

Telomerase Activity: A Marker to Distinguish Follicular Thyroid Adenoma from Carcinoma¹

Christopher B. Umbricht, Motoyasu Saji, William H. Westra, Robert Udelsman, Martha A. Zeiger, and Saraswati Sukumar²

Breast Cancer Program, Oncology Center [C. B. U., S. S.], Division of Surgical Oncology and Endocrine Surgery [M. S., R. U., M. A. Z.], and Department of Pathology [W. H. W.], The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract

The inability to distinguish microinvasive follicular thyroid cancer from benign follicular tumors preoperatively presents an important surgical dilemma. We examined 44 follicular tumors and found telomerase activity in all 11 follicular carcinomas and in 8 of 33 benign follicular tumors. It was undetectable in 22 normal thyroid tissues adjacent to the tumors. Telomerase activity may thus provide a diagnostic marker distinguishing benign from malignant follicular thyroid tumors. The ability to identify invasive follicular thyroid tumors could avert over 14,000 thyroidectomies annually in the United States, thereby significantly decreasing morbidity and health care costs.

Introduction

Estimates of the prevalence of thyroid nodules in the general population range from 4% as detected by palpation to 67% as detected by high resolution ultrasonography (1). The introduction of FNA³ for the evaluation of thyroid nodules has had a major impact on clinical management, leading to a 35–75% reduction in the number of patients requiring surgery (1). Nevertheless, 10% of FNAs are classified as suspicious for carcinoma, and the patients need to undergo surgery to clarify their diagnosis. Of 28,992 thyroid resections performed in the United States in 1995, as many as 17,557 were for benign uninodular thyroid tumors (2). Follicular neoplasms represent the majority of suspicious FNAs, 80% of which are postoperatively diagnosed as benign (1, 3). The difficulty in the diagnosis of follicular neoplasms of the thyroid stems from the fact that microinvasive follicular carcinomas differ from follicular adenomas only in that they exhibit either capsular or vascular invasion, a feature impossible to assess on FNA. Even intraoperatively, frozen section evaluation of follicular neoplasms often cannot distinguish benign from malignant (4). Because of this, patients with asymptomatic follicular adenomas of the thyroid often undergo unnecessary surgery, and conversely, patients with follicular carcinomas may initially undergo inadequate surgery. Therefore, markers capable of differentiating benign from malignant follicular neoplasms would have a significant impact on the management of these patients. Follicular neoplasms of the thyroid have been evaluated by cytogenetic and loss of heterozygosity studies (5–7). Four of five investigations on follicular carcinomas showed some abnormality of the short arm of chromosome 3, whereas two studies of follicular adenomas showed abnormalities in both 3p and 11q.

Finally, activation of one of the three *ras* oncogenes appears to be a fairly common and early event (5) but is detected in adenomas as well as in carcinomas. More importantly, less than 50% of follicular carcinomas in any published series show these changes (6, 7). Thus, no molecular markers that can reliably distinguish adenomas from carcinomas have yet been found. Recently, activation of the ribonucleoprotein telomerase has been found in a wide variety of carcinomas (8–11). Telomerase is an enzyme that maintains the stability and integrity of chromosomal ends composed of telomeres (12). Although telomerase activity is repressed in almost all nonneoplastic somatic cells, reactivation of the enzyme appears to be a necessary event for the sustained growth of most human malignant neoplasms (13). Accordingly, detection of telomerase activity may be a useful strategy for distinguishing benign from malignant lesions, especially when the distinction is not straightforward on morphological grounds alone (14, 15). We have analyzed telomerase activity in follicular thyroid lesions to determine whether this assay could be useful in distinguishing between benign follicular lesions and follicular carcinomas.

