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• ESOPHAGEAL CANCER •

The abnormal expression of retinoic acid receptor-β, *p*53 and Ki67 protein in normal, premalignant and malignant esophageal tissues

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Abstract

AIM: **Esophageal cancer remains a significant health problem worldwide. It is important to investigate alterations in expression of retinoic acid receptor-β, *p*53 and Ki67 proteins in esophageal carcinogenesis.**

METHODS: **To find biomarkers for early identification of esophageal cancer, we analyzed the retinoic acid receptor-β, *p*53 protein and the proliferation marker Ki67 in surgical specimens of normal, mildly, and severely dysplastic and malignant esophageal tissues by *in situ* hybridization of RNA and immunohistochemistry.**

RESULTS: **RAR-β was expressed in 94.3%(33/35) of normal mucosae, 67.8%(19/28) of the mild, 58.1% (18/31) of the severe lesions and 53.2%(116/218) of tumor samples. RAR-β mRNA was expressed in 62.7%(42/67), 55.1%(43/78) and 29.2%(7/24) of well, moderated and poorly differentiated SSCs. The *p*53 and Ki67 proteins were 5.9%(2/34) of the normal mucosa. P53 and Ki67 stained positively in 10.7% (3/28) and 21.4% (6/28) of mild dysplasia, and 51.6% (16/31) and 58.1% (18/31) of severely dysplasia respectively. Samples from esophageal cancer showed no higher levers of *p*53 and Ki67 expression than seen in severely dysplastic lesions. There was significant difference of RAR-β, *p*53 and Ki67 expression between normal mucosa and dysplatic tissue or esophageal cancer.**

CONCLUSION: **Loss of RAR-β expression and accumulation of *p*53 and Ki67 proteins may serve as biomarkers for early identification of esophageal cancer in the high-risk populations.**

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INTRODUCTION

Esophageal cancer remains a significant health problem in the world because the 5-year survival rate is low[1]. Chemoprevention is very important for the esophageal cancer. But it remains a problem to identify the premalignant esophageal tissues in the people with high risk. In this study, we investigate the expression of retinoic acid receptor-â (RAR-â), Ki67 and *P*53 protein in normal, premalignant, and maligant esophageal tissues to find biomarkers for early identification of the disease.

Retinoids, a group of nature and synthetic analogues of vitamin A, can modulate cell growth and differentiation[2]. Retinoids are known to exert their biological effects by binding to specific nuclear retinoid receptors, which belong to a steroid/thyroid hormone-receptor superfamily. The nuclear retinoid receptors are divided into retinoic acid receptors (RARs) and retinoid X receptors (RXRs), both with three subtypes (α,β,γ)[3]. Retinoids can suppress or reverse epithelial carcinogenesis and to prevent the development of invasive cancer in many animal models[4-6]. In a Chinese clinical trial using the synthetic retinoid N-4-(ethoxycarbophenyl) retinamide, the incidence of cancer in the treatment group with severe esophageal dysplasia was 43.2%, that was lower than of the group treated with placebo[7]. Our previous study demonstrated that all-trans RA can induce esophageal cancer cell lines to undergo apoptosis, which was associated with the expression and upregulation of RAR-β[8]. In this study, we tried to explore the earliest loss of RAR-β expression.

Deletion or mutation of tumor suppressor genes may be a key event in carcinogenesis. The human *p*53 gene encodes a nuclear protein that may be vital in the regulatory control of cell proliferation[9,10]. Loss of wild-type *p*53 function is considered to be a key event in the induction of malignant transformation in many cancers,including esophageal cancers[11-14].

Ki67 is a nuclear antigen expressed during G1, S, M and G2 periods of cell cycle. The expression level of Ki67 indicated the status of cell proliferation. Some studies have showed that Ki67 protein could be a biomarker to identify the high-risk precancerous tissue[15,16]. The present study was undertaken to determine the accumulation of *p*53 and Ki67 protein in normal, premalignant, and malignant esophageal tissues to gain insight into the possible involvement of them in the early stage of esophageal carcinogenesis.

