, negative; (B) p15, negative; (C) p14, negative; (D) skp2, negative; (E) ki-67, negative; (F) bcl-2, positive. EID, from top to bottom: (A) p16, positive; (B) p15, positive; (C) p14, negative; (D) skp2, positive, (E) ki-67, positive; (F) bcl-2, positive. Well-differentiated ESCC, from top to bottom: (A) p16, positive; (B) p15, positive; (C) p14, positive; (D) skp2, positive, (E) ki-67, positive; (F) bcl-2, positive. Poorly differentiated ESCC, from top to bottom: (A) p16, positive; (B) p15, positive; (C) p14, positive; (D) skp2, positive, (E) ki-67, positive; (F) bcl-2, positive. ESCC, esophageal squamous cell carcinoma; EID, esophageal intraepithelial dysplasia.

Results

As shown in Fig. 1, $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and bcl-2 expression was observed in the cytoplasm of esophageal epithelial cells with no evidence of nuclear staining, while skp2 and ki-67 showed predominant nuclear staining at different portion of epithelial.

As shown in Table II, overall, $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ showed almost complete negative expression in the normal esophageal epithelium, while marked positive expression was observed in the EID tissues and different pathological lesions of the ESCC tissues. More specifically, the $p14^{ARF}$ expression was negative in most of the normal esophageal tissues (85%), but diffuse positive staining was observed in the EID (82%) and ESCC (100%) tissues, including 9 cases of EID and 60 cases of ESCC. The $p16^{INK4a}$ staining was similar to that of $p15^{INK4b}$, with only 2 cases of normal esophageal tissue having weakly positive and most of the EID and ESCC tissues showing marked staining (73 and 88%, respectively). $p15^{INK4b}$ showed positive staining in 73% of the EID and 92% of the ESCC specimens, while no positive staining was observed in the normal esophageal tissue specimens.

A total of 46 male and 14 female ESCC patients participated in this study, 53% of whom had lymph node metastasis. The TNM stages of these patients are listed in Table I. As shown in Table III, a statistically significant correlation was observed between $p16^{INK4a}$ expression and the degree of differentiation. The poorly differentiated ESCC showed stronger $p16^{INK4a}$ staining compared with the well-differentiated ESCC ($P<0.05$). $p16^{INK4a}$ expression was observed in

Table II. Comparison of the immunohistochemical staining results for $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ in the ESCC tissue specimens and corresponding normal esophageal tissue specimens.

	$p14^{ARF}$			$p15^{INK4b}$			$p16^{INK4a}$		
	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value
Normal (20)	17	3	<0.001	20	0	<0.001	18	2	<0.001
EID (11)	2	9		3	8		3	8	
ESCC (60)	0	60		5	55		7	53	

ESCC, esophageal squamous cell carcinoma; EID, esophageal intraepithelial dysplasia.

Table III. Immunohistochemical expression of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ and their correlation with the clinicopathological parameters of the ESCC patients.

	$p14^{ARF}$			$p15^{INK4b}$			$p16^{INK4a}$		
	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value
Gender									
Male	0	46	-	2	44	0.141	4	42	0.410
Female	0	14		3	11		3	11	
Differentiation									
Well	0	17	-	3	14	0.261	5	12	0.025
Poor	0	43		2	41		2	41	
Lymph node metastasis									
No	0	28	-	4	24	0.275	4	24	0.851
Yes	0	32		1	31		3	29	
TNM stage									
I	0	6	-	2	4	0.088	0	6	1.000
IIA	0	22		2	20		3	19	
IIB	0	14		1	13		2	12	
III	0	18		0	18		2	16	

95% of the poorly differentiated ESCC and only 71% of the well-differentiated ESCC tissues. The positive expression of $p15^{INK4b}$ was observed in 95% cases of poorly differentiated ESCC and 82% cases of well-differentiated ESCC. The positive staining of $p14^{ARF}$ was found in all the ESCC cases. Although there is no statistical evidence to verify that cases with lymph mode metastasis had a more marked immunostaining of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$, the positive staining ratios are high in these specimens. There was no statistical correlation between the expression of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ between the genders and among the TNM stages.

