

Simian Virus 40-Like Sequences from Early and Late Regions in Human Thyroid Tumors of Different Histotypes

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Simian virus 40 (SV40) sequences were investigated in human thyroid tumors of different histotypes, Graves' disease thyroid specimens, normal thyroid tissues, and peripheral blood mononuclear cells (PBMC) of healthy donors. Specific SV40 large T antigen (Tag) sequences were detected, by PCR and filter hybridization, in human thyroid tumors with a frequency ranging from 66% in papillary thyroid carcinomas (PTC) to 100% in anaplastic thyroid carcinomas (ATC). SV40 was revealed in 60% and 100% of normal thyroid tissues adjacent to PTC and ATC, respectively, but in only 10% of control normal thyroid tissues (NTT) from patients affected by multinodular goiter. Thyroid tissues from patients affected by the Graves' disease were found to be SV40 positive with a frequency of 20%. In agreement with previous investigations, the

presence of SV40 sequences was detected in 25% of PBMC of healthy individuals. SV40 Tag mRNA was detected by RT-PCR, whereas the viral oncoprotein was revealed by immunohistochemistry with a specific monoclonal antibody. The high prevalence of SV40 footprints in human thyroid tumors indicates that the oncogenic virus may participate as a cofactor in the onset/progression of specific human thyroid cancers. Detection of SV40 sequences in NTT adjacent to thyroid cancers suggests that the viral infection may spread from transformed cells to normal cells surrounding the tumor. The presence of the SV40 footprint in PBMC implies that blood cells are vectors of the virus in other tissues of the host. (*J Clin Endocrinol Metab* 88: 892–899, 2003)

SIMIAN VIRUS 40 (SV40) large T antigen (Tag) sequences have been detected with different prevalences by PCR and filter hybridization in human brain tumors (1–6), pleural mesotheliomas (7–11), bone tumors (6, 12–16), pituitary (17) and thyroid neoplasms (18), and lymphoproliferative disorders and lymphomas (19–23). Previous studies carried out by Southern blot hybridization technique with papillary thyroid and bone tumor DNAs confirmed the SV40 specificity of these sequences and assessed whether the viral DNA can be integrated into the human genome (14, 18). Moreover, SV40 Tag-coding sequences were detected in normal lung and pituitary tissues (9, 17), peripheral blood mononuclear cells (PBMC) of patients affected by osteosarcomas (15), PBMC from blood donors (2, 3, 6, 15, 19, 21), and sperm fluids of healthy individuals (3). SV40-neutralizing antibodies were found in human sera (24), and SV40 sequences have been detected in PBMC and brain tissue of monkeys (25). In most studies SV40 sequences were detected by PCR amplification, suggesting that SV40 infection in human tumors and normal tissues commonly results in a low viral load. Indeed, by a

semiquantitative PCR assay 10^{-4} – 10^{-2} SV40 genome equivalents/cell were determined in DNA samples from human lymphoproliferative disorders and PBMC of blood donors (19). SV40 reactivation, by transfection of SV40-positive human DNA into permissive monkey cells, was reported in only a single case (26), in agreement with the observation that in human cells, which are semipermissive, SV40 replicates poorly (27), reaches low viral titers (19, 28), and generates heterogeneous defective genomes at a high rate (6, 29).

SV40 transforms to the neoplastic phenotype cells from different species, including human cells (30, 31), and induces in rodents specific neoplasms, such as ependymomas, choroid plexus papillomas, osteosarcomas, soft tissue sarcomas, lymphomas, and mesotheliomas (32). SV40 immortalization, transformation, and oncogenicity are mediated by the Tag oncoprotein. The Tag of SV40 is a nuclear multifunctional phosphoprotein of 94 kDa that displays adenosine triphosphatase and helicase activities and induces viral and cellular DNA replication (33). Tag binds to p53, p105Rb1 and p130Rb2 tumor suppressor gene products and to p300 and p400 transcription coactivators, abolishing their functions (34). SV40 is clastogenic and mutagenic, inducing numerical and structural chromosome aberrations and gene mutations in human cells (35–37). SV40 Tag/p53 and SV40 Tag/pRb complexes were detected in human mesothelioma (38, 39)

Abbreviations: ATC, Anaplastic thyroid carcinoma; GD, Graves' disease; mab, monoclonal antibody; MTC, medullary thyroid cancer; NTT, normal thyroid tissue; PBMC, peripheral blood mononuclear cells; PTC, papillary thyroid carcinoma; RXR α , retinoid X receptor- α ; SV40, simian virus 40; Tag, large T antigen; TR α 1, thyroid hormone receptor- α 1.

and in brain tumor samples (5), thus adding further support to a role for SV40 in human tumorigenesis. The evidence that SV40 Tag sustains immortalization of human neoplastic cells is supported by experiments showing the induction of apoptosis in SV40 Tag-positive mesothelioma cells transfected with antisense Tag sequences (40).

