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Telomerase Repeat Amplification Protocol (TRAP): A New Molecular Marker for Parathyroid Carcinoma

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Telomerase results to be active in human germ, stem cells, several malignant cell tumors and in immortalized cell lines. In order to investigate if molecular mechanisms other than *Rb* gene inactivation can be helpful to diagnose malignancy of parathyroid tumors, we decided to investigate the presence of active telomerase in homogenates from different pathological parathyroid tissues (hyperplastic, adenomatous, carcinomatous, and normal) and primary cell cultures. The TRAP assay was performed to detect this activity in histologically characterized normal, hyperplastic, adenomatous, and carcinomatous human parathyroid tissues, primary cell lines, and one metastatic tissue from parathyroid carcinoma. Only malignant parathyroid glands and the metastatic tissue were TRAP positive. Our findings suggest that telomerase expression could represent an important molecular mechanism underlying the acquisition and progression of an aggressive phenotype of epithelial parathyroid cells and it may help to predict their malignant potential. The TRAP assay is easy to perform and it could become an additional tool to be included in the harmamentarium for the molecular diagnosis of parathyroid carcinoma.

Progressive shortening of telomeres represents one of the molecular mechanisms developed by cells to avoid uncontrolled proliferation. Telomere length constitutes the biologic clock regulating the normal cell life span through maintenance of chromosome integrity. Human germ and stem cells, the majority of tumor and immortalized cell lines express telomerase, the specific enzyme capable to prevent the shortening of telomeres, which is not detected in most normal adult tissues. In the majority of human cancers telomerase activity is up-regulated or reactivated and correlation between cancer and telomerase expression has been demonstrated (1), the latter occurring probably as a late genetic event following earlier events, such as inactivation of tumor suppressor genes (2). Normal parathyroid tissue exhibits a very low cell proliferative activity and parathyroid carcinoma represents a disease rare (less than 1% of parathyroid tumors) and difficult to diagnose. Recently, loss of heterozygosity (LOH) studies, both in sporadic and familial forms of parathyroid carcinoma (3–5), showed allelic loss at 13q12.3-32 region where *Rb* and *BRCA2* genes colocalize (4), suggesting a possible role of these suppressor genes in the pathogenesis of such malignancy and offering potential markers for differential diagnosis. In order to investigate if also telomerase activity could be involved in uncontrolled proliferation of parathyroid cancer cells we performed TRAP assay in histologically characterized normal, hyperplastic, adenomatous, and carcinomatous human parathyroid tissues.

MATERIALS AND METHODS

Human parathyroid tissue and cell cultures. In this study pathological parathyroid glands were obtained from patients undergoing neck surgery, 4 for primary hyperparathyroidism, 2 for uremic hyperparathyroidism and 1 for parathyroid carcinoma. Normal parathyroid tissue was obtained from a patient who underwent surgery for thyroid carcinoma. From these tissues primary cultures were obtained. In a patient with parathyroid carcinoma cryopreserved skin metastatic tissue was available. All surgical specimens have been collected after obtaining informed consent from the patients.

Cell culture procedures. Parathyroid tissue was minced into small fragments and digested at 37°C for two hours with 1.2 mg/ml collagenase type II (Sigma, Milan, Italy) in a mixture (1:1) Dulbecco's modified Eagle's medium–nutrient mixture Ham's F-12 (DME-F12) (Sigma, Milan, Italy). The suspension was dispersed mechanically by aspiration into a serological pipette and successively centrifuged at 500 × g. The pellet was resuspended in DME-F12 medium containing 5% calf serum (Irvine Scientific, Santa Ana, CA), 1% Nutridoma (Boehringer Mannheim, Germany), 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 100 u/ml penicillin and 100 μg/ml streptomycin and filtered through