Materials and Methods

Tissue Samples. All cases undergoing thyroidectomy for suspected follicular thyroid cancer for which frozen tissue was available in our tissue bank were collected for this study. None of the tumors recurred after thyroidectomy. Tissue blocks from the center of thyroid nodules were obtained at the time of surgery by a staff pathologist and stored at -80°C with a coded identifier. All cases with a final pathological diagnosis of medullary, papillary, or mixed thyroid cancer were excluded from this analysis. Adjacent normal thyroid tissue was obtained when possible. The tissue blocks were embedded in OCT, and two consecutive 10- μm cryostat sections were obtained and immediately frozen in liquid nitrogen and stored at -80°C . One slide was stained with H&E and reviewed with the study pathologist in conjunction with the paraffin-embedded sections for evidence of invasive growth and the presence of lymphocytic infiltrates. The tissue of the other cryostat section was suspended in 50 μl of telomerase lysis buffer [$1\times$ TLB = 0.5% (3-[3-cholamidopropyl-dimethyl-ammonio]-1-propane sulfonate, 10 mM Tris-HCl (pH 7.5), 1 mM MgCl_2 , 1 mM EGTA, 5 mM β -mercaptoethanol, 0.1 mM (4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochlorine, and 10% glycerol], and extracts were prepared as described (16). Protein concentrations were determined by Bradford assay (17). Cytological samples from FNAs were obtained after adequate diagnostic samples had been made by expelling the residual aspirate on a glass slide and snap freezing the sample in liquid nitrogen. Extracts were then prepared by overlaying the frozen samples with 50 μl of TLB and recovering the lysate by aspiration.

Telomerase Assay. Aliquots of tissue extract containing 0.5 μg of protein, with and without inactivation with RNase A (16), were used for the telomerase assays. Standard TRAP assays were performed as described (16). All assays contained 10 ag of the 150-bp ITAS for detection of Taq polymerase inhibitors (18). When ITAS inhibition was detected, the assay was modified to include an extraction with phenol:chloroform:isoamyl alcohol at 25:24:1, followed by ethanol precipitation (19) before the PCR amplification step. PCR products were electrophoresed in a 10% nondenaturing polyacrylamide gel. The DNA ladders were visualized using a Molecular Dynamics Phosphorimager after a

Received 2/27/97; accepted 4/20/97.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported in part by Johns Hopkins Oncology Center funds (to S. S.) and by Grant 9610 from the Susan G. Komen Foundation (to C. B. U.).

² To whom requests for reprints should be addressed, at Johns Hopkins Oncology Center, 370 Ross Building, 720 Rutland Avenue, Baltimore, MD 21205-2196. Phone: (410) 614-2484; Fax: (410) 614-4073; E-mail: saras@welchlink.welch.jhu.edu.

³ The abbreviations used are: FNA, fine-needle aspiration; TRAP, telomere repeat amplification protocol; ITAS, internal telomerase assay standard; ag, attogram (10^{-18}g).

12-h exposure. Detectable telomerase activity was defined as a hexanucleotide ladder of three or more bands not present in matched buffer controls. The assays were performed without knowledge of sample diagnoses.

Statistical Analysis. The tissue samples were divided into two groups: samples with no detectable telomerase activity; and samples with detectable telomerase activity. The following parameters were used for univariate analysis: presence of invasive carcinoma, tumor size, presence of oxyphilic (Huerthle cell) histological subtype, patient age, and sex. All *P*s refer to either Fisher's Exact test for tables or Wilcoxon's Rank Sum test for nonparametric data, where appropriate (20). The analysis was performed using the JMP statistical software package (SAS Institute, Inc.) on a Macintosh microcomputer.

