Amplification of the human epidermal growth factor receptor 2 gene in differentiated thyroid cancer correlates with telomere shortening

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Received December 18, 2012; Accepted February 11, 2013

DOI: 10.3892/ijo.2013.1848

Abstract. The human epidermal growth factor receptor 2 (HER2) proto-oncogene plays an important role in the development and progression of breast and gastric cancer. Monitoring of the HER2 status and treatment with trastuzumab was performed initially in breast cancer, and subsequently in gastric cancer. However, the HER2 status of thyroid cancer remains unexplored. Telomere alteration and telomerase activity have been observed in most human cancers and are known to be a feature of malignancy. The aims of this study were to clarify the HER2 status of thyroid cancer and to examine any correlations to various characteristics of malignancy. We investigated 69 cases of differentiated thyroid cancers with reference to: i) telomere length as measured using tissue quantitative fluorescence *in situ* hybridization (Q-FISH), ii) expression of human telomerase reverse transcriptase (hTERT) as determined by immunohistochemistry (IHC), and iii) overexpression of the HER2 protein as determined by IHC and amplification of the HER2 gene as determined by fluorescence *in situ* hybridization (FISH). The telomeres of thyroid cancers, especially follicular carcinomas, were significantly shorter compared to those of adjacent normal tissues. Positivity for hTERT expression and HER2 amplification were observed in approximately 70 and 22% of thyroid cancers, respectively. Our data demonstrated that telomeres in HER2-positive cancers were significantly shorter compared to those in HER2-negative cancers. These results suggest that highly malignant differentiated thyroid cancer can be detected by monitoring HER2 status and telomere shortening, and that trastuzumab therapy may be effective for refractory thyroid cancer.

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Key words: telomere, tissue quantitative fluorescence *in situ* hybridization, telomerase, human epidermal growth factor receptor 2/neu, differentiated thyroid cancer

Introduction

Human epidermal growth factor receptor 2 (HER2), also known as erbB2 and HER2/neu, is a proto-oncogene located at 17q12-21.32, playing an important role in the development and progression of human tumors, most notably breast cancer. This gene encodes a 185-kDa transmembrane protein with tyrosine kinase activity known to be involved in signal transduction during cell growth (1,2). Amplification of the gene has been identified in approximately 25-30% of human breast tumors (3,4). Amplification of HER2 and concomitant overexpression of the protein is considered to be an important biological marker of poor prognosis, more aggressive disease, and an increased risk of recurrence (3-5), as well as being a useful indicator of response to anti-HER2 therapy, for example using trastuzumab. To assess whether patients are likely to benefit from trastuzumab, monitoring of HER2 status has become routine practice using two techniques that are often used together: detection of gene amplification and protein overepression using fluorescence *in situ* hybridization (FISH) and immunohistochemistry (IHC), respectively.

Most studies have reported that the HER2 positivity rate in gastric cancer is about 15-25% (6-9), and that HER2 positivity in gastric cancer is associated with poorer prognosis, more aggressive disease and shorter survival (7,9-14). Trastuzumab treatment has been shown to be clearly beneficial for patients with gastric cancer showing HER2 gene amplification and/or protein overexpression (15). Breast cancer was the first malignancy for which HER2 gene amplification or protein overexpression was detected and trastuzumab therapy applied, and this was later extended to gastric cancer. However, no

detailed study has yet investigated HER2 gene amplification and protein overexpression in thyroid cancer.

Telomeres, which are located at the ends of chromosomes, are composed of a repeated DNA sequence (TTAGGG) and specific binding proteins. These structures protect chromosome ends and prevent them from being recognized as DNA double-strand breaks (16). The telomere repeat sequence becomes shortened by each cell division, DNA damage due to oxidative stress or through changes in telomere-associated proteins (17,18). It has been proposed that telomere shortening is an important biological factor involved in carcinogenesis, cell senescence, cell replication, cell immortality and aging (19-21). Thus, as a biological marker, telomere length reflects malignant potential, and might also be associated with genetic instability and the degree of malignancy risk (22). The enzyme telomerase is a reverse transcriptase that maintains chromosome ends by addition of the DNA sequence repeat TTAGGG to the end of a DNA strand. The enzyme is composed of a telomerase RNA component (TERC), and a protein component called human telomerase reverse transcriptase (hTERT), along with specific accessory proteins. The telomerase synthesizes the telomeric DNA, and counteracts progressive shortening of the telomere (23). Some cancers, including thyroid cancer (24), and their precursor lesions have been reported to show telomere shortening (25-29), telomerase activation (27) and expression of hTERT mRNA (30).