SV40 is a monkey virus that was believed to be transmitted to humans only under exceptional situations in natural infection (32). SV40-contaminated vaccines (32), in particular antipolio vaccines, were administered to hundreds of millions of humans worldwide between 1955 and 1963 (34). However, the presence of this viral agent in humans, before the introduction of SV40-contaminated vaccines, cannot be discarded (41).

Malignant thyroid cancer encompasses a spectrum of different histotypes ranging in aggressiveness from the slow-growing, indolent papillary thyroid cancer (PTC) up to the rapidly fatal anaplastic thyroid cancer (ATC). Moreover, thyroid C cells may give rise to so-called medullary thyroid cancer (MTC), which has a malignant potential between papillary and follicular thyroid cancer (42). Specific oncogenes have been shown to be involved in thyroid carcinogenesis; *ras* (43) and peroxisome proliferator-activated receptor- γ 1 mutations have been reported in follicular thyroid cancer (44). The most important genetic alteration of PTC is the rearrangement of the *RET* proto-oncogene, producing several chimeric oncogenes, named *RET/PTC*. These rearrangements are present in nearly 50% of naturally occurring PTC and in nearly 80% of radiation-induced PTC (45–47). Tyrosine receptor kinase rearrangements are found in PTC at a low frequency (48), whereas the *MET* oncogene is overexpressed in 50% of the cases (49). ATC, the most aggressive thyroid cancer, is characterized by the presence of *p53* inactivating mutations in 22–83% of the cases (50). MTC can be either sporadic or hereditary, and the main genetic alterations of the diseases are somatic or germline *RET* gene point mutations, respectively (51, 52). Graves' disease (GD) is an organ-specific autoimmune disorder characterized by the presence of TSH receptor-stimulating antibodies leading to continuous stimulation of the follicular cells and consequent hyperthyroidism (53).

In a previous investigation, in a small proportion of papillary thyroid carcinomas, SV40-like DNA sequences have been found integrated into the genomic tumor DNA (18). These data were obtained by the low sensitive Southern blot hybridization technique, and they were limited to the papillary histotype.

In the present study we analyzed by the more sensitive PCR technique a new large series of human thyroid tumors of different histotypes and other benign thyroid disease samples. We also studied normal thyroid tissues (NTT) from patients affected by multinodular goiter and PBMC from blood donors. Three different SV40 genomic regions, corresponding to the Tag amino (N)-terminal, regulatory, and VP1 structural protein carboxyl (C)-terminal sequences, were analyzed. The SV40 specificity of different regions, amplified by PCR, was investigated by filter hybridization with internal oligoprobes and was further assessed by DNA sequencing. Tag expression was revealed by RT-PCR and immuno-

histochemistry with the specific monoclonal antibody (mab) Pab 101.

Materials and Methods

Patients, clinical specimens, and cell lines

In this study 109 patients, 80 females and 29 males, between 30 and 84 yr of age, were enrolled. Twenty-seven primary PTC, 2 lymph node metastases, and 10 NTT adjacent to the carcinoma were from 29 patients affected by PTC. Eighteen primary MTC and 2 lymph node metastases were obtained from 20 patients affected by MTC. Twenty primary ATC and 10 NTT near the tumor were from 20 ATC patients, whereas 20 GD thyroid tissues were from 20 GD patients. Twenty NTT were from 20 patients affected by multinodular goiter. In addition, 20 PBMC samples from individuals, relatives of patients affected by sporadic MTC, were analyzed. All samples were obtained from patients after informed consent.

Four human thyroid carcinoma cell lines, designated FRO and ARO from anaplastic carcinoma, WRO from follicular carcinoma, and NPA from poor differentiated papillary carcinoma, provided by Dr. J. Fagin (University of Cincinnati, Cincinnati, OH), were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 0.1 mg/liter streptomycin, 1 mM sodium pyruvate, and nonessential amino acids.

DNA purification

Fresh tissues were immediately frozen and kept at -80°C . Each sample was cut, minced, and digested with sodium dodecyl sulfate (1%) and proteinase K (500 $\mu\text{g}/\text{ml}$), followed by extraction with a mixture of phenol-chloroform-isoamyl alcohol (25:24:1). DNA was precipitated with ethanol/sodium acetate (0.2 M), resuspended in TE buffer (10 mM Tris-HCl, pH 8, and 1 mM EDTA), and stored at -20°C (6). Sections from formalin-fixed, paraffin-embedded tissues were extracted using a commercial kit (QIAGEN, Milan, Italy) following the manufacturer's instructions.

PCR, filter hybridization, and DNA sequence analyses

SV40 DNA from wild-type VA-45-54-1 (our laboratory) and 776 (Sigma-Aldrich, Milan, Italy) strains (34) were used as controls in PCR amplification and DNA sequence experiments. Each DNA sample was first tested for suitability for PCR by amplification of *p53* gene (exons 7–8) sequences (Table 1). Only positive samples were further investigated for amplification of SV40 sequences. All experiments were carried out in triplicate at the Section of Endocrinology, University of Pisa. Precautions to avoid PCR contamination were carefully taken. In the first step of our analysis, all samples were screened for the highly conserved SV40 Tag sequences of 172 bp, coding for the N-terminal portion of the oncoprotein (1) using the SV40 detection kit (Poisys Research, Trieste, Italy; Table 1). Positive samples were further analyzed for regulatory and VP1 C-terminal sequences (Table 1).