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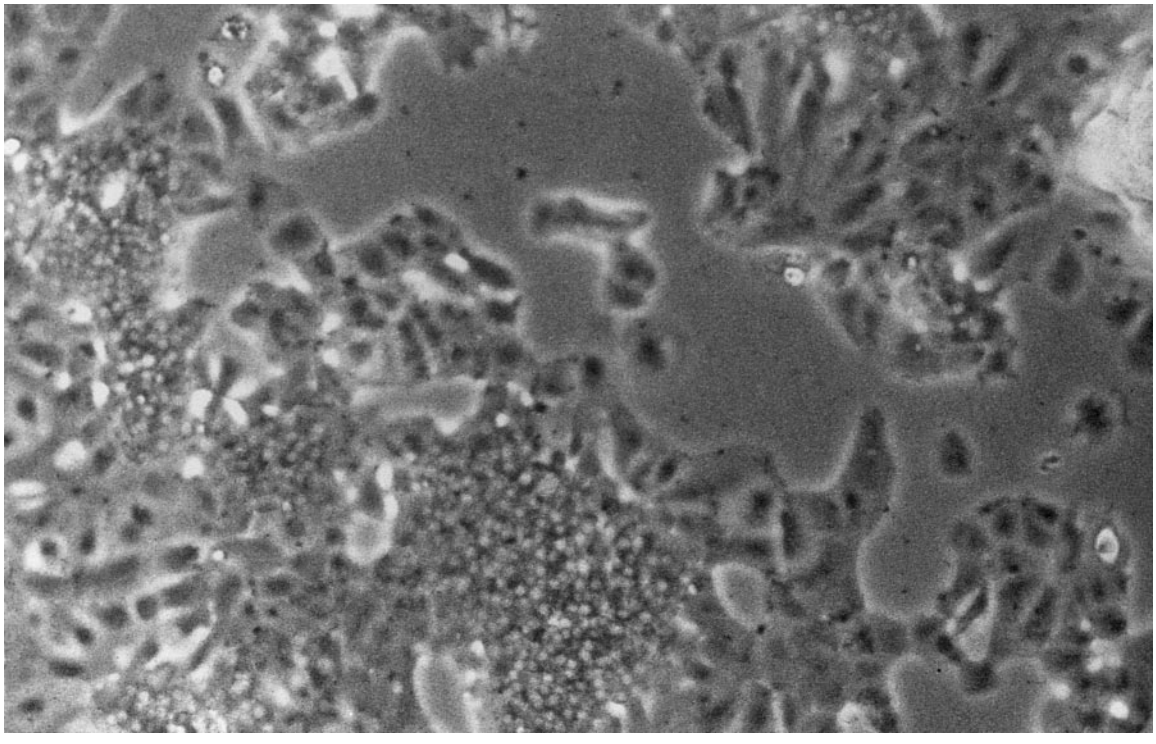

FIG. 1. Phase contrast light microscopy of human parathyroid cells in culture. Cells in the cluster exhibited a typical epithelial-like morphology with round elements in the center (×100).

60-μm and 150-μm mesh screens (Sigma, Milan, Italy). Cells harvested through the 150-μm filter were collected and distributed into 100 mm plastic tissue culture dishes in growth medium. Cells were cultured at 37°C in a humidified 95% air/5% CO_2 atmosphere.

Morphological investigations. Phase-contrast light microscopy was employed to examine the growth of the cell monolayer and the morphological characteristics of the cells. Moreover, approximately 5×10^6 cells were collected and prepared for transmission electron microscopy (TEM). Cells pellets were fixed in 4% cold glutaraldehyde in 0.1 M cacodilate buffer and postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer at room temperature. The pellet was dehydrated in a graded acetone series, passed through propylene oxide and embedded in Epon 812. Ultrathin sections were cut and stained with uranyl acetate and alkaline bismuth subnitrate, and examined under a Siemens Elmiscop 102 electron microscope at 80 kV.

TRAP assay. The TRAP assay was performed according to the method described by Kim *et al.* (1) with some modifications (6). MCF-7 cells were used as positive control (1, 7). The TRAP assay was performed in homogenates of parathyroid tissues and of cell cultures. Total protein concentration in the homogenate was measured using the Bradford protein assay method (8). The PCR product was resolved by electrophoresis on 10% nondenaturing polyacrylamide gel, which was then autoradiographed. Telomerase activity was determined to be positive when a 6-bp ladder pattern of bands, each representing the addition of a hexanucleotide telomeric repeat was observed after 24 h exposure at −80°C. A dilution series of control telomerase extracts (12 μg, 6 μg, 1.2 μg and 0.6 μg of total protein) were always examined in parallel to give a titration curve for normalizing experimental variations.