The aim of this study was to clarify whether HER2 gene amplification and protein overexpression are detectable in differentiated thyroid cancer, and to investigate any correlations between HER2 status and feature of malignancy such as telomere shortening or hTERT expression. For this purpose, we measured telomere lengths in thyroid cancer using quantitative fluorescence *in situ* hybridization (Q-FISH), which allowed us to estimate the telomere lengths of individual cells in each section. We examined the expression of hTERT and overexpression of HER2 protein by IHC using an anti-hTERT polyclonal antibody and an anti-c-erbB2 monoclonal antibody, respectively. Furthermore, we performed FISH to detect amplification of the HER2 gene in differentiated thyroid cancer.

Materials and methods

Tissue specimens. We examined a total of 69 thyroid tumors, including 61 papillary carcinomas and 8 follicular carcinomas. All samples were collected after obtaining informed consent from the patients, who underwent thyroidectomy at the Kanaji Thyroid Hospital, Tokyo, Japan. Tumor and adjacent normal tissues were obtained from each patient and stored at -80˚C until fixation. The tissues were then fixed for 2 h in 10% buffered formalin solution and embedded in paraffin according to standard processing procedures. They were then sliced into sections 4 μm thick for FISH and IHC analysis.

Tissue Q-FISH. Tissue Q-FISH was performed as described previously (31,32). In brief, tissue sections were deparaffinized and treated with 0.2 N HCl, 1 M sodium thiocyanate at 80˚C, 1% pepsin at 37˚C, and 10 mg/ml RNase at 37˚C. A peptide nucleic acid (PNA) telomere probe conjugated to Cy3 (telo C Cy3 probe: 5'-CCCTAACCCTAACCCTAA-3', Fasmac, Kanagawa, Japan) and a PNA centromere probe conjugated to fluorescein isothiocyanate (FITC) (Cenp 1 probe: 5'-CTTCGTT GGAAACGGGT-3', Fasmac) were applied to each section. The nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, St. Louis, MO).

FISH images were recorded by a CCD camera attached to an epifluorescence microscope (Eclipse 90i, Nikon, Tokyo, Japan) equipped with a triple band-pass filter set for DAPI/FITC/Cy3 (61000v2m, Chroma Technology Corp., Rockingham, VT) and a x40 objective lens (Plan Fluor x40/0.75, Nikon). Microscope control and image acquisition were performed using Image-Pro Plus software (version 6.3, Media Cybernetics, Bethesda, MD). The captured images were analyzed as described previously using our original software (32), 'Tissue Telo', which allows manual identification of nuclear regions from the composite color image: DAPI (blue channel), FITC (green) and Cy3 (red). Fluorescence intensities of telomere signals (Cy3) and centromere signals (FITC) for each nucleus were measured, and then the telomere-centromere ratio (TCR) was calculated, as there is no guarantee that the entire nucleus will be captured within any given tissue section.

IHC staining. The expression of hTERT protein was determined by IHC staining using rabbit antiserum against hTERT prepared by a colleague (O.Y.). It has been confirmed that telomerase activity is included in the immune precipitate obtained with this rabbit antiserum (33). The tissue sections were pre-treated with Tris-EDTA buffer solution (pH 9.0) at 95˚C. After incubation with the primary antibody for 2 h, visualization was performed using a polymer IHC detection system (Envision Kit, Dako Japan, Kyoto, Japan). The expression of HER2 protein was determined using anti-c-erbB2 antibody (Dako, Glostrup, Denmark) in accordance with the manufacturer's instructions. The degree of HER2 staining was scored as 0, 1, 2 or 3 according to the criteria for gastric cancer (34), because there are no established criteria for thyroid cancer, and the staining pattern of thyroid cancer was found to be similar to that of gastric cancer, as described below. The HER2 score was judged by a pathologist (M.K.).

