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Overexpression of human telomerase RNA is an early event in oesophageal carcinogenesis

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Abstract Telomerase, the ribonucleoprotein enzyme that elongates telomeres, is repressed in normal human somatic cells but is reactivated during tumour progression. The purpose of this study was to investigate the localization of human telomerase RNA (hTR) expression in human oesophageal dysplasia and cancer by using in situ mRNA hybridization (ISH) with avidin–biotin staining. Ki-67 immunoreactivity was also examined. We analysed 51 squamous cell carcinomas, 9 dysplasias and 60 normal mucosae. The integrity of the mRNA in each sample was verified by using a poly $d(T)_{20}$ probe. Seventy-six samples (63%) showed no mRNA degradation; these included 30 carcinomas, 7 dysplasias and 39 normal mucosae. At the single-cell level, high levels of hTR expression were found in the cytoplasm and especially in the nucleus. Most (>90%) cancer cells demonstrated high levels of hTR expression in 29 (97%) of the 30 tumours. Most dysplastic cells also showed high levels of hTR in all 7 dysplastic cases. In all 39 normal mucosae, most basal cells indicated high levels of hTR expression, which were also seen in infiltrating lymphocytes. The distribution of hTR-expressing cells was similar to that of Ki-67-positive cells. These data suggest that overexpression of hTR may be correlated with the proliferative activity that defined by Ki-67 immunoreactivity and is an early event in carcinogenesis of the oesophagus.

Key words Human telomerase RNA · In situ mRNA hybridization · Oesophageal carcinoma · Oesophageal dysplasia

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Introduction

Telomerase is a ribonucleoprotein that synthesizes telomeric DNA located at chromosomal ends using a segment of RNA within its molecule as a template [9, 22]. Telomeres are composed of many hundreds of tandem repeats of the sequence TTAGGG, and they protect chromosomes, preventing fusions, recombination and degradation [4]. In one normal somatic cell division, chromosomes lose 50–200 base pairs of the telomeric sequence, and thus telomeres shorten progressively with age [10]. In contrast, immortalized and carcinoma cells show no loss of telomere length during cell division [7]. Telomerase activity has been demonstrated in tumours of various organs, but not in normal somatic cell lines or non-neoplastic tissues adjacent to the carcinomas [14, 32]. These facts suggest that telomerase activity is directly involved in protection against telomere shortening and cell death.

The RNA component of human telomerase (hTR) was recently cloned [1]. We have reported that 81% cases expressed hTR at higher levels in gastric carcinomas than in the corresponding mucosae, although all tumour specimens and the corresponding mucosa expressed various levels of hTR on Northern blot analysis [20]. Furthermore, we analysed hTR expression in gastric precancerous and cancerous lesions by using in situ mRNA hybridization (ISH) [11].

Oesophageal carcinoma occurs with a high frequency in Japan and certain areas of China [26]. The development of oesophageal squamous cell carcinoma is a multistep and progressive process, an early indicator of which is an increased proliferation of epithelial cells, including basal cell hyperplasia and dysplasia, which are regarded as precancerous lesions. In this process, multiple genetic alterations and overexpression of growth factor–receptor systems are involved [31], including amplification of *epidermal growth factor receptor (EGFR)*, *cyclin D1*/*hst-1*/*int-2* gene [18, 37], loss of heterozygosity (LOH) at multiple chromosomal loci (e.g., 3p, 5q, 9p, 9q, 13q, 17p, 17q) [2, 29] and mutation of the *p53* gene [12].

However, there are no reports on hTR expression in oesophageal lesions. The purpose of this study was to investigate the localization of hTR expression in oesophageal dysplastic and cancerous lesions by using ISH.

Materials and methods

Surgically or endoscopically removed oesophageal tissues were obtained from Hiroshima University Hospital from 1993 to 1996. The definitions used in histological classification of the oesophageal lesions were those laid down in the criteria of the Japanese Society for Oesophageal Diseases [13]. The samples analysed were 51 squamous cell carcinomas (20 well-differentiated, 19 moderately differentiated and 9 poorly differentiated squamous cell carcinomas, and 3 carcinomas in situ). Nine cases were dysplastic, with 8 moderate dysplasias and 1 severe dysplasia. Sixty corresponding normal mucosae were also examined.