TP53 PCR-single strand conformation polymorphism (SSCP) analysis. The SSCP technique (9) was used for its high sensitivity (90–100%) to detect TP53 mutations through the analysis of gene fragments mobility shift and DNA sequencing. In parathyroid carcinoma specimens TP53 gene mutations screening kit (ANALITICA Padova, Italy) was used to detect the presence of any kind of mutation at exons 5, 6, 7 and 8 of TP53 gene, according to the manufacturer's instructions. Briefly, 100 ng of DNA from the nuclear pellet was used in PCR reactions with five different pairs of primers (supplied in the kit) designed to span exons 5 to 8. Concentrations of $MgCl_2$ were 1.5 mM for primers A, B, C and E and 2 mM for primer D. Annealing temperatures were 55°C for primers A and E and 60°C for primers B, C and D. Cycle steps of PCR were 2′ at 95°C, 40 cycles 35 s at 94°C, 40 s at the relative annealing temperature, 40 s at 72°C and a final extension step at 72°C. PCR products were analyzed on a 2% agarose gel for specificity of reaction. Two microliters of PCR product were added with 6 μl of denaturing loading buffer (95% v/v formamide, 10 mM EDTA, 0.1% Basic Fuchsin, 0.01% Bromophenol Blue), denatured at 95°C for 5′ and loaded on a 12% acrilamide gel in TBE. Running conditions were 5 mA for 14 to 18 h in TBE 1X at 20°C through water recycling. At the end of the run the gels were silver stained as it follows: 10% ethanol for 5′ 1% nitric acid for 3′, 2 mM silver nitrate for 20′, three washes with 0.019% formaldehyde in sodium carbonate 0.28 M, 10% acetic acid for 2′.

RESULTS

Morphological Appearance of Human Parathyroid Cells in Primary Culture

At 24 h, nearly all cells were adherent and displayed clusters containing round and polygonal epithelial-like cells (Fig. 1), rarely surrounded by isolated spindle-shaped cells. Cells maintained in growth medium reached the confluence in about four days. TEM analysis was performed on primary culture and revealed a cell layer with cytoplasm containing numerous mitochondria, Golgi apparatus and a well developed rough

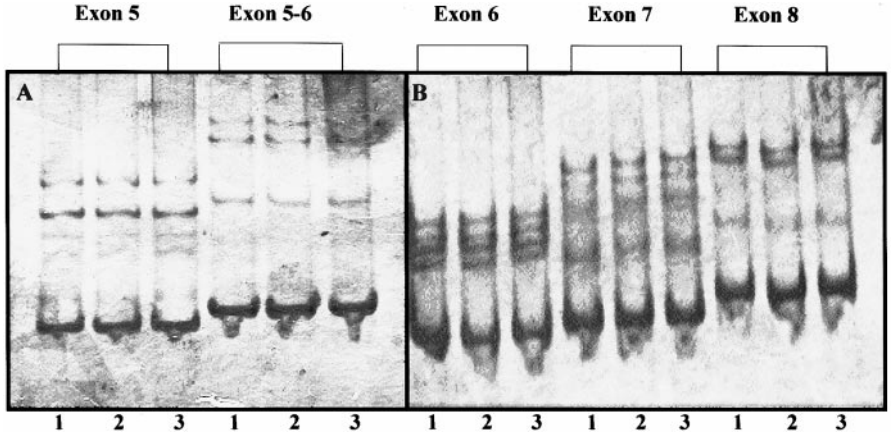

FIG. 2. Mutational analysis of TP53 exons 5 to 8 (A and B). Lane 1 depicts the negative control (normal patient constitutive DNA), lanes 2 and 3 represent respectively parathyroid carcinomatous tissue and skin metastasis from a different parathyroid carcinoma. The migration pattern was identical for the two samples compared to the normal control, indicating that no mutation is present in exons 5 to 8 of the TP53 gene.

endoplasmic reticulum. Typically electron-dense round inclusions, corresponding to secretory granules were observed.