HER2 FISH. We demonstrated HER2 FISH in 3 cases with a HER2 IHC score of 0 or 1, and in all cases with a HER2 IHC score of 2 or 3. HER2 FISH was performed using the Histra HER2 FISH kit (Jokoh, Tokyo, Japan), which employs two DNA probes. The probe specific for the HER2 gene was labeled with rhodamine, and an α satellite probe targeting the centromere region of chromosome 17 (CEP17) was labeled with FITC. The assay was performed in accordance with the manufacturer's instructions. In brief, specimens were incubated in pretreatment solution (99˚C for 20 min) and then digested with protease (37˚C for 10 min). The DNA probe was applied and hybridized to each section overnight at 37˚C. The slides were then washed, counterstained with DAPI, and observed by fluorescence microscopy. In each of the specimens of papillary carcinoma, the total numbers of HER2 and CEP17 signals were counted in 20 tumor cell nuclei, and the ratios of HER2 signals to CEP17 signals were calculated according to the ASCO/CAP criteria (35). Polysomy 17 was defined as the occurrence of a centromere copy number of three or more for chromosome 17 per cell.

Statistical analysis. Correlations between HER2 IHC score and FISH ratio were analyzed using the Spearman's rank correlation coefficient. We used an agglomerative hierarchical clustering approach with Ward's method to divide the data for the HER2 FISH ratio into two groups. The significance of differences was examined by TCR with Welch's t-test. The significance of differences in age and tumor size was examined using Student's t-test. The significance of differences in sex, TNM classification and hTERT expression was examined using Fisher's exact probability test. Differences at $P<0.05$ were considered significant.

Results

Tissue Q-FISH. Telomere signals (small red spots within nuclei) of tumor cells in papillary carcinoma (Fig. 1A-2 and -4) and follicular tumors were weaker than those in adjacent normal follicular epithelial cells (Fig. 1A-1 and -3). The mean TCR of papillary carcinoma and follicular carcinoma cells was significantly less than that of normal follicular epithelial cells and fibroblasts (Fig. 2, $P<0.05$). In addition, the mean TCR of follicular carcinoma cells was significantly less than that of papillary carcinoma cells (Fig. 2, $P<0.05$). The peak TCR in papillary carcinoma cells was 1 to 2, and that in follicular carcinoma cells was close to 1. The peak TCR in normal follicular epithelial cells was 1.5 to 3.5, and that in fibroblasts was 2 or more, and the peak TCR in normal follicular epithelial cells and fibroblasts had a very wide distribution (Fig. 2).

hTERT expression. hTERT protein was strongly expressed in the nuclei and nucleoli of cancer cells (Fig. 1B-2). hTERT expression was confirmed not only in cancer cells but also lymphocytes, especially those in lymph follicles. hTERT protein was not expressed in normal follicular epithelial cells (Fig. 1B-1). More than 10% of tumor cells were considered positive for hTERT expression (Fig. 1B-2). hTERT expression was detected in all of the follicular carcinomas and in 40 (66%) of the papillary carcinomas.

Table I. Correlation between HER2 expression and HER2 FISH.

	FISH ratio	
HER2 (protein expression)	<1.3 (negative, n=23)	≥1.3 (positive, n=14)
0	3	0
1	3	0
2	13	4
3	4	10

HER2 status. When the degree of HER2 staining was scored as 0, 1, 2 or 3 according to the criteria for gastric cancer, basolateral or lateral cell membranes of papillary carcinoma were strongly stained with a score of 3 (Fig. 1C-4). Seventeen of the papillary carcinomas had an IHC score of 2 and 14 had a score of 3 (Table I). One case of follicular carcinoma had an IHC score of 2, and 2 cases had a score of 3 (data not shown). Amplification of the HER2 gene was confirmed by FISH in cells of differentiated thyroid cancer (Fig. 1D-1, -2 and -3); the HER2 FISH ratio was significantly correlated with the IHC score and FISH ratio (Fig. 3, $P<0.001$). Cluster analysis of the FISH ratio separated the cancers into two groups (showing relative strong and weak amplification), with a borderline of 1.3 (Fig. 3, dotted line). Fourteen of the 32 papillary carcinomas with an IHC score of 2 or 3 showed a FISH ratio of ≥1.3 (Table I). One of the 3 follicular carcinomas with an IHC score of 2 or 3 showed a FISH ratio of ≥1.3 (data not shown). All of the cases with an IHC score of 0 or 1 showed a FISH ratio of <1.3 (Fig. 3, Table I). When the cases positive and negative for HER2 amplification were compared, a

Table II. Characteristics of the patients with papillary thyroid carcinoma.