A specific antisense oligonucleotide DNA probe was designed that was complementary to the hTR transcript [5]. The sequence of the antisense probe for hTR used in this study was 5′-CAC′GGC′GCC′TAC′GCC′CTT′CTC′AGT′TAG-3′. First, the specificity of the oligonucleotide sequence was determined by a Genome Data Base search using the Oligo program (National Biosciences, Plymouth, Minn.), showing 100% homology with the hTR sequence and minimal homology with nonspecific mammalian gene sequences. Second, the specificity of the oligonucleotide sequence was confirmed by Northern blot analysis. A $d(T)_{20}$ oligonucleotide was used to verify the integrity and lack of degradation of mRNA in each sample. The DNA probe was synthesized with six biotin molecules at the 3' end (Research Genetics, Huntsville, Ala.) [6]. The lyophilized probe was reconstituted to a stock solution at 1 µg/µl in 10 mM Tris (pH 7.6) and 1 mM EDTA. The stock solution was diluted 1:200 with Probe Diluent (Research Genetics) before use.

Formalin-fixed, paraffin-embedded tissues 4 µm thick were placed on silane-treated ProbeOn Plus slides. The slides were dewaxed and dehydrated with Autodewaxer and Autoalcohol (Research Genetics) followed by digestion with pepsin [25] and then hybridized as described below.

ISH was performed as described previously [8, 15–17]. ISH was carried out by using the MicroProbe manual staining system (Fisher Scientific) [27]. Hybridization of the probe was carried out for 45 min at 45°C, and then the slides were washed three times for 2 min each time with 2×standard saline citrate at 45°C. The slides were then incubated with alkaline phosphatase-labelled avidin for 30 min at 45°C, rinsed with alkaline phosphatase enhancer for 1 min and incubated with a chromogen substrate for 15 min at 45°C. A positive reaction in this assay stained red. If the staining was weak in positive controls, incubation with a chromogen substrate for 15 min at 45°C was added. Controls for endogenous alkaline phosphatase included treatment of the samples in the absence of the biotinylated probe and use of chromogen alone.

To check the specificity of the hybridization signal, followed controls were performed, (i) RNAse pretreatment of tissue sections, (ii) substitution of the antisense probe with a biotin-labelled sense probe, (iii) competition assay with unlabelled antisense probe, and (iv) no probe. All four control treatments have no signals or markedly decreased signals. The integrity of the mRNA in the samples was verified by using a poly $d(T)_{20}$ probe. Samples that the RNA was degraded were eliminated.

Ki-67 immunohistochemistry was performed as described previously [36]. Anti-Ki-67 monoclonal antibody (MIB-1) was obtained from Medical and Biological Laboratories (Nagoya, Japan).

Results

The integrity of the mRNA in each sample was verified by using a poly $d(T)_{20}$ probe. There were 76 (63%) samples that had an intense histochemical reaction, indicating that the mRNA was not degraded. These specimens were 30 carcinomas (10 well-differentiated, 12 moderately differentiated and 7 poorly differentiated squamous cell carcinomas and 1 carcinoma in situ); 7 dysplasias (6 moderate dysplasias and 1 severe dysplasia); and 39 normal mucosae.

Next, we examined the expression of hTR in oesophageal dysplastic and cancerous lesions by using ISH. High levels of hTR expression were found in most (>90%) cancerous cells in 29/30 (97%) cancerous tissues. One moderately differentiated squamous cell carcinoma showed low levels of hTR expression, and less than 10% of cancer cells showed high levels of hTR expression. There were no differences in hTR expression levels among the grades of differentiation of carcinomas. Most dysplastic cells showed high levels of hTR expression in all 7 dysplastic tissues. In all 39 normal mucosae, most basal cells had high levels of hTR expression (Table 1, Fig. 1). At the single-cell level, high levels of hTR expression were found in the cytoplasm and especially in the nucleus. The distribution of hTR-expressing cells was similar that of Ki-67-positive cells. In normal cells, not only basal cells but also infiltrating lymphocytes showed high levels of hTR expression. The distribution of hTR-expressing lymphocytes was different from that of L26 (B-cell marker) positive cells or UCHL-1 (T-cell marker) positive cells, immunohistochemically (data not shown).

Table 1 hTR overexpression in esophageal lesions

Histology	Distribution of hTR overexpression	No. cases
Squamous cell carcinoma		
Well differentiated	>90% of cancerous cells	10/10 (100%)
Moderately differentiated	>90% of cancerous cells	11/12 (92%)
	<10% of cancerous cells	1/12 (8%)
Poorly differentiated	>90% of cancerous cells	7/7 (100%)
Carcinoma in situ	>90% of cancerous cells	1/1 (100%)
Dysplasia		
Severe	>90% of dysplastic cells	1/1 (100%)
Moderate	>90% of dysplastic cells	6/6 (100%)
Normal mocosa	>90% of basal cells	39/39 (100%)