DNA was amplified for 35 cycles in a total volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 200 μM deoxy-NTP, 50 pmol of each primer, and 1 U of *Taq* DNA polymerase (Amersham Pharmacia Biotech, Milan, Italy). Primers, oligoprobes, annealing temperatures, and PCR product size are reported in Table 1. Ten microliters of each PCR reaction were loaded on 2% agarose gel and electrophoresed in $1\times$ TAE (40 mM Tris acetate and 1 mM EDTA, pH 8), stained by ethidium bromide, and photographed. DNA was transferred to nylon membranes and cross-linked to filter by UV irradiation (3, 6). All filters were hybridized to SV40-specific internal oligoprobes (Table 1) at 42°C in $5\times$ SSC (3 M NaCl and 0.3 M sodium citrate), 0.1% sodium dodecyl sulfate, block solution, and 0.5% dextran sulfate (Amersham Pharmacia Biotech). Oligoprobes were 3'-end labeled employing the enhanced chemiluminescence labeling kit and revealed by a chemiluminescent reagent (Amersham Pharmacia Biotech) (6). The stringency of the final wash was adjusted according to the melting temperature. Filters were exposed to x-ray films (Kodak, Rochester, NY) for 15–60 min. PCR-amplified products were DNA-sequenced by Sanger's technique with the USB Sequenase kit (Amersham Pharmacia Biotech) or auto-

TABLE 1. Oligonucleotides used as primers in PCR and as probes in filter hybridization

SV40 DNA regions	Oligonucleotides ^a	Reference position ^b	T ^c	Size (bp)
Tag NH2	PYV for: 5'-TAGGTGCCAACCTATGGAACAGA-3'	nt 4574–4552	54	172
	PYV rev: 5'-GAAAGTCTTTAGGGTCTTCTACC-3'	nt 4403–4425		
	SV probe: 5'-ATGTTGAGAGTCAGCAGTAGCC-3'	nt 4452–4473		
Regulatory	RA1: 5'-AATGTGTGTGTCAGTTAGGGTGTG-3'	nt 266–245	55	314
	RA2: 5'-TCCAAAAAGCCTCCTCACTACTT-3'	nt 5195–5218		
	R probe: 5'-TTAGTCAGCCATGGGGCGGAGA-3'	nt 29–50		
VP1 COOH	LA1: 5'-GGGTGTTGGGCCCTTGTGCAAAGC-3'	nt 2251–2274	67	294
	LA2: 5'-CATGTCTGGATCCCCAGGAAGCTC-3'	nt 2545–2522		
	L probe: 5'-TTAACAGGAGGACACAGAGGGTGGATGG-3'	nt 2432–2460		
Human p53 gene, exons 7–8	For: 5'-ATCCTGAGTAGTGGTAATCT-3'	nt 14441–14460	55	151
	Rev: 5'-TACCTCGCTTAGTGCTCCCT-3'	nt 14591–14572		

^a Oligonucleotides used as primers in PCR and as probes in filter hybridizations.

^b nt, Reference nucleotide positions in SV40 strain 776 (71) and human p53 gene (European Molecular Biology Laboratory/GenBank accession no. X54156).

^c T, PCR annealing temperature (C).

matically with an ABI PRISM 310 apparatus (PE Applied Biosystems, Foster City, CA) (6).

RT-PCR and immunohistochemistry

RNA was extracted from frozen tissue samples with RNazol according to the manufacturer's protocol (Tel-Test, Friendswood, TX), whereas from formalin-fixed paraffin-embedded sections that were deparaffinized with xylol and digested with proteinase K (Roche, Milan, Italy), the RNA was purified with a mixture of phenol/chloroform/isoamyl alcohol (25:24:1). The concentration of RNA was determined by spectrophotometry. Five micrograms of cytoplasmic RNA were treated with 10 U deoxyribonuclease I (Amersham Pharmacia Biotech) for 20 min at 37 C, purified with phenol/chloroform, and precipitated with ethanol/0.3 M sodium acetate. RNA was resuspended in diethylpyrocarbonate-treated water and retrotranscribed with 500 ng random examers, 20 U AMV retrotranscriptase (Promega Corp., Milan, Italy), 80 U RNasin (Promega Corp.), and 100 μM deoxy-NTP, buffer 1× [50 mM Tris-HCl (pH 8.3), 50 mM KCl, 10 mM MgCl₂, 10 mM dithiothreitol, and 0.5 mM spermidine] in 100 μl. cDNA was then PCR amplified with primers specific for the Tag N-terminal region using an annealing temperature of 54 C and 45 cycles (3, 6) (Table 1).