As shown in Table IV, there was positive staining for the proliferation marker skp2 in 64% of the EID and 72% of the ESCC cases but only 10% of the normal esophageal tissues. The analysis of ki-67 revealed a markedly high level of expression in the EID (91%) and ESCC (100%) cases, while there was a low incidence of positive staining in the basal epithelial cells of the normal tissue (20%). The bcl-2 immunostaining revealed that 36 ESCC cases (60%) and only 2 (10%) of the normal esophageal tissues had positive bcl-2 expression. As shown in Table V, cells that showed a positive expression of bcl-2 were found in 15 (88%) of the well-differentiated and 21 (49%) of the poorly differentiated ESCC cases. The variant expression of bcl-2 may be due to the heterogeneity of the cells.

Discussion

The incidence of esophageal carcinoma rises exponentially with age, beginning from the fourth decade of life and peaking at the age of 75 years. Evidence suggests that cellular senescence is involved in causing organismal aging. Senescence, the process by which cells permanently withdraw from the cell cycle in response to diverse stress, may also contribute to this age-related disease (23). The senescence marker p16 was found to be upregulated with age in the progenitor cells of the mouse brain, bone marrow and pancreas. However, cellular senescence has two roles in cells. On one hand, senescence is associated with aging, which significantly increases the transformation of epithelial tumors. On the other hand, it is acknowledged that senescence is crucial in the prevention of

Table IV. Comparison of the immunohistochemical staining results for bcl-2, skp2 and ki-67 in the ESCC tissue specimens and corresponding normal esophageal tissue specimens.

	bcl-2			skp2			ki-67		
	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value
Normal (20)	18	2	<0.001	18	2	<0.001	16	4	<0.001
EID (11)	0	11		4	7		1	10	
ESCC (60)	24	36		17	43		0	60	

ESCC, esophageal squamous cell carcinoma; EID, esophageal intraepithelial dysplasia.

Table V. Immunohistochemical expression of bcl-2, ki-67 and skp2 and their correlation with the clinicopathological parameters of patients.

	bcl-2			ki-67			skp2		
	Negative	Positive	P-value	Negative	Positive	P-value	Negative	Positive	P-value
Gender									
Male	19	27	0.709	0	46	-	13	33	1.000
Female	5	9		0	14		4	10	
Differentiation									
Well	2	15	0.005	0	17	-	9	8	0.028
Poor	22	21		0	43		8	35	
Lymph node metastasis									
No	16	12	0.011	0	28	-	10	18	0.235
Yes	8	24		0	32		7	25	
TNM stage									
I	3	3	0.472	0	6	-	4	2	0.223
IIA	6	16		0	22		6	16	
IIB	7	7		0	14		3	11	
III	8	10		0	18		4	14	

cancer, which is achieved by inducing reversible proliferative arrest mediated by ARF/p53 and irreversible proliferative arrest mediated by the concomitant actions of INK4a/Rb and the p53 pathway (4,24). In this process, the main barrier to the overgrowth of cancer is the derepression of the INK4a/ARF locus (25,26). Overall, cellular senescence has two roles: cancer protection in the young and age promotion in the old. To the best of our knowledge, epithelial cells are the origin of most carcinomas, resulting from either the mutation of an oncogene in an epithelial cell or, in a paracrine manner, by an adjacent senescent fibroblast cell, converging on the promotion of epithelial tumorigenesis. However, whether senescence occurs in epithelial cells during transformation is unknown.

To date, evidence suggests that $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ are widely downregulated in several solid tumors (27), including hepatic (28), breast, urinary bladder, pancreatic and esophageal carcinomas and gliomas (29). The INK4a/ARF locus encodes two significant tumor suppressors, $p16^{INK4}$ and ARF, which share the same exons but encode different reading frames. $p16^{INK4a}$ is an inhibitor of CDK4 and CDK6 and acts by imposing a G_1 cell cycle arrest. The INK4a/ARF locus has been regarded as a controller of cancer evolution which is expressed at low levels in most tissues in young organisms but becomes derepressed with age. The inactivation of senescence markers, due to homozygous deletion or hypermethylation of the genes which encode them, may be a significant mechanism in the dysfunction of the Rb and p53 growth regulation pathways during ESCC development (30). However, it has been reported that the senescence pathway remains intact in a large number of prostate cancer and cervical squamous carcinoma cases (31) even if the ki-67 index indicates increased proliferation in these cancers. Zhang *et al* found that the senescence markers $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$ and DCR2 were expressed more frequently in prostate carcinomas than in benign tissues (32) and Meng *et al* identified activated senescence markers in colon cancer cells (33). Moreover, Schwarze identified an alteration in the p16/pRb pathway in the majority of primary prostate cancers *in vitro* (34). In our ESCC samples, the expression of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ increased during ESCC progression and was also associated with greater age, poor differentiation and,

to some degree, a high tumor stage. That is, although cancer cells generally lose their ability to undergo senescence and apoptosis, certain tumor cells trigger senescence in response to severe DNA damage or other stimuli (16,35). Therefore, senescence may play a role in cancer development.