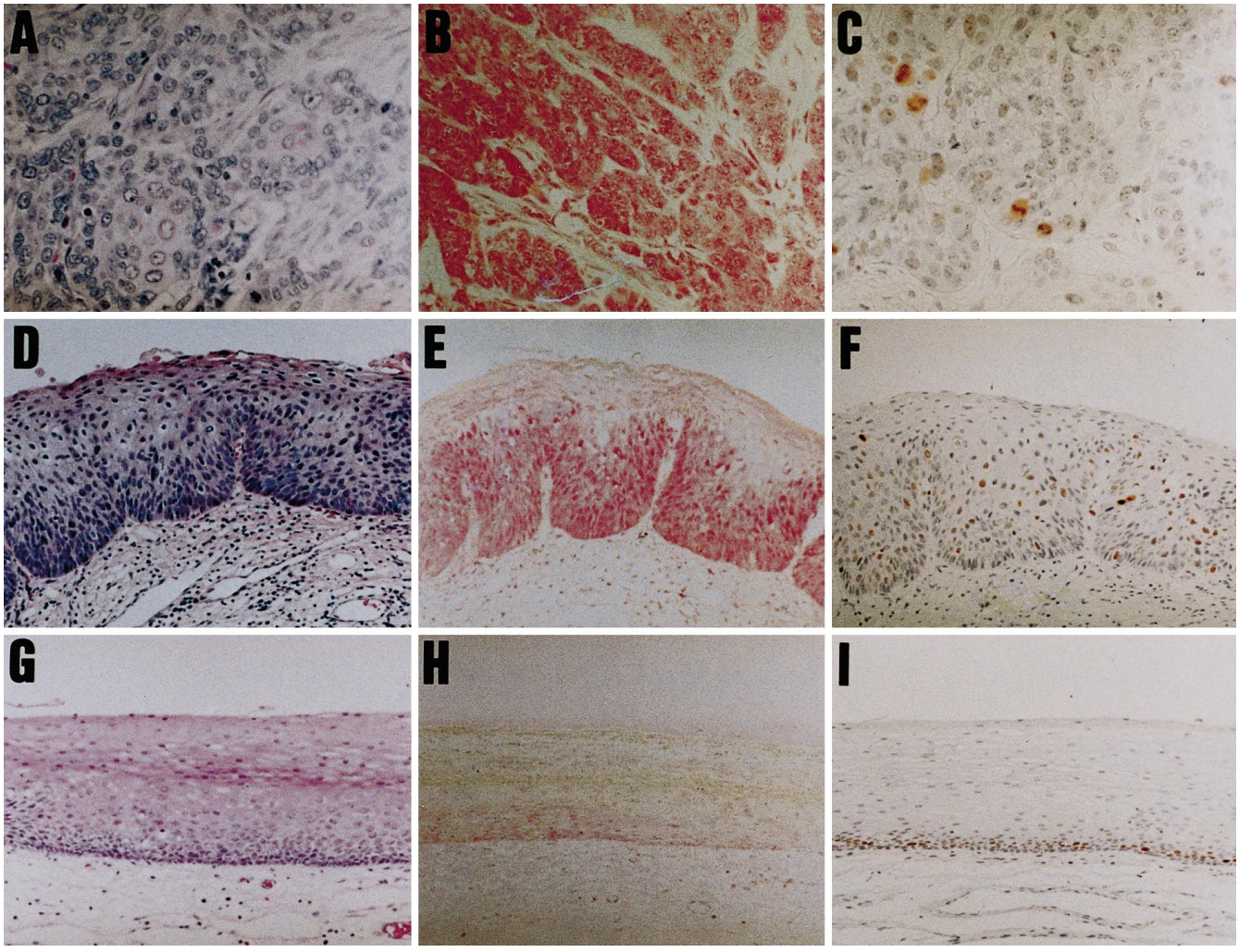

Fig. 1A–I ISH for hTR expression in oesophageal lesions. **A**, **D**, **G** Microscopic view of the well-differentiated squamous cell carcinoma, moderate dysplasia, and normal epithelium. HE, original magnification: **A** ×200, **D**, **G** ×100. **B**, **E**, **H** ISH with the antisense hTR oligonucleotide probe in the well-differentiated squamous cell carcinoma, moderate dysplasia, and normal epithelium. Original magnification: **B** ×200, **E**, **H** ×100. Cells that were almost cancerous and dysplastic cells showed high levels of hTR expression. In normal mucosa, basal cells and infiltrating lymphocytes indicated high levels of hTR expression. **C**, **F**, **I** Ki-67 immunoreactivity in the well-differentiated squamous cell carcinoma, moderate dysplasia, and normal epithelium. Original magnification: **C** ×200, **F**, **I** ×100