	HER2 IHC 0-1 or FISH negative	FISH positive	P-value
Age (average ± SD)	51.2±15.0	47.4±16.3	0.42[a]
Sex			0.83[b]
Male	10	4	
Female	37	10	
TNM stage			0.55[b]
I-III	32	10	
IVA	15	4	
Tumor size (average ± SD)	21.3±10.2	22.8±11.6	0.26[a]
TCR (average ± SD)	2.6±1.8	2.3±1.7	9.20×10^{-10}[c]
hTERT expression			0.06[b]
Negative	19	2	
Positive	28	12	

[a]Student's t-test, [b]Fisher's exact probability test, [c]Welch's t-test.

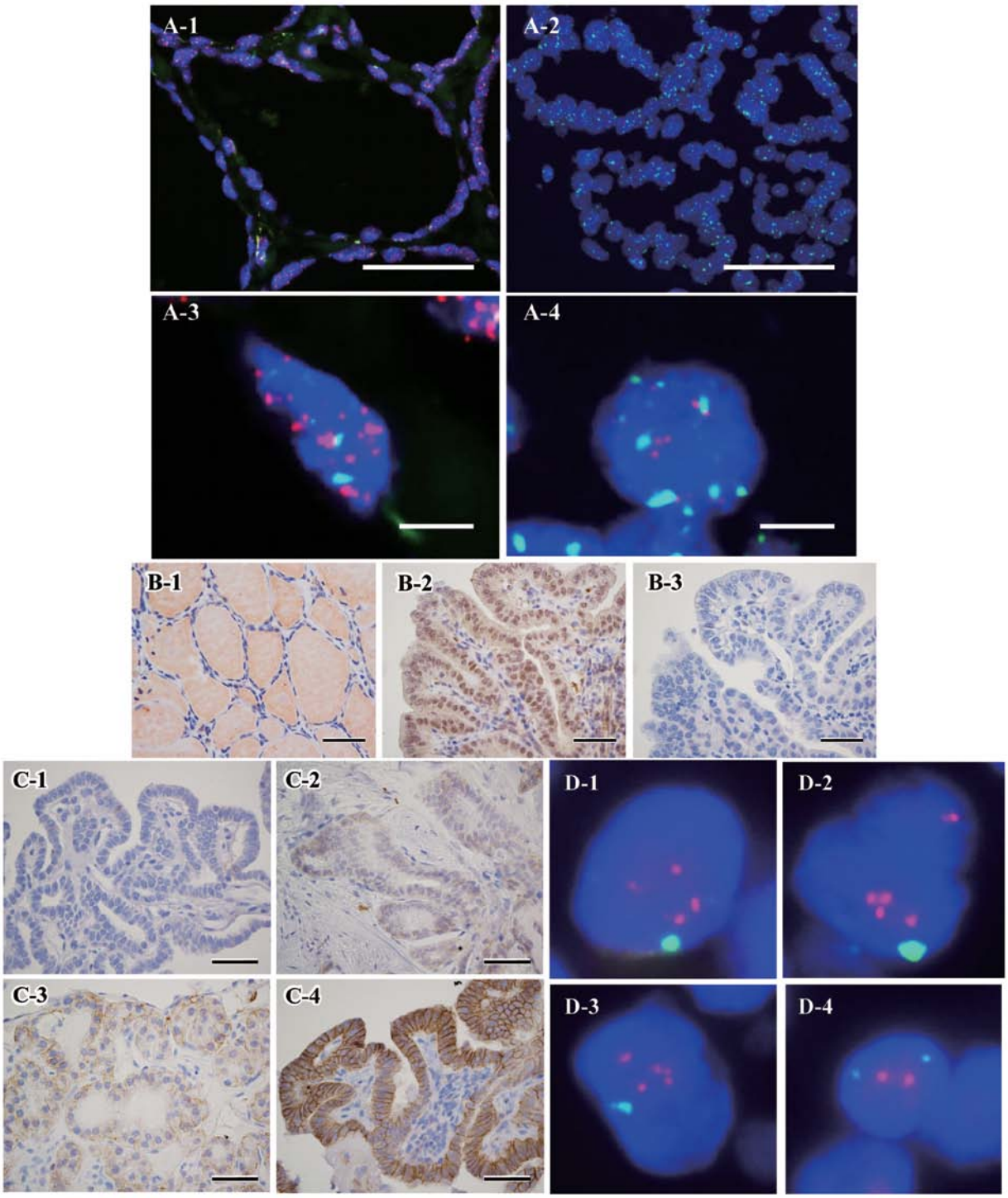

Figure 1. (A) Representative tissue Q-FISH images of normal follicular epithelial cells (A-1 and -3) and papillary carcinomas (A-2 and -4). The nuclei are stained fluorescent blue (DAPI). Red spots (Cy3) and green spots (FITC) within nuclei indicate telomere and centromere signals, respectively. The scale bar is 50 and 5 μm in A-1, -2 and A-3, -4, respectively. Telomere signals of cancer cells are clearly weaker than those in adjacent normal follicular epithelial cells. (B) Immunohistochemical (IHC) staining of follicular epithelial cells (B-1) and papillary carcinomas (B-2) using a polyclonal rabbit antiserum against hTERT. B-3 shows negative control staining against B-2, using rabbit normal serum. The nuclei and nucleoli of cancer cells are stained strongly, but the nuclei of follicular epithelial cells are not stained (scale bars represent 50 μm). (C) HER2 IHC staining of papillary carcinomas. HER2 