MATERIALS AND METHODS

Tissue specimens

Three hundred and twelve tissue specimens were obtained from the Ci County, Hebei Province and the Cancer Hospital, China, respectively. The esophageal specimens of normal mucosae (*n*=35) used in this study were obtained from a clinical chemopreventive trial in Ci County with a high prevalence of esophageal cancer. These samples were reviewed by pathologists and considered to be morphologically normal epithelium. There were 59 specimens of dysplatic lessions and 218 specimens of esophageal cancer from the Cancer Hospital, China. Of these dysplastic lessions, there were 28 cases of mild dysplastic lessions and 31 cases of severe dysplastic lesions. Of these esophageal cancers, there were 169 cases of squamous cell carcinoma (SCC), 29 cases of adenocarcinoma (AC) and 20 cases of adenosquamous cell carcinoma (AC-SSC). There were 67 well differentiated SSCs, 78 moderately differentiated and 24 poorly differentiated SSCs among the SSCs. All samples were

routinely fixed in 10% buffered formalin, embedded in paraffin, and cut into 4ìm sections. One of each of these sections was stained with hematoxylin and eosin for classification.

In Situ Hybridization of RNA

Levels of RAR-β expression were measured by using a previously described method of nonradioactive *in situ* hybridization. The quality and specificity of the digoxigenin-labeled anti-sense and sense riboprobes were determined using Northern blotting, and the specificity of the binding of antisense riboprobes was verified using negative control sections. Briefly, the tissue sections were treated with 0.2 N HCl and proteinase K, respectively, after deparaffinization and rehydration. The slides were then postfixed with 4% paraformaldehyde and acetylated in freshly prepared 0.25% acetic anhydride in a 42℃ for 1h with a hybridization solution containing 50% deionized formamide, 2X standard saline citrate, 2X Denhardt's solution, 10% dextran sulfate, 400μg/ml yeast tRNA, 250μg/ml salmon-sperm DNA, and 20mm dithiothreitol in diethylpyrocarbinate-treated water. Next the slides were incubated in 50μl per slide hybridization solution containing 20ng of a freshly denatures dig-cRNA probe at 42℃ for 4h. After that, the slides were washed for 2h in 2XSSC containing 2% normal sheep serum (NSS) and 0.05% Triton X-100 and then for 20minutes at 42℃ in 0.1XSSC. For color reaction, the slides were incubated for 30min at 23℃ in 0.1mol/L maleic acid and 0.15mol/L NaCl (PH 7.5, buffer 1) containing 2% NSS and 0.3% Triton X-100 and then incubated overnight at 4℃ with a sheep anti-digoxigenin antibody. After being washed in buffer 1 twice, the color was developed in a chromogen solution (45μl of nitroblue tetrazolium and 35μl of an X-phosphate solution in 10ml of buffer 2, which consisted of 0.1M Tris, 0.1M NaCl, and 0.05M $MgCl_2$ (PH9.5)) for 6h with occasional observation for color development. The slides were then mounted with cover glass in Aqua mounting medium.

Immunohistochemistry

The immunohistochemical detection of *p*53 protein and the proliferation marker Ki67 was performed using a modified ABC technique. Briefly, tissue sections were deparaffinized in xylene and rehydrated in a series of ethanol solutions (100-50%). The sections were then microwaved for 15min to retrieve antigens in 0.02M citric acid solution. The endogenous peroxidase activity was blocked by incubation in a 1% methanolic hydrogen peroxide solution for 30min. This was followed by preincubation with 20% normal horse serum to minimize nonspecific binding of the second antibody. The sections were then incubated at 23℃ for 4h with monoclonal mouse anti-*p*53 or Ki 67 antibody from Vector Laboratories (Burlingame, Calif., USA) both diluted at 1 ∶ 50 in PBS. After being washed three timed in PBS, the sections were incubated with biotinylated horse anti-mouse IgG (H+L) (Vector) for 30min at 23℃ and then incubated with the ABC kit (Vector) for 30min in the dark. This was followed by incubation with 3-amino-9-ethylcarbazole (Sigma Chemical, St. Louis, Mo., USA) solution were finally mounted with Aqua mount medium under coverslips. Control sections were incubated with normal mouse IgG instead of primary antibodies or with the second antibody only.

