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RESEARCH ARTICLE

Lost expression of thyroid hormone receptor-β1 mRNA in esophageal cancer

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Abstract **Thyroid hormone receptors (TR), ligand-mediated transcription factors, regulate cell growth, differentiation, and apoptosis. In humans, two different genes encode TR-α and TR-β and they are often co-expressed in various tissues at different levels. To explore the role of TR in esophageal cancer, we analyzed expression of TR-β1 mRNA (most abundantly expressed in the majority of normal cells) in normal and malignant esophageal tissue specimens using *in situ* hybridization. The TR-β1 mRNA was detected in 92.3% (96 of 104) of normal esophageal mucosa, whereas TR-β1 mRNA was only detected in 55.8% (58 of 104 cases) of esophageal squamous cell carcinoma specimens ($P < 0.00001$). Expression of TR-β1 mRNA was associated with well-differentiated cancers ($P < 0.001$). Furthermore, we determined whether the loss of heterozygosity (LOH) in TR-β gene locus would be responsible for the lost TR-β1 expression. Analysis of 73 esophageal tissue specimens generated 39 informative cases, 17 of which showed LOH (43.6%) but only 9 of these 17 cases were correlated with lost TR-β1 expression. This study demonstrated that expression of TR-β1 mRNA was lost in esophageal cancer tissues, which may be due to multiple mechanisms.**

Keywords esophageal cancer, thyroid hormone receptor, *in situ* hybridization, LOH

Introduction

Thyroid hormone receptors (TRs) regulate cell growth, differentiation, and apoptosis through their binding to promoter regions of the target genes (Sakurai et al., 1990; Lazar, 1993; Brent, 1994; Mangelsdorf et al., 1995; Chamba et al., 1996). These receptors are members of the steroid hormone receptor superfamily (Mangelsdorf et al., 1995) and were first discovered as cellular homologs of the viral oncogene v-erbA that binds to T3 with great affinity and specificity (Sap et al., 1986; Weinberger et al., 1986). The latter has ability to block the differentiation of erythroid precursors and to promote cell proliferation together with v-erbB oncogene. In human beings, two different genes encode TR-α and TR-β, each of which includes two isoforms (α1, α2, β1, and β2, respectively) that are often co-expressed in a variety of tissues at different levels (Lazar, 1993; Chamba et al., 1996). For example, TR-α1 is most abundantly expressed in the skeletal muscle and brown fat and TR-α2 is particularly prominent in the brain, while TR-β1 is present in the most cell types and TR-β2 is mainly found in the adult pituitary gland and nervous system (Wood et al., 1994). Although it remains to be determined whether each of these isoforms serves a distinct function, recent studies demonstrated that the over-expression of TR-β1 in neuronal cell line Neuro-2a blocked its proliferation (Lebel et al., 1994) and induced the morphological and functional differentiation, indicating the role of TR-β1 in cell differentiation (Lebel et al., 1994; Nagasawa et al., 1997). TRβ1 also appears to play an important role in development of several types of human cancers, such as breast cancer (Li et al., 2002).

Esophageal cancer is characterized by an advanced pathological stage at diagnosis and poor prognosis (Blot, 1994; Cohen and Rudin, 2002; Xu, 2007). Genetic alterations in esophageal cancer are frequently exhibited in chromosome 3p, 5q, 9p, 9q, 13q, 17p, 17q and /or 18q (Cohen and Rudin, 2002). Alterations of p53 and p16 and overexpression of EGFR and TGF-α are reported to be associated with esophageal carcinogenesis (Cohen and Rudin, 2002; Xu, 2007). We demonstrated frequent loss of RAR-β expression in surgical specimens of esophageal cancer patients and this loss may be responsible for the resistance of esophageal cancer cells to retinoid treatment (Qiu et al., 1999; Xu et al.,

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1999). Furthermore, our previous study demonstrated that loss of heterozygosity of the TR-β gene (THRB) in esophageal cancer (Qiu et al., 2000). In this study, we analyzed the expression of TR-β1 in surgical specimens from esophageal cancer patients and correlated TR-β1 expression with loss of heterozygosity (LOH) of TR-β gene.