expression was divided into four depending on staining intensity: score 0 (C-1), no reaction; score 1 (C-2), barely visible (at high magnification); score 2 (C-3), weak staining (at low magnification); score 3 (C-4), strong staining (at low magnification). Lateral and basolateral epithelial cell membranes were stained strongly with a score of 3 (scale bars represent 50 μm). (D) Representative HER2 FISH images of cells with (D-1, -2 and -3) and without (D-4) HER2 gene amplification. Red spots (rhodamine) and green spots (FITC) within nuclei indicate the HER2 gene and CEP17 signals, respectively.

significant difference in mean TCR was observed between them (Table II). Telomeres in the group positive for HER2 amplification were significantly shorter than those in the negative group (Table II). No significant differences in HER2 amplification were observed in terms of age, sex, stage, tumor size and expression of hTERT (Table II).

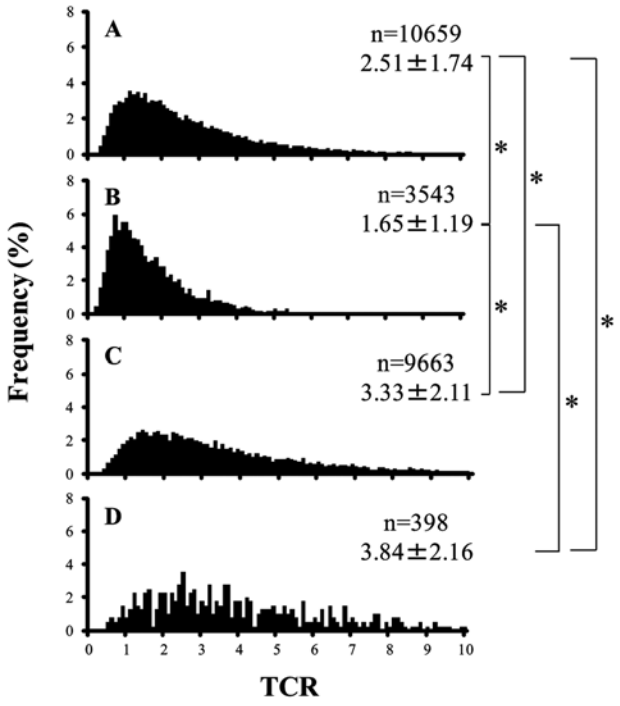

Figure 2. The distribution of TCR in each cell type. (A) Papillary carcinoma, (B) follicular carcinoma, (C) normal follicular epithelial cells, and (D) fibroblasts. Number of analyzed nuclei (n) and mean TCR ± standard deviation are indicated in each histogram. Asterisks indicate a significant difference. The peak of TCR distribution in A and B was significantly shorter than in C and D.