Review and scoring of the sections

The stained sections were reviewed and scored independently by two investigators using an Olympus microscope. The sections stained for nuclear retinoid receptors were assigned to positive or negative staining categories only. Positively staining means that 10% or more epithelial cells stained positively. Sections stained for *p*53 protein and Ki67 were assigned scores of 0-3, with 0 meaning no staining; 1, weak positive, the mounts of positive cells are less than 10%; 2, positive, the mounts of positive cells are more than 10% but less than 50%; and 3, strong positive, the mounts of positive cells are more than 50 %. Scores 0 and 1were counted as negative and scores 2 and 3 as positive cases. Statistical analysis was performed using fisher's exact test to determine the association between normal or dysplastic tissues and tumors. P values were generated using Statistica version 3.0a for a Macintosh computer (StatSoft, Tulsa, Okla., USA).

RESULTS

RAR-β expression

Our priliminary study showed that RAR-α, RAR-γ and RXR-α were expressed in all of the esophageal samples without significant difference, so we excluded RAR-α, RAR-γ and RXR-α from this study and only investigate the expression of RAR-β. In 33 of 35 samples of normal mucosae, RAR-β was expressed, whereas RAR-β were detected in only 67.8%(19/28) and 58.1% (18/31) of the mild and severe lesions, respectively. In 116 of 218 tumor samples, RAR-β was expressed (Table 1). The expression of RAR-β decreased significantly between normal mucosae and dysplastic tissues or esophageal cancers (Table 2). But it was similar among the three subtypes of esophageal cancer (Table 1). However RAR-β expression was associated with the degree of squamous cell differentiation: RAR-β mRNA was expressed in 62.7%(42/67) of well differentiated SSCs, but in only 55.1%(43/78) of moderated differentiated SSCs, and in 29. 2%(7/24) of poorly differentiated SSCs (Table 3).

Table 1 Expression of the biomarkers in all kinds of esophageal tissues

Biomarker	%(positive/total)					
	Normal	Mild Dysplasia	Severe dysplasia	SSC	AC	AC-SSC
RAR-β	94.3	67.9	58.1	54.4	51.7	45.0
	(33/35)	(19/28)	(18/31)[a]	(92/169)	(15/29)	(9/20)
P53	5.7	10.7	51.6	56.8	62.1	60.0
	(2/35)	(3/28)	(16/31)[b]	(96/169)	(18/29)	(12/20)
Ki67	5.9	21.4	58.1	62.1	58.6	65.0
	(2/34)	(6/28)	(18/31)[c]	(105/169)	(17/29)	(13/20)

[a]$P<0.01$ *vs* χ^2 test between normal tissues and severe dysplastic tissues. [b]$P<0.0001$ *vs* χ^2 test between normal tissues and severe dysplastic tissues. [c]$P<0.00002$ *vs* χ^2 test between normal tissues and severe dysplastic tissues.

Table 2 Expression of the biomarkers in normal, dysplastic,and malignant esophageal tissues

Biomarker	%(positive/ total)		
	Normal	dysplasia	tumor
RAR-β	94.3(33/35)	62.7(37/59)[a]	53.2(116/218)[b]
P53	5.7(2/35)	32.2(19/59)[c]	57.8(126/218)
Ki67	5.9(2/34)	40.7(24/59)[d]	61.9(135/218)

[a]$P=0.016$ *vs* χ^2 test between normal tissues and dysplastic tissues. [b]$P<0.0003$ *vs* χ^2 test between normal tissues and tumor tissues. [c]$P=0.006$ *vs*χ^2 test between normal tissues and dysplastic tissues.[a a d]$P<0.0008$ *vs* χ^2 test between normal tissues and dysplastic tissues.

Table 3 Expression of RAR-β in the three subtypes of esophageal squamous cell carcinomas

Tumor differentiation	%(positive/total)
Well differentiated SCC	62.7(42/67)
Moderately differentiated	55.1(43/78)
Poorly differentiated	29.2(7/24)[a]

[a]$P<0.005$ *vs* χ^2 test between well differentiated and poorly diffentiated SSCs.