Results

A total of 66 frozen samples of follicular neoplasms, hyperplastic nodules, and normal thyroid tissue were obtained to prepare whole-cell extracts. Standard TRAP assays were performed as described (16, 18) on 44 thyroid tissues, the FNAs of which showed a follicular lesion (11 follicular carcinomas, 23 follicular adenomas, and 10 hyperplastic nodules), and on 22 matched normal thyroid tissue specimens obtained from 8 cases of carcinoma and 14 cases of benign follicular lesions. The data are summarized in Table 1, and representative results of TRAP assays are presented in Figs. 1 and 2. All 11 carcinomas were positive for telomerase activity, showing the characteristic ladder of hexamer telomere repeats. Five of 23 follicular adenomas (22%) and 3 of 10 hyperplastic nodules (30%) were also positive. All normal thyroid tissue samples were negative. As illustrated in Fig. 2, no appreciable difference was apparent between telomerase-positive benign and malignant lesions. In this series, the telomerase assay had a specificity of 76% and a sensitivity of 100%. The predictive value of a positive test was 58%, and the predictive value of a negative test was 100% (20). Of the parameters evaluated by univariate analysis, there was a statistically significant correlation between detectable telomerase activity and increasing tumor size ($P = 0.001$) and patient age ($P = 0.027$) by the Wilcoxon Rank Sum test (Ref. 20; data not shown). The sample size was insufficient for multivariate analyses. Two potential problems with the telomerase assay in these tumors are: (a) false-positive results, presumably due to lymphocytic infiltrates that have known detectable telomerase activity (21, 22); and (b) false-negative results seen when PCR inhibitors are present in the tissue extracts. In our study, three of the five follicular adenoma samples that were positive for telomerase activity contained lymphocytic infiltrates throughout the gland. In all three cases, telomerase activity was also detectable in the extranodular tissue containing lymphocytic infiltrates (Fig. 2, Lanes 18–22). Notably, none of the follicular carcinomas in this series had lymphocytic infiltrates. Fig. 3 illustrates the second problem, that of false-negative results attributed to PCR inhibitors. These can be distinguished from true-negative results by the concurrent loss of the ITAS control product. PCR inhibition, masking telomerase activity, was encountered in three follicular carcinomas and one hyperplastic nodule, which was reversed in each case by phenol extraction (19) prior to PCR amplification. Mixing experiments were performed to further evaluate the presence of soluble inhibitors. Tissue extracts that showed inhibited amplification of the ITAS control product blocked telomerase activity

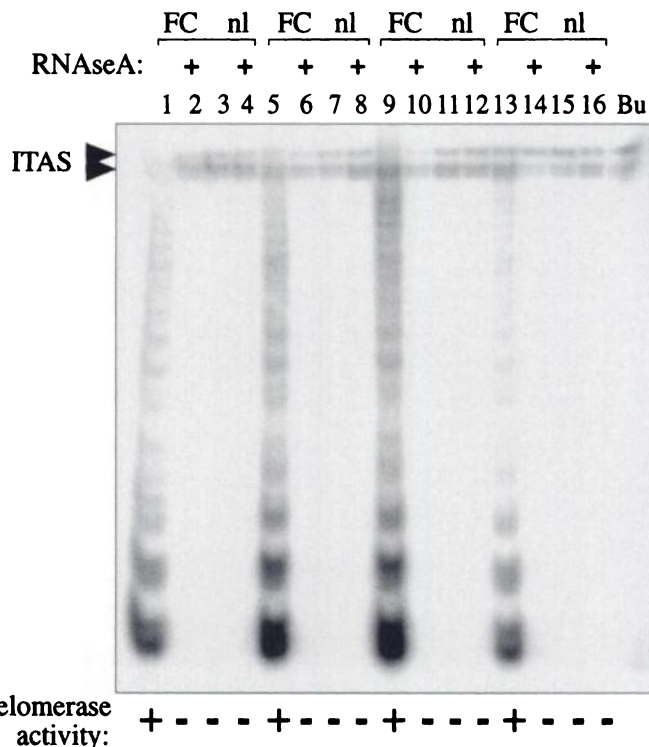


Fig. 1. Telomerase activity in follicular thyroid cancer. Clarified tissue lysates (0.5 μg protein) prepared from a 10-μm frozen section from follicular carcinoma (FC) and adjacent normal thyroid (nl) were subjected to standard TRAP assays (16, 18). Bu, telomerase lysis buffer without tissue lysate. Inactivation of extracts by pretreatment with RNase A is indicated by + at the top of the lanes. Radioactive PCR products were separated by electrophoresis in a nondenaturing 10% polyacrylamide gels and visualized by Phosphorimager (Molecular Dynamics) scanning after a 12-h exposure. The assay result is indicated at the bottom of the figure. Arrowheads, amplification products of a 150-bp ITAS, included in each reaction to detect inhibitors of the PCR reaction.