Materials and methods

Esophageal cancer specimens

Esophageal tissue specimens from 104 esophageal cancer patients were obtained from Guangzhou Nanfang Hospital, China. These specimens also included distant normal squamous mucosae. All samples were routinely fixed in 10% buffered formalin, embedded in paraffin, and cut into 4 μm sections. One each of these sections was stained with hematoxylin and eosin for classification as described in our previous studies (Qiu et al., 1999, 2000).

In situ hybridization

Human TR-β1 cDNA was described by Ribeiro et al. (1992) and its *Hin*d III and *Bam*H1 fragment was subcloned into pCMX vector. To detect TR-β1 mRNA in tissue sections, the sense and antisense probes of TR-β1 were made by incorporation of digoxigenin-UTP with our previously described method of nonradioactive *in situ* hybridization (Qiu et al., 1999; Xu et al., 1999). The specificity of the digoxigenin-labeled anti-sense TR-β1 riboprobe was verified using negative control sections and its sense probe. Briefly, the tissue sections first underwent the treatment with 0.2 M HCl and proteinase K, respectively after deparaffinization and rehydration. The sections were then postfixed with 4% paraformaldehyde and acetylated in freshly prepared 0.25% acetic anhydride in a 0.1 M triethanolamine buffer. The sections were then prehybridized at 42°C with a hybridization solution containing 50% deionized formamide, 2× standard saline citrate, 2× Denhardt's solution, 10% dextran sulfate, 400 μg/mL yeast tRNA, 250 μg/mL salmon-sperm DNA, and 20 mM dithiothreitol in diethylpyrocarbonate-treated water. Next, the sections were incubated in 50 μL per slide hybridization solution containing 20 ng of a freshly denatured the sense or antisense probe at 42°C for 4 h. After that, the sections were washed for 2 h in 2× SSC containing 2% normal sheep serum (NSS) and 0.05% Triton X-100 and then for 20 min at 42°C in 0.1× SSC. For color reaction, the sections were incubated for 30 min at 23°C in 0.1 M maleic acid and 0.15 M NaCl (pH 7.5, buffer 1) containing 2% NSS and 0.3% Triton X-100 and then incubated overnight at 4°C with a sheep anti-digoxigenin antibody. After washed in buffer 1 twice, the color was developed in a NBT solution for 4 h with occasional observation for color development. The sections were then mounted with coverslips in an Aqua-mounting medium (Fisher, Houston, TX).

Analysis of LOH using PCR amplification of microsatellite marker

Genomic DNA from tissue sections was extracted as described previously (Qiu et al., 2000). Briefly, tissue sections were deparaffinized in xylene twice for 10 min each and followed by treated in 100% ethanol twice briefly. By compared to the H&E sections under the microscope, the normal cells including epithelial and stroma cells and tumor cells were then dissected separately from three consecutive sections of each case and then put into Eppendorf tubes. These samples were treated with proteinase K (Roche, Indianapolis, IN) at 54°C overnight. Genomic DNA was then extracted by phenol/chloroform and dissolved in 20 to 50 μL TE buffer (pH 8.0). After these, LOH was analyzed by using PCR amplification of microsatellite marker. Specifically, 100 ng genomic DNA was used for each PCR amplification. THRB marker was used to detect LOH (Research Genetics, Inc. Huntsville, AL). The PCR amplification was performed in a final volume of 20 μL containing 1.5 mM $MgCl_2$, 50 mM KCl, 20 mM Tris-HCl (pH 8.4), 100 μM dNTP, 2.5 U Taq DNA polymerase (Promega, Madison, WI), and 0.2 μM primers. The reaction was initiated with a 3 min incubation at 95°C, followed by 35 amplification cycles (95°C for 30 s, 60°C for 1 min, and 72°C for 1 min) and a final extension step (72°C for 10 min and then storage at 4°C). The PCR products from each sample were then separated on 8% polyacrylamide-urea-fomamide gel. The gel was then carefully removed from the electrophoresis apparatus and positive signal was detected by using silver staining (Qiu et al., 2000). At last, the stained gel was dried on Speed Gel Vacuum Model SG 210D (Savant Instruments, Inc., Farmingdale, NY) for 2 h.