TP53 Mutational Analysis

Migration pattern was identical for the two samples compared to the normal control indicating that no mutation is present in exons 5 to 8 of the TP53 gene (Fig. 2).

TRAP Assay

The parathyroid carcinoma, the metastatic tissue and the primary cell cultures from parathyroid carcinomatous tissue exhibited a very high telomerase activity as compared to the positive control (1). The amplified telomerase products were heterogeneous in length and created a ladder pattern of bands each representing the addition of a hexanucleotide telomeric repeat by telomerase (Fig. 3). A reduced signal intensity of each pattern of bands was observed to correlate to the different dilutions of samples and the distribution of the bands did not change in serially diluted extracts (Fig. 3). This band pattern was not observed when the extracts were pre-treated with RNase A, Proteinase K or heat or when the PCR primers or cell extracts were not included (data not shown).

DISCUSSION

Parathyroid carcinoma is a very rare disease occurring in 0.5–2% of cases exhibiting clinical features of primary hyperparathyroidism. It is difficult to properly identify the spectrum of clinical presentation, degree of malignancy and prognosis of parathyroid carcinoma and, therefore, the major problem in these patients is failure to perform adequate resection of the carcinomatous parathyroid tissue. Loco-regional lymphonodes metastasis can be already present at the moment of diagnosis. Although, several histological markers have been used to differentiate benign from malignant parathyroid lesions, most of them resulted to be of complex applicability, making difficult the prediction of the clinical prognosis by histopathological criteria without clinical evidence (10).

The possibility that genetic markers may become prognostic indicators for parathyroid carcinoma has been taken in serious consideration. Evidence supports that complete inactivation of the *RB* tumor suppressor gene may be a key factor in the pathogenesis of parathyroid carcinoma (3). Clonal analysis of sporadic and familial forms of parathyroid carcinoma unraveled

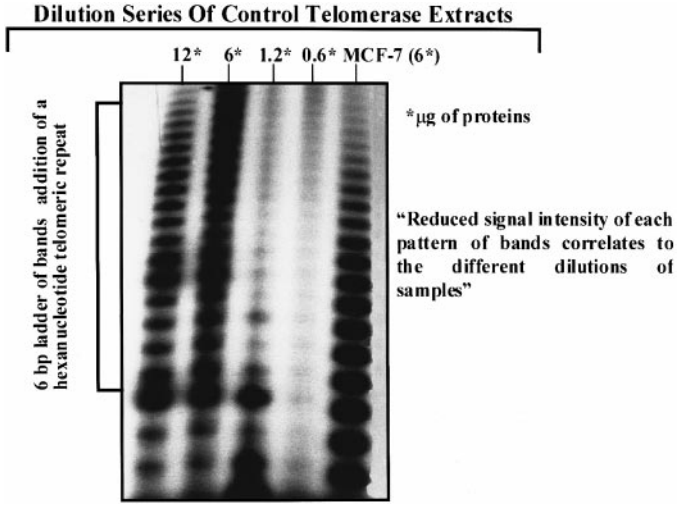

FIG. 3. TRAP assay. The amplified telomerase products exhibited heterogeneity of length creating a ladder pattern of bands each representing the addition of a hexanucleotide telomeric repeat by telomerase. Protein concentrations from cell lysate are depicted in the upper part of the figure (*).

presence of gross deletions on chromosome 13q12.3-32 with consequent loss of one copy of *Rb* and *BRCA2* tumor suppressor genes, both mapping in this region (4). Other genes are also being assessed for their potential contribution to parathyroid carcinomatosis. In fact, a small subgroup of parathyroid carcinomas exhibited allelic loss at the TP53 locus and abnormal expression of p53 protein, even though SSCP mutational analysis of TP53 5-9 exons failed to reveal any mutation of the gene (11).

In our series were unable to detect any mutation in 5 to 8 exon sequences, where 90% of mutations associated to various human neoplasias have been located (12). However, DNA mutations of p53 gene in exons 2–4 and 9–11, in which frame-shift insertions/deletions occur at higher rate than in exons 5–8 (13) cannot be excluded.

Moreover, in breast cancers tissues with high levels of telomerase activity p53 protein accumulation was shown without p53 mutations (14), suggesting the latter to be not necessary for malignant tissue transformation.