in samples known to be telomerase positive. The inhibition was reversed by phenol extraction (data not shown). These results underscore the importance of a careful pathological review of the tissue for evidence of inflammatory infiltrates and of removal of inhibitors in ITAS-negative samples to allow reliable interpretation of a telomerase assay. Using telomerase as a diagnostic marker for malignancies of the thyroid will require the adaptation of this assay to clinical specimens such as FNAs of the thyroid (9). To determine whether telomerase activity could be detected in FNAs of the thyroid, we performed a pilot study on six FNAs suspicious for thyroid cancer. Telomerase activity was detectable in four of six FNAs (see Fig. 4). The positive FNAs were later confirmed to be from papillary carcinomas of the thyroid; the two negative samples were from benign adenomas.

Discussion

In this study, we applied a molecular test to a medical problem that has defied histopathological resolution. Telomerase activity was detected in 100% of follicular thyroid carcinomas but was undetectable in 76% of benign thyroid lesions. Six of 31 cases (19%) of benign follicular tumors without lymphocytic infiltrates had detectable telomerase activity. Telomerase activity may reflect a highly proliferative phenotype, a notion supported by the fact that all of the telomerase-positive benign lesions were over 2.5 cm in size. Alternatively, some histologically benign lesions may have malignant potential, because follicular adenomas and a subset of hyperplastic nodules are clonal and may be precursor lesions of follicular carcinomas (5, 23–26). Because the distinction of benign adenomatous lesions from carcinoma rests upon the histological detection of tissue invasion, the

Table 1 Telomerase activity in follicular thyroid tumors

	Total	Telomerase activity		
		+	-	% positive
Follicular carcinoma	11	11	0	100
Follicular adenoma	23	5	18	22
Hyperplastic nodule	10	3	7	30
Normal thyroid tissue	22	0	22	0

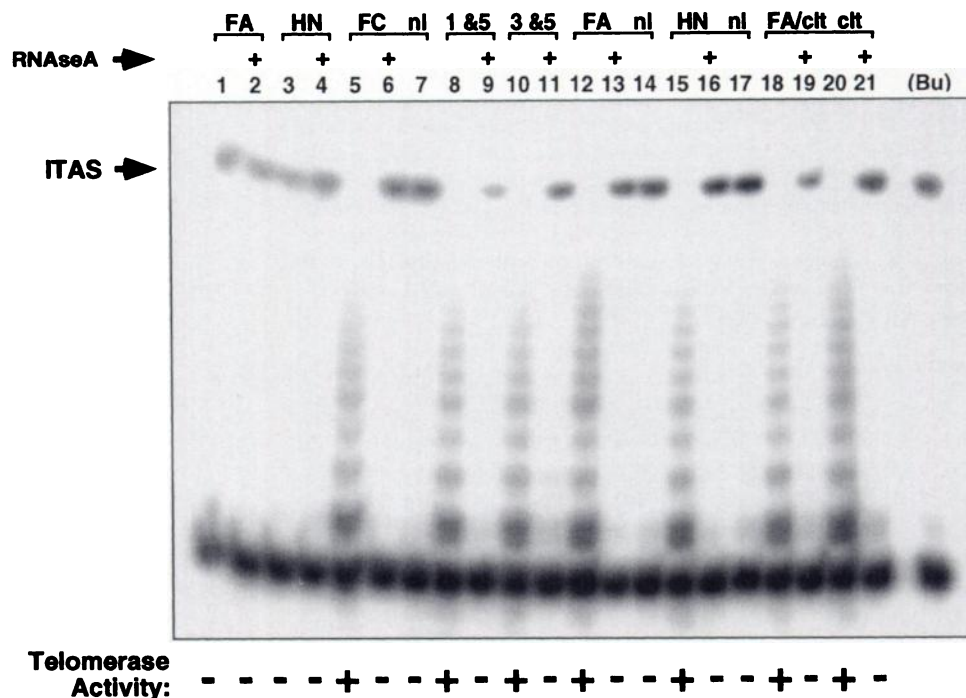


Fig. 2. Telomerase activity in follicular thyroid lesions. Samples from follicular adenoma (FA), hyperplastic nodules (HN), follicular carcinoma (FC), and adjacent normal thyroid, (nl) were assayed for telomerase activity as in Fig. 1. Lanes 8-11 show the results of mixing experiments of extracts used in Lanes 1, 3, and 5. *ctl*, a tissue sample showing histological evidence of chronic lymphocytic thyroiditis in a follicular adenoma as well as in surrounding tissue. *Bu*, telomerase lysis buffer without tissue lysate. Inactivation of extracts by pretreatment with RNase A is indicated by + at the top of the lanes. The PCR products were analyzed as in Fig. 1. Arrow, amplification products of 10 ag of the ITAS control.