Review, scoring, and statistical analysis

The stained sections were reviewed and scored with an Olympus microscope. The sections were signed as positive or negative staining. The positive staining means 10% or more epithelial cells stained positive. LOH was documented from the dried gel and defined as a more than 50% reduction of intensity by visual inspection by two investigators. Statistical chi-square (χ^2) test was performed to determine the association between normal or distant normal tissues and tumors and Linear Correlation and Regression tests to determine the correlation of LOH with TR-β1 expression. P value < 0.05 was considered statistically significant, which was generated by using Statistica version 4.0 for PowerMac (StatSoft, Tulsa, OK).

Results

Expression of TR-β1 in normal esophageal epithelium

Normal esophageal mucosae from 104 esophageal cancer

patients were used for the study and were from both the sectioned margin (distant normal) and adjacent to the cancer cells (adjacent normal). These tissue sections were hybridized *in situ* with dig-labeled TR-β1 sense or antisense cRNA probes. RXR-α antisense probe was used as a positive control of the intact mRNA in the tissue specimens. One of represented figures is shown in Fig. 1A, with positive staining for antisense probe and no staining for sense probe, indicating the probe is specific. Of the 104 cases, 96 cases were stained positively.

Reduction of TR-β1 mRNA in esophageal cancers

A total of 104 esophageal cancer specimens were analyzed for TR-β1 mRNA using *in situ* hybridization and 58 cases (55.8%) were stained positively (Fig. 1B and C). Compared to normal esophageal mucosa, expression of TR-β1 mRNA was reduced in esophageal cancer specimens and the reduction is statistically significant ($P < 0.00001$ by McNemar test). Further analyses of the data show that expression of TR-β1 mRNA was associated with tumor differentiation ($P =$