Immunohistochemistry was carried out in samples previously found to be SV40⁺ to investigate the expression of SV40 Tag oncoprotein. SV40 Tag was analyzed with the mab Pab 101 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which recognizes a specific C-terminal epitope of the viral oncoprotein (3). All tissues were fixed in 10% formalin, dehydrated, and paraffin-embedded at the Section of Pathology, University of Pisa. Thin sections (5 μm) were deparaffinized with xylol and rehydrated with ethanol solutions. All slides were subjected to antigen retrieval using 10% citrate buffer in a microwave oven, as indicated by the supplier (Milestone, Bergamo, Italy). Washes were performed with PBS for 5 min. Endogenous peroxidase activity was blocked with 5% H₂O₂ for 15 min. Tissue sections were incubated with the purified Tag mab, diluted 1:100, at room temperature for 1 h, and subjected to avidin and biotin block for 20 min each, to streptavidin-peroxidase for 10 min, and to diaminobenzadine chromogen substrate for 5 min. The sections were then counterstained with hematoxylin. COS-7 monkey cells expressing SV40 Tag and normal thyroid glands (SV40-negative) were used in each experiment as SV40-positive and SV40-negative controls, respectively (3).

Statistical analysis

Data were analyzed by the univariate statistics test for difference between two independent groups. The prevalence of SV40 Tag N-terminal coding sequences in each thyroid tumor type was compared with the control represented by NTT from patients affected by multinodular goiter. *P* value less than 0.05 was considered statistically significant.

Results

PCR analysis of SV40 sequences in human thyroid tumors, NTT, and PBMC from donors

In this investigation human thyroid tumors of different histotypes, NTT from patients affected and not affected by thyroid carcinomas, GD thyroid tissues, and PBMC from blood donors were analyzed by PCR for sequences of three different SV40 genomic regions (Table 1). In the first step, DNA samples were analyzed by PCR for the conserved SV40 Tag N-terminal coding sequences by the PYV set of primers, which efficiently amplify these viral sequences (1, 3) (Table 1). Tumor samples were found SV40-positive with high prevalence, ranging from 66% of PTC to 100% of ATC (Fig. 1 and Table 2). A similar prevalence, ranging from 60–100%, was detected in the corresponding NTT surrounding the thyroid tumors, whereas in the thyroid tissue from patients affected by multinodular goiter and GD thyroid specimens, the frequency of SV40 sequences was of 10% and 20%, respectively (Fig. 1 and Table 2). PBMC from blood donors were also SV40 positive at the 25% (Fig. 1 and Table 2). Four human thyroid carcinoma cell lines used as a control, designated FRO and ARO from anaplastic carcinoma, WRO from follicular carcinoma, and NPA from poor differentiated papillary carcinoma, were all SV40 negative (Table 2). The different frequencies of SV40 Tag N-terminal coding sequences detected in PTC, MTC, and ATC compared with NTT are statistically significant (Table 2).

Samples found positive for SV40 Tag N-terminal coding sequences were further investigated for the SV40 regulatory and VP1 regions. The SV40 regulatory region (Table 1) was detected in 13 of 19 (68%) PTC, 15 of 18 (83%) MTC, and 15 of 20 (75%) ATC (Table 2). Sixteen normal thyroid tissues adjacent to the tumor, obtained from 6 PTC and 10 ATC patients, respectively, were positive for the SV40 regulatory region, whereas 4 GD thyroid specimens were all negative for the same region (Table 2). The SV40-VP1 sequences were detected in samples positive for the Tag-coding sequences with a prevalence similar to that in the regulatory region (Table 2). To assess further the specificity of SV40 sequences, 7 samples (5 PTC, 1 MTC, and 1 thyroid tissue from a GD specimen) that showed strong signals in hybridization experiments were DNA se-