telomerase-positive samples could represent a step in follicular carcinogenesis before tissue invasion has yet occurred. Under these circumstances, telomerase activity may indicate a potential for malignant progression in a follicular lesion that morphologically appears

benign. The universal presence of telomerase activity in follicular thyroid cancer suggests that telomerase activation may be a necessary step in the malignant progression of follicular neoplasms of the thyroid. In this study, we did not attempt to quantify telomerase

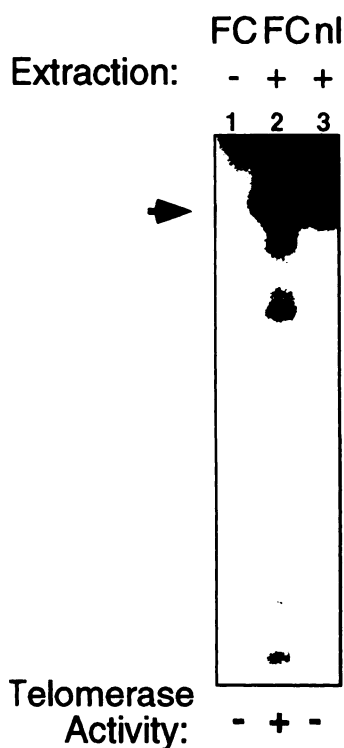


Fig. 3. Masking of telomerase activity by inhibitors of the PCR reaction. Phosphor-imager scan of TRAP assay of tissue extracts from a case of follicular carcinoma (FC) and adjacent normal thyroid tissue (nl) without (Lane 1) and with (Lanes 2 and 3) phenol extraction. When PCR inhibition was detected by the loss of the ITAS control band (arrow, Lane 1), the telomerase-dependent primer elongation products of the TRAP assay underwent phenol:chloroform:isoamyl alcohol (25:24:1) extraction and ethanol precipitation (19). The precipitated telomerase elongation products were then resuspended in TRAP buffer and amplified by PCR as in the standard TRAP assay.