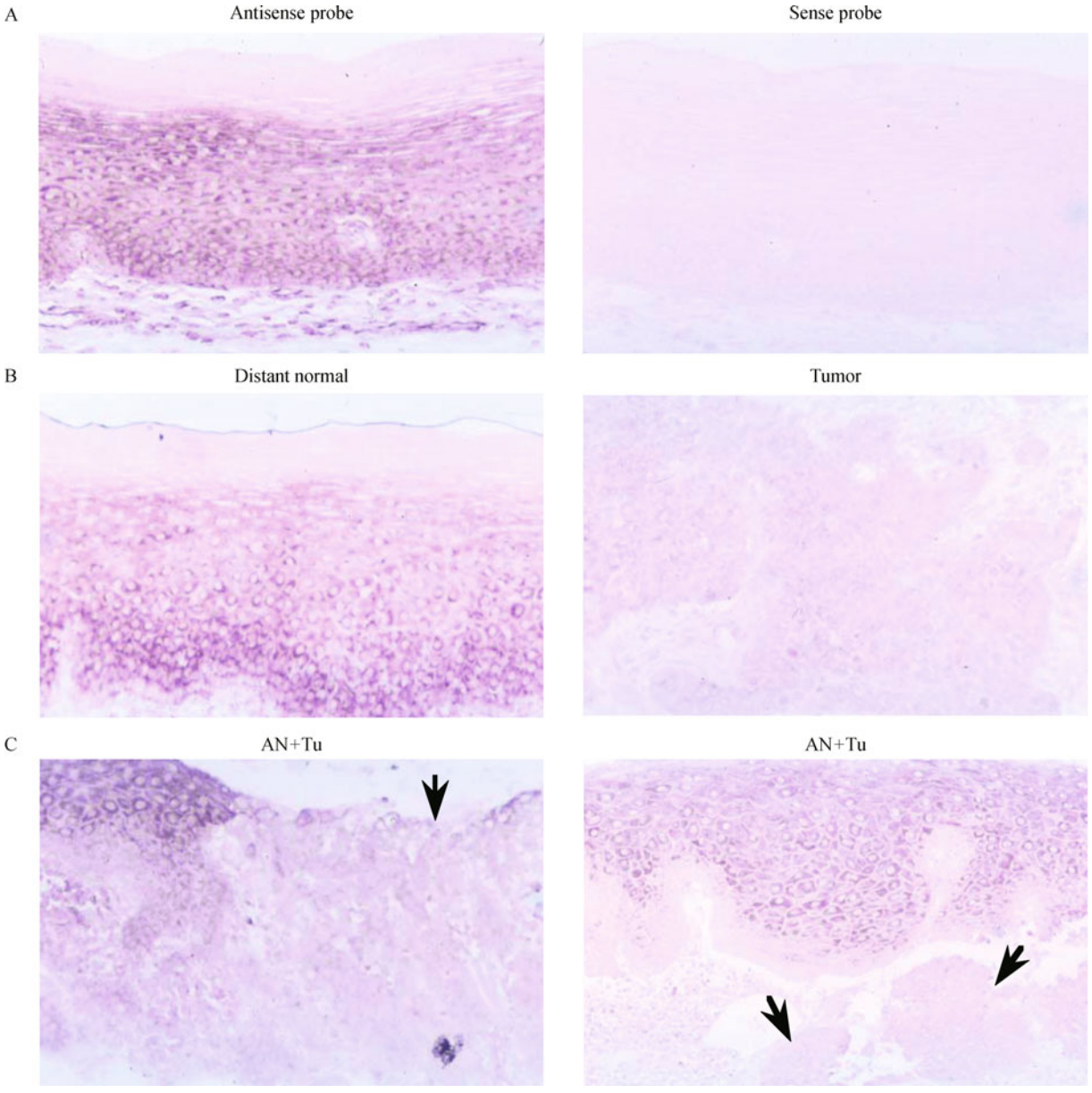

Figure 1 Expression of TR-β1 mRNA in esophageal tissue specimens. A: Detection of TR-β1 mRNA expression in distant normal tissue sections of formalin-fixed and paraffin-embedded surgical specimens using *in situ* hybridization with antisense or sense digoxigenin-labeled riboprobe; B: Differential expression of TR-β1 in distant normal and cancerous esophageal tissues; C: Differential expression of TR-β1 in adjacent normal (left or top) and cancerous (the arrows) esophageal tissues.

0.0054). However, there was no association of TR-β1 mRNA expression with other clinicopathological data of these patients, e.g., age, or tumor size or stages (data not shown). In addition, the positivity of TR-β1 mRNA was equally distributed in male (44/86), while 14/21 female patients expressed TR-β1 mRNA.

LOH in TR-β gene locus

Next, we detected loss of heterozygosity in TR-β gene localized at the chromosome 3p24 using a microsatellite marker THRB. We analyzed a total of 73 paired cases of normal and cancerous esophageal tissues and generated 39 informative cases, which in turn yielded 17 cases positive for LOH. In comparison with TR-β expression, we found that only 9 of LOH-positive cases contributed to loss of TR-β expression and 8 cases with TR-β expression occurred in LOH-negative tissues (Fig. 2 and Table 1), indicating some other mechanism may also be responsible for the loss of TR-β expression in esophageal cancers.

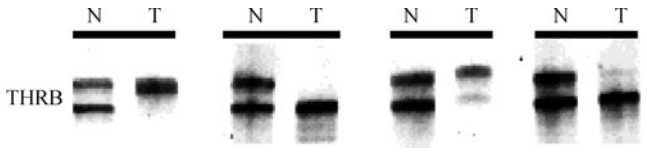

Figure 2 LOH analysis of TR-β gene in paired normal and cancerous esophageal tissues. Microsatellite marker THRB was used to detect LOH in TR-β gene locus. LOH was considered when loss of a band or significant decrease in the band intensity. N: normal; T: tumor tissues.

Discussion

In the present study, we detected expression of TRβ1 mRNA in normal and malignant esophageal tissues using *in situ* hybridization. Our data showed that TR-β1 mRNA was expressed in 92.3% of normal esophageal mucosa, whereas its expression was decreased to 55.8% in esophageal cancers. The lost TRβ1 mRNA expression associated with tumor de-differentiation. However, LOH in chromosome 3p21-24 (TR-β gene locus; Sakurai et al., 1990) only partially contributed to loss of TR-β expression in esophageal cancer. The present study demonstrated that lost TR-β1 expression might contribute to esophageal cancer development, although the molecular bases of the lost TR-β expression need further investigation.