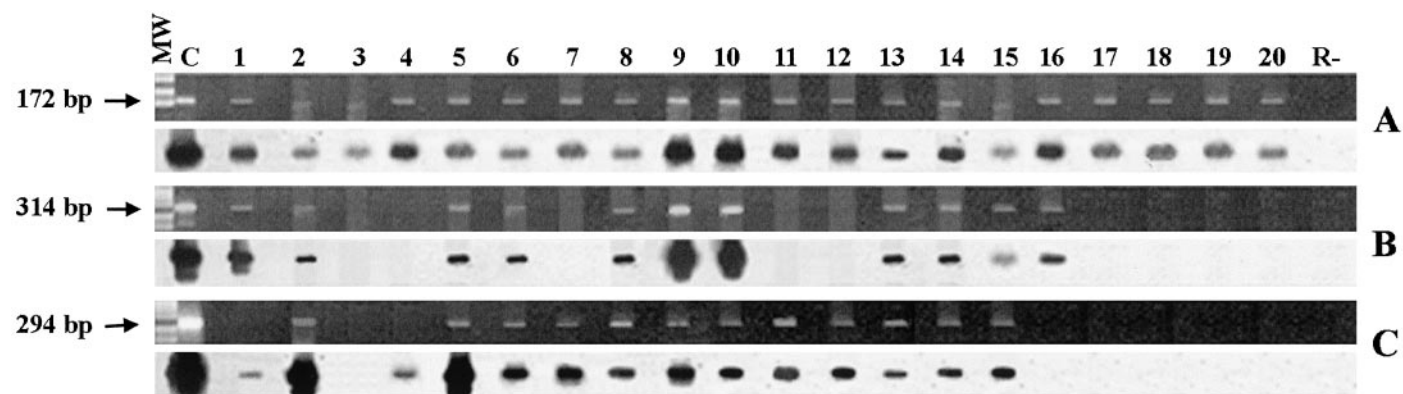


FIG. 1. Agarose gel electrophoresis of PCR-amplified SV40 regions stained by ethidium bromide and hybridization with specific internal oligoprobes. MW, Molecular weight markers (marker IV, Roche). Lane C, Positive control represented by SV40 DNA, strain 776. Lanes 1–3, PTC samples. Lanes 4–6, MTC samples. Lanes 7–10, ATC samples. Lanes 11 and 12, GD samples. Lanes 13 and 14, NTT from PTC samples. Lanes 15 and 16, NTT from ATC specimens. Lanes 17 and 18, NTT from multinodular goiter samples. Lanes 19 and 20, PBMC (Table 2). Lane R–, negative control of the PCR reaction without DNA template. A, Tag N-terminal region amplified with primers PYV.for-PYV.rev (top) and hybridized with the internal SV oligoprobe (bottom). The arrow indicates the product size obtained by PCR (172 bp). B, Regulatory region amplified with primers RA1-RA2 (top) and hybridized with the internal R oligoprobe (bottom). The arrow indicates the product size obtained by PCR (314 bp). C, VP1 region amplified with primers LA1-LA2 (top) and hybridized with the internal L oligoprobe (bottom). The arrow indicates the product size obtained by PCR (294 bp; see Table 1).

TABLE 2. SV40-like sequences in human thyroid tumors, tumor cell lines, normal tissues, and PBMC

Tissues and cell lines ^a	Tag N-terminal sequences ^b	Regulatory sequences ^b	VP1 sequences ^b	Statistical analysis ^c
Primary thyroid carcinoma				
Papillary	19/29 (66)	13/19 (68)	12/19 (63)	$P = 0.02$
Medullary	18/20 (90)	15/18 (83)	16/18 (88)	$P = 0.01$
Anaplastic	20/20 (100)	15/20 (75)	15/20 (75)	$P = 0.01$
Thyroid tumor cell lines	0/4			
GD tissue	4/20 (20)	0/4	4/4 (100)	
Normal thyroid tissues				
Adjacent to PTC	6/10 (60)	6/6 (100)	6/6 (100)	
Adjacent to ATC	10/10 (100)	10/10 (100)	7/10 (70)	
Patients with multinodular goiter	2/20 (10)	0/2	0/2	
PBMC	5/20 (25)	ND	ND	

ND, Not determined.

^a Total specimens: 129 biopsies, 4 cell lines, and 20 PBMC.

^b SV40 DNA sequences from different genomic regions were detected by PCR amplification (Table 1). Positive samples/samples analyzed, and percentage (%) are indicated.

^c Prevalence of SV40 Tag N-terminal coding sequences, in different thyroid tumor type, vs. control represented by NTT from patients affected by multinodular goiter.

quenced for the three regions investigated, *i.e.* Tag N-terminal, regulatory, and VP1 C-terminal sequences. DNA sequencing showed a complete identity with the SV40 wild-type strain 776. Only a single DNA sample from a GD tissue showed a silent point mutation, a C to T transition, at nucleotide 2482 of VP1 C-terminal coding sequences (Fig. 2).

Tag expression by RT-PCR and immunohistochemistry

Tag expression was investigated at the mRNA level by RT-PCR analysis, followed by filter hybridization with the specific internal oligoprobe SV (Table 1). The Tag transcript was searched in 24 thyroid cancer specimens previously found to be SV40-positive by PCR, whereas 30 samples found to be SV40 negative were used as negative controls (Table 3). Nine of 13 PTC and 8 of 11 ATC samples showed the expression of the mRNA specific of SV40 Tag, whereas none of the negative controls was positive for the Tag transcript (Fig. 3 and Table 3).

The SV40 Tag oncoprotein was analyzed by immunohistochemistry in 9 PTC and 8 ATC specimens found positive by RT-PCR for the specific Tag mRNA, whereas the negative controls were 30 SV40⁻ thyroid tissues (Table 3). Immunoreactive samples, 3 PTC and 8 ATC specimens (Table 3), showed SV40 Tag staining mainly in the cell cytoplasm. The percentage of SV40 Tag-positive cells in PTC and ATC specimens varied from sample to sample. In PTC samples T335 and T375, only 30% of the cells stained positively (Fig. 4B), whereas in PTC sample T405, the majority of cells showed a strong cytoplasmic signal (Fig. 4C). Similarly, the 8 ATC samples found positive for the Tag oncoprotein showed weak (5 specimens), medium (2 specimens), and strong (1 specimen) cytoplasmic staining (Fig. 4D). None of the negative controls immunoreacted for SV40 Tag oncoprotein (Table 3).