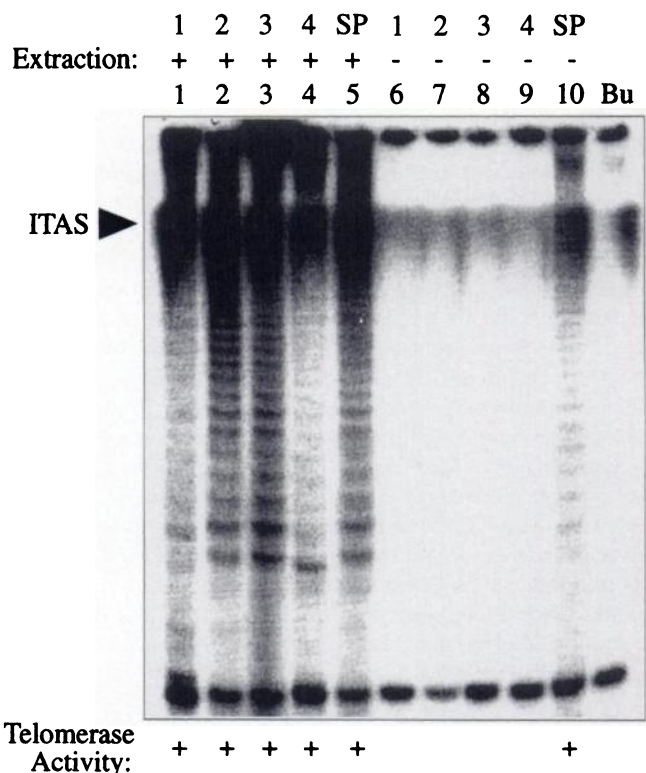


Fig. 4. Telomerase activity in FNAs of thyroid cancer. Phosphorimager scan of TRAP assay of extracts from FNA samples of four papillary carcinomas (Lanes 1-4 and 6-9) with (Lanes 1-5) and without (Lanes 6-10) phenol extraction. Lanes 5 and 10 (SP) are from a tissue block of a follicular thyroid cancer. *Bu*, buffer control. Telomerase activity was undetectable in the standard assay, with partial loss of the ITAS control (arrowhead) but was detectable after phenol extraction. Four FNAs from nonneoplastic thyroid samples, including two follicular adenomas, showed no detectable telomerase activity (data not shown).

activity in the thyroid tumors, although this may provide some additional information on the biology of these tumors. In our experience, the TRAP assay and its current modifications (27, 28) remains too vulnerable to systematic errors, such as variations in tissue procurement and freezing, tissue composition, PCR inhibition, and substrate competition with the internal PCR control (Fig. 2). Although a positive telomerase result was not 100% specific in this series, the ability to obviate unnecessary and extensive surgery for 76% of the patients with benign follicular lesions that have no detectable telomerase activity has great clinical potential. More importantly, the fact that this assay is highly sensitive in the detection of follicular thyroid cancer may allow a more definitive initial operation for patients with suspected follicular thyroid lesions. This requires adaptation of the telomerase assay to preoperative clinical samples. We have confirmed the feasibility of performing telomerase assays in thyroid FNAs. Thus, the measurement of telomerase in FNAs suspicious for follicular neoplasms could significantly limit the number and extent of up to 76% of the 17,600 thyroid resections performed annually in the United States for benign thyroid tumors (2). Therefore, such a test may ultimately be integrated into the preoperative evaluation of patients who harbor a thyroid nodule.