From the present observations it appears clear that telomerase activity segregates with a parathyroid malignant phenotype and even though expression of telomerase activity *per se* is uncapable to immortalize human cells, combination with cell cycle disrupting events it may enhance the immortalizing process (7). Therefore, telomerase activity could represent an important molecular mechanism underlying the acquisition and progression of an aggressive phenotype of epithelial parathyroid cells and it may help to predict their malignant potential. Being the TRAP assay sufficient to determine the presence/absence of telomerase in tissues where expression in normal cells is lacking or extremely poor, telomerase activity assayed by the TRAP protocol could be used by pathologists in the examination of parathyroid tissue specimens and of cells obtained from the parathyroid fine needle aspiration (15).

In conclusion, TRAP assay is an easy and sensitive assay that could become an additional tool to include in the screening of parathyroid proliferative disorders. The recent cloning of the RNA component and of the catalytic sub-unit of telomerase opened new opportunities in both analysis and detection of tissue telomerase-RNA (hTR) and -protein (hTERT) components (16). This approach could make possible in the near future retrospective analysis in fixed parathyroid tissues (17). Finally, the understanding of the enzyme regulatory mechanisms and the development of novel anti-telomerase cancer drugs could open novel therapeutic avenues in a number of malignancies, including parathyroid carcinoma.

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REFERENCES

1. Kim, N. W., Piatyszck, 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. (1994) *Science* **226,** 2011–2015.
2. Shay, J. W. (1997) *J. Cell. Physiol.* **173**(2), 266–270.
3. Cryns, V. L., Thor, A., Xa, H. J., Hu, S. X., Wierman, M. E., Vickery, A. L. Jr., Benedict, W. F., and Arnold, A. (1994) *N. Engl. J. Med.* **330**(11), 757–761.
4. Pearce, S. H., Trump, D., Wooding, C., Sheppard, M. N., Clayton, R. N., and Thakker, R. V. (1996) *Clin. Endocrinol.* **45**(2), 195–200.
5. Yoshimoto, K., Endo, H., Tsuyuguchi, M., Tanaka, C., Kimura, T., Iwahana, H., Kato, G., Sano, T., and Itakura, M. (1998) *Clin. Endocrinol.* **48**(1), 67–72.
6. Kiyono, T., Scott, A. F., Koop, J. I., McDougall, J. K., Galloway, D. A., and Klingelhutz, A. J. (1998) *Nature* **396,** 84–88.
7. Soule, H. D., Vazquez, J., Long, A., Albert, S., and Brennan, M. (1973) *J. Natl. Cancer Inst.* **51,** 1409–1416.
8. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
9. Hayashi, K., and Yandell, D. W. (1993) *Hum. Mutat.* **2**(5), 338–346.
10. Dionisio, P., Stramignoni, E., Passaroni, G., Pucci, A., Valentim, M., Berto, I. M., Portigliatti Barbos, M., Cadario, A., Gasparri, G., and Bajardi, P. (1996) *Nephron* **74**(4), 720–723.
11. Cryns, V. L., Rubio, M. F., Thor, A. D., Louis, D. N., and Arnold, A. (1994) *J. Clin. Endocrinol. Metab.* **78**(6), 1320–1324.
12. Hollstein, M., Sidransky, D., Vogelstein, B., and Harrism, C. C. (1991) *Science* **253,** 49–53.
13. Perwez Hussain, S., and Harris, C. C. (1998) *Cancer Res.* **58,** 4023–4037.
14. Roos, G., Nilsson, P., Cajander, S., Nielsen, N. H., Arnerlov, C., and Landberg, G. (1998) *Int. J. Cancer* **79**(4), 343–348.
15. Aogi, K., Kitahara, K., Buley, I., Backdahl, M., Tahara, H., Sugino, T., Tarin, D., and Goodison, S. (1998) *Clin. Cancer Res.* **4**(8), 1965–1970.
16. McKenzie, K. E., Umbricht, C. B., and Sukumar, S. (1999) *Mol. Med. Today* **5,** 114–122.
17. Soder, A. I., Going, J. J., Kaye, S. B., and Keith, W. N. (1998) *Oncogene* **16,** 979–983.