Discussion

In our study, carried out by PCR, filter hybridization, and DNA sequence analyses, SV40 sequences from different viral

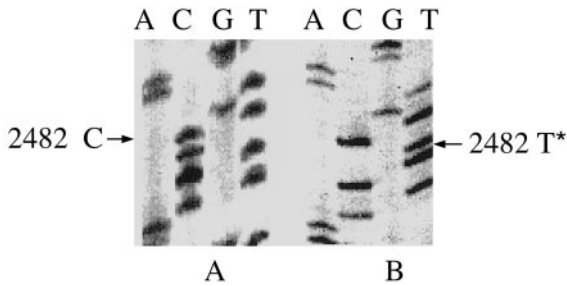


FIG. 2. DNA sequence analysis of the PCR amplified products from control DNA of SV40 wild-type strain 776 (A) and from a GD thyroid tissue (B). The SV40 DNA sequence is shown, from top to bottom, from nt 2474–2488, corresponding to the Tag carboxyl-terminal sequences. The SV40 DNA sequences in the SV40 wild-type strain 776 and in the GD sample were identical, except for the presence of a C to T transition at nucleotide 2482 (arrow), which is a silent point mutation.

TABLE 3. SV40 Tag mRNA expression and SV40 Tag oncoprotein in human thyroid tumors

Thyroid tissue	Tag mRNA ^a	Tag oncoprotein ^b
PTC	9/13 (69)	3/9 (33)
ATC	8/11 (73)	8/8 (100)
Negative control ^c		
PTC	0/10	0/10
GD	0/10	0/10
NTT	0/10	0/10

^a SV40 Tag mRNA was investigated by RT-PCR in specimens previously found SV40⁺ by PCR (Tables 1 and 2). Positive samples/samples analyzed, and percentage (%) are indicated.

^b SV40 Tag oncoprotein was investigated by immunohistochemistry with the mab Pab 101.

^c Negative controls are SV40⁻ specimens, *i.e.* 10 PTC, 10 GD, and 10 NTT from patients affected by multinodular goiter.

regions were detected in human thyroid tumors of different histotypes, in nonneoplastic and normal thyroid tissues, as well as in PBMC from blood donors. The use of this sensitive technique disclosed a much higher prevalence of SV40 sequences in human thyroid tumors compared with a previous study carried out in PTC by Southern blot hybridization (18). In the present investigation, SV40 sequences have been found with at high prevalence in primary PTC, MTC, ATC specimens, and NTT surrounding the tumors, whereas thyroid tissues of patients affected by GD and multinodular goiter and PBMC samples from blood donors were SV40 positive at a lower frequency. Statistical analyses indicate that the prevalence of SV40 Tag N-terminal coding sequences in each different thyroid tumor *vs.* NTT is statistically significant. In addition, the prevalence of SV40 Tag sequences in thyroid cancers correlates with the malignancy. DNA sequence analysis indicated that the different SV40 regions, amplified by PCR, belong to SV40 wild-type 776 strain and not to other simian, human, or recombinant polyomaviruses.

In this study, in agreement with previous reports, some, but not all, investigated SV40 genomic regions were detected in human thyroid specimens (6, 12, 15, 19, 54, 55). Indeed, it has been reported that 1) the primers employed vary in PCR amplification efficiency for different SV40 sequences present at low viral DNA load; 2) the SV40 sequences could not be detected because of mutations, deletions, or strain variations; and 3) the SV40 genomes could belong to defective viral

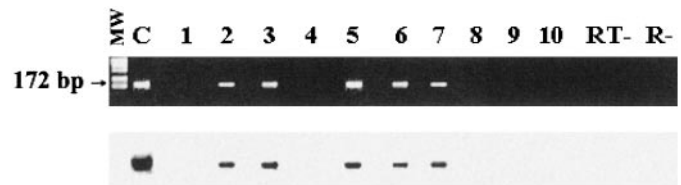


FIG. 3. Agarose gel electrophoresis of the RT-PCR-amplified early region stained by ethidium bromide (top) and filter hybridization with the specific internal SV oligoprobe (bottom). MW, Molecular weight markers (marker IV, Roche). Lane C, Positive control cDNA from COS-7 cells expressing the Tag. Lanes 1–3, PTC samples. Lanes 4–7, ATC samples. Lanes 8–10, Negative controls from PTC, GD, and NTT samples, respectively. Lane RT-, Negative control without RNA. Lane R-, Negative control without cDNA template. The arrow indicates the product size obtained by RT-PCR (172 bp).

DNA molecules that may occur in human cells even at low multiplicity of infection. It is also possible that some SV40 regions are not present in our samples (6, 12, 15, 19, 54, 55).