skp2 is a member of the Skp1-Cullin-F-box protein (SCF) complex and considered to be a proto-oncogene, as its overexpression causes increased proliferation and metastasis, at least in part through increased p27 proteolysis (24). Wang *et al* found that phosphorylation at Ser72 is crucial for the ability of the skp2 protein to promote cell proliferation and tumorigenesis, through several complementary mechanisms (36). skp2 also induces cells to undergo a mitotic division cycle by degrading p27 in early G_1 (37). The results of our immunostaining assays revealed that skp2 is activated at the dysplasia stage of esophageal tumorigenesis and maintains a high level of expression in most ESCCs (38). The uninhibited proliferation eventually leads to the development of ESCC.

The nuclear antigen ki-67, which is markedly expressed in the S and M phases of the cell cycle (39-41), was used to estimating cell growth. bcl-2 is a proto-oncogene which has been identified as a biologically significant inhibitor of apoptosis, whose overexpression leads to cell proliferation (42-44). In our data, a high expression of ki-67 was associated with the high expression of skp2 and the low expression of bcl-2, which acts as a marker of proliferation (27,45). We detected the expression of bcl-2 in EID and ESCC tissues and half of the ESCC cases showed a paradoxical loss of bcl-2, which indicated a poor prognosis. The high expression of bcl-2 in EID showed its anti-apoptotic function by blocking p53-mediated G_1 arrest (46). The paradoxical loss of bcl-2 in ESCC may not be explained as a result of chemo-radiotherapy, since none of the cases in our database were treated prior to surgery. However, the same results were obtained in cervical and endothelial carcinoma, indicating that other mechanisms may be involved.

In general, the role of senescence in tumorigenesis attracts a great deal of attention, particularly concerning the functions of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$. According to previous studies, tumorigenesis in old age not only reflects the accumulation of oncogenic mutations but also stromal alteration (47). Campisi (48) addresses these issues as good citizen and bad neighbors. The epithelial cells in squamous carcinomas always show senescence and apoptosis. Senescence serves as a powerful barrier for tumorigenesis in epithelial cells. However, in ESCC, the high expression of $p14^{ARF}$, $p15^{INK4b}$ and $p16^{INK4a}$ provides evidence for the activation of senescence in the epithelial rather than stromal cells. Further studies are required to explain this phenomenon. However, the expression of markers of senescence and proliferation, including $p14^{ARF}$, $p15^{INK4b}$, $p16^{INK4a}$, bcl-2, skp2 and ki-67 in ESCC and promalignancies and a loss of their expression in normal tissue and neoplasm may augment routine histological diagnostic methods for difficult cases.