Discussion

Overexpression of hTR may be correlated with the proliferative activity defined by Ki-67 immunoreactivity and seems likely to be an early event in carcinogenesis according to our ISH studies. Recently, hTR was cloned and telomerase inhibition through the expression of antisense hTR transcript has been found to lead to a cell crisis, suggesting that telomerase antagonism is an important new strategy for the suppression of tumour growth [5]. Since the hTR is a proven target for anticancer therapy, it is clearly important to investigate hTR expression in human cancerous and precancerous lesions. Oesophageal carcinoma is one of the most malignant gastrointestinal cancers, with a high frequency of lymph node metastasis even in its early stages. Clarifying the mechanism of oesophageal carcinogenesis is crucial, and we focused on the hTR expression.

Few researchers have reported high levels of hTR expression in malignant tissues but not in nonmalignant tissues by using the ISH technique [28, 30, 34, 35]. In these reports, however, ISH was performed with radioisotope-labelled probes. We have developed an ISH method with avidin–biotin staining to analyse the localization of hTR expression in tissues and have reported on hTR expression in gastric precancers and cancers [11]. We showed that overexpression of hTR is an early event in gastric carcinogenesis. In the lung, Yashima et al. [35] reported that overexpression of hTR was found in dysplastic and cancerous lesions of the bronchus and concluded that overexpression of hTR is an early event in lung carcinogenesis.

Takubo et al. [33] reported that 87% of oesophageal carcinomas had telomerase activity and 23% of normal oesophageal mucosae showed detectable telomerase ac-

tivity. Most oesophageal cancers also exhibit intense telomerase activity. However, there are no reports of hTR expression in oesophageal dysplastic and cancerous lesions, and our study is the first to reveal hTR expression in oesophageal lesions. Most (>90%) cancer cells had high levels of hTR expression in 29 (97%) of the 30 tumours, and only 1 carcinoma showed low levels of hTR expression, where less than 10% of tumour cells showed high levels of hTR expression. There was no difference in hTR expression levels among the grades of differentiation of carcinomas. Most dysplastic cells demonstrated high levels of hTR expression in all 7 dysplastic tissues. In all 39 normal mucosae, most basal cells showed high levels of hTR expression. The distribution of hTR-expressing cells was similar to that of Ki-67-positive cells. These data imply that overexpression of hTR may be correlated with the proliferative activity that is defined by Ki-67 immunoreactivity and may be an early event in carcinogenesis in the oesophagus.

In transgenic mouse models, differential regulation of telomerase activity and telomerase RNA during tumorigenesis was reported. Mouse telomerase RNA (mTR) levels were up-regulated in early preneoplastic stages, although telomerase activity was detected in the only late-stage tumours and mTR levels did not parallel the amount of telomerase activity [5]. In Man, it is also reported that the high levels of hTR expression were observed in tumour tissues or cell lines that lacked telomerase activity and that it did not parallel the increase in telomerase activity [3]. We have also reported that 81% of gastric carcinomas expressed hTR at higher levels than in the corresponding mucosae, although all the tumour specimens and the corresponding mucosa expressed various levels of hTR on Northern blot analysis [20]. In this study, we did not use Northern blot analysis, and the correlation between hTR expression and telomerase activity in oesophageal lesions is therefore unclear.

The human telomerase catalytic subunit gene, hTRT/hEST2/TERT has recently been cloned [21, 23] and is expressed at high levels in telomerase-positive tissues, although undetectable in telomerase-negative tissues [24]. The message is up-regulated at the same time as telomerase is activated during the immortalization of cultured cells and down-regulated during cellular differentiation. It is suggested that the induction of hTRT/hEST2/TERT mRNA expression is required for telomerase activation, and Kolquist et al., using ISH, have reported that hTRT/hEST2/TERT mRNA is expressed in malignant tissues, in early premalignant lesions and in a subset of cells in normal breast ISH [19]. hTRT/hEST2/TERT expression appeared early during tumorigenesis, beginning with early preinvasive changes and increasing gradually during progression, both in the amount of hTRT/hEST2/TERT mRNA present within individual cells and in the number of cells expressing it within neoplastic lesions. However, hTRT/hEST2/TERT mRNA expression in oesophageal lesions has not been reported.

Our data suggest that, although the hTR component is present in normal oesophageal tissue in basal cells or infiltrating lymphocytes, it increases in expression during oesophageal carcinogenesis. Sufficient synthesis of hTR is a prerequisite for telomerase activation in tumorigenesis.

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