Tag expression was not revealed in all samples previously found to be SV40 positive by PCR. This result could be due to our conditions of RT-PCR, which did not allow detection of the SV40 Tag mRNA present in small amounts. None of the negative controls was positive for the SV40 Tag transcript. Immunohistochemistry analysis for the Tag oncoprotein carried out in thyroid cancers found positive for the Tag viral transcript shows a perfect identity in ATC specimens, whereas only 33% of PTC samples positive by RT-PCR stained positively for the Tag oncoprotein, probably because of the low amount of the viral product. In the 80% of the SV40 Tag-positive PTC and ATC samples, immunostaining was detected in only a fraction of the cells. This result is in agreement with the data from previous investigations, which detected a low SV40 load in human samples. SV40 Tag staining was mainly detected in the cytoplasm of both PTC- and ATC-positive samples. Other studies reported SV40 Tag staining in the cytoplasm (56). In the field, there is a general agreement for considering the cytoplasmic staining of a nuclear protein, such as SV40 Tag, a false positive result. However, a recent investigation reported the characterization of a cytoplasmic cell protein, named p193, belonging to the Bcl-2 family, which interacts specifically with SV40 Tag. The p193/SV40 Tag complex has been found in the cell cytoplasm of SV40-positive cells. Interestingly, it turned out that p193 is an apoptosis-promoting protein, and SV40 Tag bound to it inhibits p193 apoptotic activity (56). It is possible that in our samples the p193/Tag complex occurred, thus explaining the presence in the cytoplasm of the Tag viral oncoprotein. In this connection it should be pointed out that the apoptotic activity in thyroid tumor cells is very low (57).

It has been reported that the SV40 late promoter is regulated by the thyroid hormone receptor- α 1 (TR α 1) in combination with the retinoid X receptor- α (RXR α). The inhibition is relieved by the thyroid hormone T₃ (58, 59). The hypothesis is that TR α 1 and RXR α regulators block transcription of the late genes until the onset of viral replication (58, 59). The viral early genes are poorly transcribed when late genes are overexpressed, thus reducing the amount of Tag molecules, viral DNA copies, and virions (60). This mechanism seems cell type specific (60). TR α 1 and RXR α are present and active in follicular thyroid cells (61) and perhaps in parafollicular cells,