References

1. Pisani P, Parkin DM and Ferlay J: Estimates of the worldwide mortality from eighteen major cancers in 1985. Implications for prevention and projections of future burden. Int J Cancer 55: 891-903, 1993.
2. Sarbia M, Verreet P, Bittinger F, Dutkowski P, Heep H, Willers R and Gabbert HE: Basaloid squamous cell carcinoma of the esophagus: diagnosis and prognosis. Cancer 79: 1871-1878, 1997.
3. Hu J, Li R, Sun L and Ni Y: Influence of esophageal carcinoma operations on gastroesophageal reflux. Ann Thorac Surg 78: 298-302, 2004.
4. Shan W, Yang G and Liu J: The inflammatory network: bridging senescent stroma and epithelial tumorigenesis. Front Biosci 14: 4044-4057, 2009.
5. Stein GH and Dulic V: Origins of G1 arrest in senescent human fibroblasts. Bioessays 17: 537-543, 1995.
6. Hayflick L: Theories of biological aging. Exp Gerontol 20: 145-159, 1985.
7. Oller AR, Rastogi P, Morgenthaler S and Thilly WG: A statistical model to estimate variance in long term-low dose mutation assays: testing of the model in a human lymphoblastoid mutation ssay. Mutat Res 216: 149-161, 1989.
8. Jackson AL and Loeb LA: The mutation rate and cancer. Genetics 148: 1483-1490, 1998.
9. Bissell MJ and Labarge MA: Context, tissue plasticity, and cancer: are tumor stem cells also regulated by the microenvironment. Cancer Cell 7: 17-23, 2005.
10. Rosu-Myles M and Wolff L: p15Ink4b: dual function in myelopoiesis and inactivation in myeloid disease. Blood Cells Mol Dis 40: 406-409, 2008.
11. Zhang Z, Rosen DG, Yao JL, Huang J and Liu J: Expression of p14ARF, p15INK4b, p16INK4a, and DCR2 increases during prostate cancer progression. Mod Pathol 19: 1339-1343, 2006.
12. Zindy F, Quelle DE, Roussel MF and Sherr CJ: Expression of the p16INK4a tumor suppressor versus other INK4 family members during mouse development and aging. Oncogene 15: 203-211, 1997.
13. Shapiro GI, Edwards CD, Ewen ME and Rollins BJ: p16INK4A participates in a G1 arrest checkpoint in response to DNA damage. Mol Cell Biol 18: 378-387, 1998.
14. Stone S, Dayananth P, Jiang P, Weaver-Feldhaus JM, Tavtigian SV, Cannon-Albright L and Kamb A: Genomic structure, expression and mutational analysis of the P15 (MTS2) gene. Oncogene 11: 987-991, 1995.
15. Fukai K, Yokosuka O, Imazeki F, Tada M, Mikata R, Miyazaki M, Ochiai T and Saisho H: Methylation status of p14ARF, p15INK4b, and p16INK4a genes in human hepatocellular carcinoma. Liver Int 25: 1209-1216, 2005.
16. Collado M, Blasco MA and Serrano M: Cellular senescence in cancer and aging. Cell 130: 223-233, 2007.
17. Rinehart CA and Torti VR: Aging and cancer: the role of stromal interactions with epithelial cells. Mol Carcinog 18: 187-192, 1997.
18. Beck JC, Hosick HL and Watkins BA: Growth of epithelium from a preneoplastic mammary outgrowth in response to mammary adipose tissue. In Vitro Cell Dev Biol 25: 409-418, 1989.
19. Hayashi N and Cunha GR: Mesenchyme-induced changes in the neoplastic characteristics of the Dunning prostatic adenocarcinoma. Cancer Res 51: 4924-4930, 1991.
20. Li TY, Xu LY, Wu ZY, Liao LD, Shen JH, Xu XE, Du ZP, Zhao Q and Li EM: Reduced nuclear and ectopic cytoplasmic expression of lysyl oxidase-like 2 is associated with lymph node metastasis and poor prognosis in esophageal squamous cell carcinoma. Hum Pathol Dec 26, 2011 (Epub ahead of print).
21. Yang GZ, Li L, Ding HY and Zhou JS: Cyclooxygenase-2 is over-expressed in Chinese esophageal squamous cell carcinoma, and correlated with NF-kappaB: an immunohistochemical study. Exp Mol Pathol 79: 214-218, 2005.
22. Grace VM, Shalini JV, lekha TT, Devaraj SN and Devaraj H: Co-overexpression of p53 and bcl-2 proteins in HPV-induced squamous cell carcinoma of the uterine cervix. Gynecol Oncol 91: 51-58, 2003.
23. DePinho RA: The age of cancer. Nature 408: 248-254, 2000.
24. Zheng WQ, Zheng JM, Ma R, Meng FF and Ni CR: Relationship between levels of Skp2 and P27 in breast carcinomas and possible role of Skp2 as targeted therapy. Steroids 70: 770-774, 2005.
25. Gil J and Peters G: Regulation of the INK4b-ARF-INK4a tumour suppressor locus: all for one or one for all. Nat Rev Mol Cell Biol 7: 667-677, 2006.
26. Kim WY and Sharpless NE: The regulation of INK4/ARF in cancer and aging. Cell 127: 265-275, 2006.