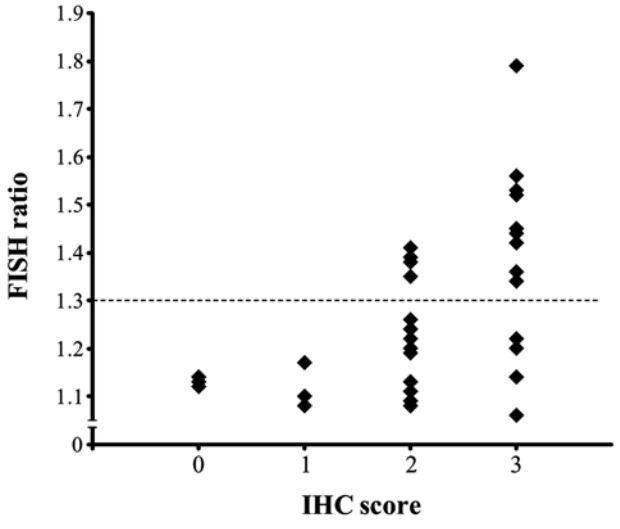

Figure 3. Scatter plot between HER2 IHC score and FISH ratio in papillary carcinomas. There is a very good correlation between the two parameters (P<0.001).

Discussion

Papillary carcinoma is the most common form of thyroid malignancy. Although it generally exhibits indolent characteristics and is associated with a favorable prognosis, cases with certain clinicopathological features can be progressive and may have a poor outcome (36). Jonklaas *et al* reported that the 15-year disease-specific survival of 3572 patients with papillary thyroid cancer was 91% for males and 96% for females (37). In a series of investigated 53,856 patients with thyroid cancer, Hundahl *et al* reported that the 10-year overall survival of those with papillary carcinoma and follicular carcinoma was 93 and 85%, respectively. Thus, follicular carcinoma had a slightly poorer prognosis (38). In the present study, therefore, we examined telomere length, hTERT protein expression and HER2 amplification status in papillary carcinomas and follicular carcinomas of the thyroid.

Southern blot analysis, which has been widely employed in previous telomere studies has certain problems: although it can measure telomere length in whole cancer tissues including lymphocytes and stem cells, it cannot exclude the subtelomere components (32). In the present study, therefore, we employed tissue Q-FISH method for determination of telomere length. We have developed a much more accurate Q-FISH-based method that can effectively estimate telomere length in different cell types using separate PNA probes for telomeres and centromeres. Tissue Q-FISH is able to measure the telomere length of cancer cells precisely, and here we obtained the telomere-centromere ratio (TCR) as a parameter representative of the telomere length. The centromere is an accurate internal control parameter, and the TCR has been shown in many previous studies to accurately reflect telomere length (31,32,39). Using Southern blot analysis, we have already shown that the telomere length of differentiated thyroid cancer cells is shorter than that in adjacent normal tissue (27). In the present study, the mean TCR of cancer cells was significantly less than that of normal follicular cells. In addition, the peak frequency of TCR for papillary carcinomas and follicular carcinomas was 1 to 2 and near 1, respectively. The TCR of normal cells had a wide distribution. Using tissue Q-FISH, Kammori *et al* (32) and Kurabayashi *et al* (39) previously investigated telomere length in esophageal and breast cancers, respectively. They found that the TCR distribution was very similar to that in our present study, the peak TCR frequency being <1 for cancer cells and >1 for adjacent normal cells, and the TCR for normal cells showing a wide distribution. The telomeres of follicular carcinoma, which has poorest prognosis among differentiated thyroid cancers, were shorter than those of papillary carcinoma. Therefore, measurement of telomere length using tissue Q-FISH appears to be useful for assessment of malignant potential. To our knowledge, this is the first report to have documented the use of tissue Q-FISH for analysis of telomere length in thyroid cancers.

For detection of hTERT protein, strong staining of nuclei and nucleoli was demonstrated by IHC. In addition, hTERT protein was recognized in lymphocytes as well as thyroid cancer cells. Using *in situ* hybridization (ISH), Kammori *et al* examined the expression of hTERT mRNA in colorectal carcinomas and follicular thyroid carcinomas, and found a pattern similar to that in the present study (24,30). hTERT protein was detected in 66% and 100% of papillary and follicular carcinomas, respectively. In a previous study, we examined the telomerase activity of thyroid cancer using TRAP, and found that papillary carcinomas and follicular carcinomas

were positive in 87.5 and 100% of cases, respectively (27). These results suggested that the expression of hTERT protein was related to the malignant potential of thyroid cancers.