A previous study showed that expression of TR-β protein was reduced in 25% (22 of 85) of primary breast cancer tissues (Li et al., 2002). Another study revealed that TR-β1 was significantly decreased in 70% of human renal clear cell carcinoma at both mRNA and protein levels, e.g., TR-β1 was 1.7 fold lower in tumors than in healthy controls (Puzianowska-Kuznicka et al., 2000). Furthermore, reduced expression of TR-β1 was found in poorly differentiated fibroblast-like osteosarcoma (Williams et al., 1994) and thyroid anaplastic cancer cells (Chen et al., 2000). Another study using eight human hepatocellular carcinoma cell lines showed that an increased invasive activity of these cells was associated with an overexpression of TR-β1. Concurrent with the downregulation of TR-β, the invasive activity of hepatocellular carcinoma cells was suppressed by the thyroid

Table 1 Comparison of LOH with TR-β expression in esophageal cancer tissue specimens

Case #	LOH	TR-β1
1	L	−
2	L	+
3	L	+
4	L	−
5	L	−
6	R	+
7	AI	+
8	R	−
9	L	−
10	R	+
11	L	−
12	L	+
13	R	−
14	R	−
15	R	+
16	R	−
17	R	+
18	L	−
19	R	−
20	R	−
21	R	+
22	L	+
23	R	+
24	L	−
25	L	+
26	L	+
27	L	+
28	L	−
29	L	−
30	R	+
31	R	+
32	R	+
33	R	+
34	R	−
35	R	+
36	L	+
37	R	+
38	R	+
39	R	−

L: LOH; R: retention; AI: allelic imbalance; −: lost; +: expressed

hormone, 3,3′,5-triiodo-L-thyronine (Lin et al., 1994, 1995). These studies suggest that aberrant expression of TR-β1 may be associated with different types of cancers. In the current study, we for the first time demonstrated the lost TR-β mRNA expression in esophageal cancer tissue specimens, which was associated with tumor de-differentiation. However, the defined function of TR-β expression in esophageal tissues remains to be determined. A previous study showed that TR-β suppressed ras-mediated transformation and tumorigenesis in the liver cells (García-Silva et al., 2011). Another study showed that TR-β plays a role in suppression of hepatocarcinoma and breast cancer metastasis and could provide a starting point for development of novel therapeutic strategies for the treatment of human cancer (Martínez-Iglesias et al., 2009).

Nevertheless, the underlying mechanisms responsible for the lost TR-β1 expression in esophageal cancers remain unknown. The current study showed that LOH could not explain all cases that lost expression of TR-β1 mRNA. A previous study demonstrated that about 30% (7 of 22) of TR-β1 gene was mutated in human renal clear cell carcinomas and that most of the mutations were localized in the hormone binding domain of the gene (Kamiya et al., 2002). Furthermore, previous studies showed that hypermethylation of the TR-β1 gene promoter was responsible for the gene silencing in breast cancer (Li et al., 2002), while TR-β1 gene promoter methylation, but not somatic mutation was found in lung cancer cell lines and TR-β1 gene promoter methylation status was significantly associated with loss of TR-β1 expression (Iwasaki et al., 2010). It, however, needs further investigation to determine the cause of lost TR-β1 expression in esophageal cancer.

Although a large body of knowledge has accumulated regarding molecular alterations associated with esophageal carcinogenesis, the defined pathogenesis of esophageal cancer remains to be defined (Cohen and Rubin, 2002; Xu, 2007). For example, a number of studies have demonstrated high frequent mutations of p53 and p16 genes, overexpression of EGFR, TGF-α, Cyclin D1 (Cohen and Rubin, 2002), and cyclooxygenase-2 (Zimmermann et al., 1999; Xu, 2002), and lost of RAR-β expression (Xu, 2007) during esophageal carcinogenesis. Development of esophageal cancer, like all other cancers, involves multiple genetic alterations (Cohen and Rubin, 2002; Xu, 2007). In the present study, we showed the lost TR-β1 expression in esophageal cancer tissues, LOH only partially contributed to the loss of TR-β1 expression. Other studies reported TR-β1 gene promoter methylation could silence TR-β1 expression in breast and lung cancers (Li et al., 2002; Iwasaki et al., 2010). Taken altogether, further investigation of the molecular alteration and molecular signaling in esophageal cancer will increase our understanding of esophageal carcinogenesis and may provide more accurate and useful biomarkers for prevention, diagnosis, treatment, or prognosis of esophageal cancer in future.

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