27. Collado M, Gil J, Efeyan A, Guerra C, Schuhmacher AJ, Barradas M, Benguria A, Zaballos A, Flores JM, Barbacid M, *et al*: Tumour biology: senescence in premalignant tumours. Nature 436: 642, 2005.
28. Jin M, Piao Z, Kim NG, Park C, Shin EC, Park JH, Jung HJ, Kim CG and Kim H: p16 is a major inactivation target in hepatocellular carcinoma. Cancer 89: 60-68, 2000.
29. Forbes S, Clements J, Dawson E, Bamford S, Webb T, Dogan A, Flanagan A, Teague J, Wooster R, Futreal PA and Stratton MR: COSMIC 2005. Br J Cancer 94: 318-322, 2006.
30. Esteller M, Corn PG, Baylin SB and Herman JG: A gene hypermethylation profile of human cancer. Cancer Res 61: 3225-3229, 2001.
31. Sano T, Masuda N, Oyama T and Nakajima T: Overexpression of p16 and p14ARF is associated with human papillomavirus infection in cervical squamous cell carcinoma and dysplasia. Pathol Int 52: 375-383, 2002.
32. Li TY, Xu LY, Wu ZY, Liao LD, Shen JH, Xu XE, Du ZP, Zhao Q and Li EM: Reduced nuclear and ectopic cytoplasmic expression of lysyl oxidase-like 2 is associated with lymph node metastasis and poor prognosis in esophageal squamous cell carcinoma. Hum Pathol: Dec 26, 2011 (Epub ahead of print).
33. Meng RD, McDonald ER 3rd, Sheikh MS, Fornace AJ Jr and El-Deiry WS: The TRAIL decoy receptor TRUNDD (DcR2, TRAIL-R4) is induced by adenovirus-p53 overexpression and can delay TRAIL-, p53-, and KILLER/DR5-dependent colon cancer apoptosis. Mol Ther 1: 130-144, 2000.
34. Schwarze SR, Shi Y, Fu VX, Watson PA and Jarrard DF: Role of cyclin-dependent kinase inhibitors in the growth arrest at senescence in human prostate epithelial and uroepithelial cells. Oncogene 20: 8184-8192, 2001.
35. Roberson RS, Kussick SJ, Vallieres E, Chen SY and Wu DY: Escape from therapy-induced accelerated cellular senescence in p53-null lung cancer cells and in human lung cancers. Cancer Res 65: 2795-2803, 2005.
36. Wang Z, Gao D, Fukushima H, Inuzuka H, Liu P, Wan L, Sarkar FH and Wei W: Skp2: A novel potential therapeutic target for prostate cancer. Biochim Biophys Acta 1825: 11-7, 2012.
37. Mani A and Gelmann EP: The ubiquitin-proteasome pathway and its role in cancer. J Clin Oncol 23: 4776-4789, 2005.
38. Dowen SE, Scott A, Mukherjee G and Stanley MA: Overexpression of Skp2 in carcinoma of the cervix does not correlate inversely with p27 expression. Int J Cancer 105: 326-330, 2003.
39. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U and Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 133: 1710-1715, 1984.
40. Gerdes J: Ki-67 and other proliferation markers useful for immunohistological diagnostic and prognostic evaluations in human malignancies. Semin Cancer Biol 1: 199-206, 1990.
41. Gerdes J, Li L, Schlueter C, Duchrow M, Wohlenberg C, Gerlach C, Stahmer I, Kloth S, Brandt E and Flad HD: Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. Am J Pathol 138: 867-873, 1991.
42. Reed JC: Bcl-2 and the regulation of programmed cell death. J Cell Biol 124: 1-6, 1994.
43. Singh BB, Chandler FW Jr, Whitaker SB and Forbes-Nelson AE: Immunohistochemical evaluation of bcl-2 oncoprotein in oral dysplasia and carcinoma. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 85: 692-698, 1998.
44. Vaux DL, Cory S and Adams JM: Bcl-2 gene promotes haemopoietic cell survival and cooperates with c-myc to immortalize pre-B cells. Nature 335: 440-442, 1988.
45. Ecker K and Hengst L: Skp2: caught in the Akt. Nat Cell Biol 11: 377-379, 2009.
46. Guillouf C, Grana X, Selvakumaran M, De Luca A, Giordano A, Hoffman B and Liebermann DA: Dissection of the genetic programs of p53-mediated G1 growth arrest and apoptosis: blocking p53-induced apoptosis unmasks G1 arrest. Blood 85: 2691-2698, 1995.
47. Bishop JM: Cancer: the rise of the genetic paradigm. Genes Dev 9: 1309-1315, 1995.
48. Campisi J: Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbours. Cell 120: 513-522, 2005.