References

- Burch, H. B. Evaluation and management of the solid thyroid nodule. *Endocrinol. Metab. Clin. North Am.*, 24: 663-710, 1995.
- Health Care Investment Analysts (HCIA). I. National Link Study, 1995. Baltimore, Maryland: HCIA, Inc., 1996.
- Gharib, H., and Goellner, J. R. Fine-needle aspiration of the thyroid: an appraisal. *Ann. Intern. Med.*, 118: 282-289, 1993.
- Chen, H., Nicol, T. L., and Udelsman, R. Follicular lesions of the thyroid: does frozen section evaluation alter operative management? *Ann. Surg.*, 222: 101-106, 1995.
- Grebe, S. K. G., and Hay, I. D. Follicular thyroid cancer. *Endocrinol. Metab. Clin. North Am.*, 24: 761-803, 1995.
- Sozzi, G., Miozzo, M., Cariani, T. C., Bongarzone, I., Pilotti, S., Pierotti, M. A., and Della Porta, G. A t(2;3)(q12-13;p24-25) in follicular thyroid adenomas. *Cancer Genet. Cytogenet.*, 64: 38-41, 1992.
- Jenkins, R. B., Hay, I. D., Herath, J. F., Schultz, C. G., Spurbeck, J. L., Grant, C. S., Goellner, J. R., and Dewald, G. W. Frequent occurrence of cytogenetic abnormalities in sporadic nonmedullary thyroid carcinoma. *Cancer (Phila.)*, 66: 1213-1220, 1990.
- Kim, N. W., Piatyszek, M. A., Prowse, K. R., Harley, C. B., West, M. D., Ho, P. L., Coviello, G. M., Wright, W. E., Weinrich, S. L., and Shay, J. W. Specific association of human telomerase activity with immortal cells and cancer. *Science (Washington DC)*, 266: 2011-2015, 1994.
- Hiyama, E., Gollahon, L., Kataoka, T., Kuroi, K., Yokoyama, T., Gazdar, A. F., Hiyama, K., Piatyszek, M. A., and Shay, J. W. Telomerase activity in human breast tumors. *J. Natl. Cancer Inst.*, 88: 116-122, 1996.
- Sugino, T., Yoshida, K., Bolodeoku, J., Tahara, H., Buley, I., Manek, S., Wells, C., Goodison, S., Ide, T., Suzuki, T., Tahara, E., and Tarin, D. Telomerase activity in human breast cancer and benign breast lesions: diagnostic applications in clinical specimens, including fine needle aspirates. *Int. J. Cancer*, 69: 301-306, 1996.
- Bednarek, A. K., Sahin, A., Brenner, A. J., Johnston, D. A., and Aldaz, C. M. Analysis of telomerase activity levels in breast cancer: positive detection at the *in situ* breast carcinoma stage. *Clin. Cancer Res.*, 3: 11-16, 1997.
- Greider, C. W. Telomere length regulation. *Annu. Rev. Biochem.*, 65: 337-365, 1996.
- Bacchetti, S., and Counter, C. M. Telomere and telomerase in human cancer. *Int. J. Oncol.*, 7: 423-432, 1995.
- Blackburn, E. H. Telomeres: no end in sight. *Cell*, 77: 621-623, 1994.
- Greider, C. W. Mammalian telomere dynamics: healing, fragmentation shortening and stabilization. *Curr. Opin. Genet. Dev.*, 4: 203-211, 1994.
- Piatyszek, M. A., Kim, N. W., Weinrich, S. L., Hiyama, K., Wright, W. E., and Shay, J. W. Detection of telomerase activity in human cells and tumors by a telomeric repeat amplification protocol (TRAP). *Methods Cell Sci.*, 17: 1-15, 1995.
- Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.*, 72: 248-254, 1976.
- Wright, W. E., Shay, J. W., and Piatyszek, M. A. Modifications of a telomeric repeat amplification protocol (TRAP) result in increased reliability, linearity and sensitivity. *Nucleic Acids Res.*, 23: 3794-3795, 1995.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual*, Ed. 2, pp. E10-E15. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1989.
- Daly, L. E., Bourke, G. J., and McGilvray, J. Interpretation and uses of medical statistics. Oxford: Blackwell Scientific Publications, 1991.
- Counter, C. M., Gupta, J., Harley, C. B., Leber, B., and Bacchetti, S. Telomerase activity in normal leukocytes and in hematological malignancies. *Blood*, 85: 2315-2320, 1995.
- Hiyama, K., Hirai, Y., Kyoizumi, S., Akiyama, M., Hiyama, E., Piatyszek, M. A., Shay, J. W., Ishioka, S., and Yamakido, M. Activation of telomerase in human lymphocytes and hematopoietic progenitor cells. *J. Immunol.*, 155: 3711-3715, 1995.
- Lemoine, N. R., Mayall, E. S., Wyllie, F. S., Williams, E. D., Goyns, M., Stringer, B., and Wynford-Thomas, D. High frequency of *ras* oncogene activation in all stages of human thyroid tumorigenesis. *Oncogene*, 4: 159-164, 1989.
- Salmon, I., Gasperin, P., Rummelink, M., Rahier, I., Rocmans, P., Pasteels, J. L., Heimann, R., and Kiss, R. Ploidy level and proliferative activity measurements in a series of 407 thyroid tumors or other pathological conditions. *Hum. Pathol.*, 24: 912-920, 1993.
- Roque, L., Gomes, P., Correia, C., Soares, P., Soares, J., and Castedo, S. Thyroid nodular hyperplasia: chromosomal studies in 14 cases. *Cancer Genet. Cytogenet.*, 69: 31-34, 1993.
- Namba, H., Matsuo, K., and Fagin, J. A. Clonal composition of benign and malignant human thyroid tumors. *J. Clin. Invest.*, 86: 120-125, 1990.
- Holt, S. E., Norton, J. C., Wright, W. E., and Shay, J. W. Comparison of the telomeric repeat amplification protocol (TRAP) to the new TRAP-eze telomerase detection kit. *Methods Cell Sci.*, 18: 237-248, 1996.
- Bodnar, A. G., Kim, N. W., Effros, R. B., and Chiu, C-P. Mechanism of telomerase induction during T cell activation. *Exp. Cell Res.*, 228: 58-63, 1996.