There are two established techniques for monitoring HER2 status: gene amplification detected by fluorescence *in situ* hybridization (FISH) and protein overepression detected by IHC. Early studies indicated that there was a close correlation between the data obtained by these two methods. In a series of 2279 patients with breast cancer, Lal *et al* found that FISH data were correlated with an IHC score of 0, 1 or 3, whereas only 25% of cases with an IHC score of 2 demonstrated gene amplification by FISH (40). On the other hand, Kammori *et al* have reported that detection of HER2 protein expression using IHC was not satisfactory for evaluation of HER2 status in breast cancer, because a number of cases showing discrepancy between IHC and FISH data were found (41). The present study revealed a significant correlation between IHC score and the FISH ratio. As the IHC staining characteristics of thyroid cancer were very similar to those of gastric cancer (lateral and basolateral epithelial cell membranes being strongly stained), we used the IHC scoring criteria for gastric cancer in this study. In cases of breast and gastric cancer, HER2 amplification is considered positive when FISH demonstrated a ratio of ≥2.0, but none of our present specimens of thyroid cancer attained this value. However, FISH would theoretically show a ratio of 1.0 for normal cells showing no HER2 gene amplification. Despite the low frequency of HER2 gene amplification, it is thought to be certainly present in thyroid cancer cells. When we performed cluster analysis of the HER2 FISH ratio, we were able to divide the cases into two groups, one showing strong amplification (FISH ratio ≥1.3) and the other showing weak amplification (FISH ratio <1.3); 23% of papillary carcinomas showed strong amplification. The HER2 positivity rate has been reported to be around 25% in breast cancers and 15-25% in gastric cancers (3,4,6-9), and that for thyroid cancer was considered to be proportional to FISH ratio of 1.3. HER2-positive cases of breast and gastric cancer are known to have a poor prognosis (3-5,7,9-14). Positivity for HER2 gene amplification was considered to be an indicator of poor prognosis in thyroid cancer, because papillary carcinomas with a HER2:FISH ratio of ≥1.3 had short telomeres and high malignant potential. Although some studies have tried to detect gene amplification and/or protein overexpression of HRE2 in thyroid cancer, they did not involve detailed examination of the FISH ratio (42-46). To our knowledge, this is the first reported study to have examined the HER2 status of thyroid cancer in detail.

Thyroid cancer generally exhibits indolent behavior and has a good prognosis (36-39). Many previous studies have suggested that short telomeres in cancer tissues are a negative prognostic indicator (47-52). In the present study, papillary carcinomas, which have a relatively good prognosis, maintained their telomere lengths and had a low rate of positivity for hTERT protein in comparison with follicular carcinomas, suggesting that measurement of telomere length by tissue Q-FISH and expression of hTERT protein using IHC would be useful for indicating the malignant potential of thyroid cancer. It was also suggested that HER2 status might have an important prognostic impact in thyroid cancer, as is the case for breast cancer and gastric cancer, because in the present series, cancers with marked HER2 gene amplification had short telomeres. When we applied the HER2 amplification criteria for breast and gastric cancer strictly to thyroid cancer, all of cases examined were judged as negative. However, the low frequency of HER2 gene amplification may not be surprising when considering that thyroid cancer exhibits indolent characteristics and very slow growth.

Currently, there is no effective chemotherapy for differentiated thyroid cancer, and radiation therapy using 131I is common in a postoperative adjuvant setting. If HER2 positivity can be detected in thyroid cancer, it may be possible to try alternative therapies such as trastuzumab for patients with intractable thyroid cancer that does not respond to radiation therapy.

In summary, the present study has identified amplification of the HER2 gene and/or overexpression of HER2 protein in approximately 23% of human papillary thyroid carcinomas, and shown that the telomeres of HER2-positive cancers are shorter than those of negative cancers. These results suggest that highly malignant thyroid cancer can be detected by monitoring HER2 status and telomere length, and that trastuzumab therapy may be effective for patients with advanced thyroid cancer.

Acknowledgements

We would like to thank Dr Mitsuyoshi Hirokawa (Kuma Hospital) for pathological diagnosis, and Miyoko Matsumoto (Kanaji Hospital) for assistance with preparation of the manuscript.

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