P53 and Ki67 expression

In contrast to RAR-β expression, the *p*53 tumor-suppressor gene and the proliferation marker Ki67 were only detected immunohistochemically in two cases each in the normal mucosa. P53 and Ki67 stained positively in 10.7%(3/28) and 21.4% (6/28) of mild dysplasia, respectively. A dramatic increase in their expression, however, was seen in 51.6%(16/31) and 58.1% (18/31) of

severely dysplastic esophageal lesions. Samples from esophageal cancers showed no higher levers of *P*53 and Ki67 expression than seen in severely dysplastic lesions. And there was no significant alteration found in the three subtypes of esophageal cancers (Table 1). However, there was significant accumulation between normal mucosae and dysplastic tissues or esophageal cancers. (Table 2).

DISCUSSION

In this study, we first analyzed the expression of RAR-β by using *in situ* hybridization, and then the *p*53 tumor-suppressor gene product and proliferation marker Ki67 protein using immunohistochemistry in normal, mild dysplastic, severely dysplastic, and cancerous mucosae. We found that RAR-β expression was progressively lost as early as in the mildly dysplastic stage of esophgeal mucosae. And RAR-β expression was significantly lost with esophageal squamous differentiation. *p*53 and Ki67 were also accumulated in the later precancerous stage of the esophagus. These results suggest that the detection of RAR-β expression and of *p*53 and Ki67 protein may be biomarkers for early identification of esophageal cancer in the high-risk populations.

It is well established that retinoids can modulate epithelial cell growth, differentiation, and apoptosis *in vitro* and *in vivo*. Retinoids can prevent abnormal squamous differentiation of epithelial cells in nonkeratinizing tissues physiologically. Retinoids can also reverse squamous metaplasia, which develops during vitamin A deficiency[2]. The abrogation in the retinoid signal pathway may be due to the loss of expression of RAR-β[17]. Indeed, in our recent study, we demonstrated that the sensitivity of esophageal cancer cells to RA was correlated not only with the constitutive expression but also with RA-induced upregulation of RAR-â[17]. Cell lines that failed to express RAR-β were resistant to RA and could form colonies in soft agar[17]. Thus, loss of RAR-β expression could contribute to the resistance to treatment with 13-cis RA in esophageal cancer. In addition, a more recent study demonstrated that RAR-β 2 knockout in F9 cells resulted in the loss of RA-associated growth arrest[18]. Various studies have clearly demonstrated that altered expression of retinoid receptors is associated with malignant transformation in human cells. Alter expression of RAR-β is a common event in different types of tumors, including head and neck, lung, and breast tumors[8,17,19,20]. In our previous study, RAR-β was expressed in all of the distant normal epithelia from esophageal cancer patients[21]. In this study, RAR-β expression is just a little low in the histologically normal mucosa. Taken together, it could be inferred that RAR-β serves as a biomarker for the early identification of esophageal cancer for chemoprevention strategy.

Previous studies demonstrated that lots of genetic alterations existed in specimens from esophageal cancer patients. For instance, *p*53 and *p*16 mutations were revealed to be early events during esophageal carcinogenesis[22, 23]. Loss of *p*53 activity could lead to malignant transformation of normal human cells[24,25]. The studies by Dr. Yang's group demonstrated that accumulation of *p*53 protein was due to *p*53 gene mutation, which occurred at very early stages of esophageal carcinogenesis[26]. Some studies have also showed that Ki67 protein could be a biomarker to identify the high-risk precancerous tissue[15,16]. In our study, both Ki67 and *p*53 proteins accumulated at early stages of esophageal carcinogenesis.

Since the RAR-β, *p*53, Ki67 expression showes significant changes at the early stage of esophageal carcinogenesis, then they could be biomakers to identify the high-risk precancerous tissue. The combination of them may improve the accuracy of identification greatly. But the underlying mechanism of the change is largely unknown, therefore more additional work is needed to investigate the mechanism and the correlation of lost expression of RAR-β, *p*53 gene protein mutation and Ki67 protein expression in esophageal cancers.

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