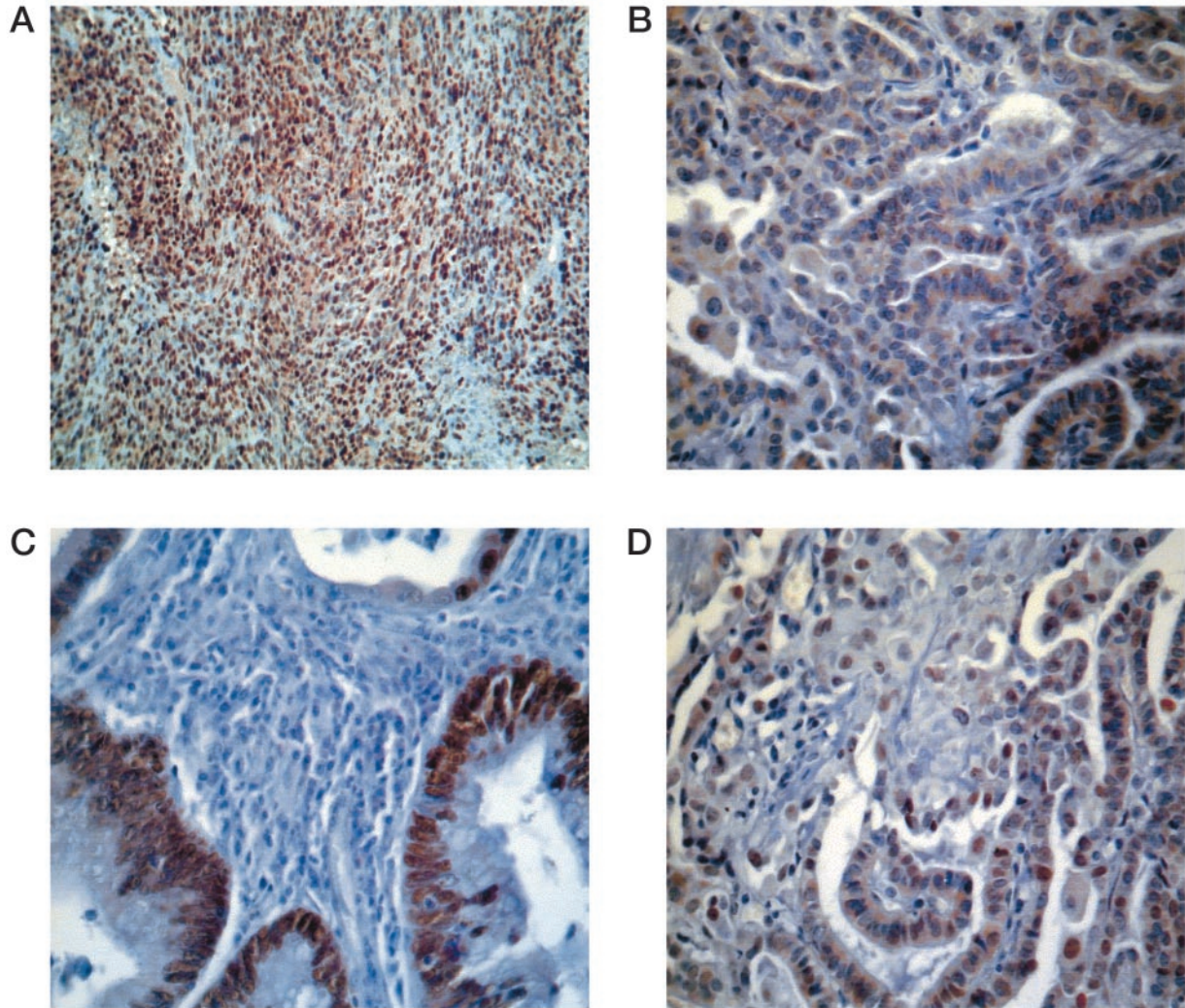


FIG. 4. Immunohistochemical analysis of SV40 Tag in thyroid carcinomas. A, COS-7 cells employed as a SV40 Tag-positive control. The Tag oncoprotein is revealed in the cell nucleus. B, PTC sample T335 with Tag cytoplasmic staining in approximately 30% of tumor cells. C, PTC sample T405 with Tag cytoplasmic staining in the majority of tumor cells. D, ATC sample A7 with a strong cytoplasmic staining revealed in cancer cells.

which are of neural origin. Recently, it has been reported that TR α 1 is present in the inactivated form, because of gene mutations, in approximately 60% of PTC (62). It is possible that the low levels of SV40 DNA replication, Tag expression, and virion production in thyroid tumor cells occur because the viral late gene promoter is not inhibited by TR α 1 and RXR α , either for the presence of thyroid hormone T₃, which relieves the TR α 1, or for the inactive TR α 1 detected in 60% of PTC.

In this study, which was mainly carried out by PCR, it was found that both neoplastic and normal thyroid tissues from patients affected by thyroid cancer are SV40 positive with high prevalence, suggesting that the SV40 infection may spread from neoplastic cells to the adjacent normal thyroid tissue.

The detection of SV40 DNA in PBMC of healthy individuals confirms the presence of these viral sequences in blood cells (2, 3, 6, 15, 19, 21). The detection of SV40 sequences in PBMC indicates that blood cells may transfer SV40 DNA/

virus to different tissues of the host. SV40 after PBMC infection, like JC and BK human polyomaviruses (32), could persist or remain latent for a long period in these cells.

The putative role of SV40 in human tumors is still a matter for investigation and discussion (32, 34). SV40 sequences were detected with different frequencies by several investigators in six different human tumors (brain and bone tumors, mesotheliomas, lymphomas, pituitary adenomas, and thyroid carcinomas) (32, 34, 54), whereas Tag oncogene expression was revealed in human tumor samples by different techniques (32, 34). These data suggest that SV40 is not a simple passenger virus in human tumor cells. Our data indicate that different SV40 DNA regions may be present with different prevalences. As human cells are considered semi-permissive, and SV40 generates in these cells defective DNA molecules at a high rate (6, 28), it is possible that SV40 regions absent in a fraction of our samples reflect the presence of incomplete genomes. Moreover, the presence of SV40 sequences in thyroid specimens is not always coupled to the

Tag detection, as observed before in other human tumors (34).

SV40 has a number of characteristics indicating that it may cooperate as a cofactor for the development or progression of human tumors (32, 34). SV40 cooperating with the *c-ras*-activated oncogene and the catalytic subunit of the telomerase transforms *in vitro* human fibroblasts (63) and astrocytes (64), and it induces malignant tumors in rodents and transgenic mice (32, 34). Moreover, SV40 Tag activates vascular endothelial growth factor expression (65, 66), and hepatocyte growth factor generating hepatocyte growth factor-scatter factor/*c-met* autocrine and paracrine loops which drive cell proliferation and invasiveness of both Tag-positive and Tag-negative cells (67). In this context it is worth reminding that hepatocyte growth factor-scatter factor/*c-met* and vascular endothelial growth factor are found overexpressed in PTC (68–70). Taken together our data and the results of other investigations suggest that the different transforming activities of SV40 may operate in human thyroid tissues during a persistent infection.

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References

- Bergsagel DJ, Finegold MJ, Butel JS, Kupsky WJ, Garcea RL 1992 DNA sequences similar to those of simian virus SV40 in ependymomas and choroid plexus tumors of childhood. *N Engl J Med* 326:988–993
- Martini F, De Mattei M, Iaccheri L, Lazzarin L, Barbanti-Brodano G, Gerosa M, Tognon M 1995 Human brain tumors and simian virus 40. *J Natl Cancer Inst* 87:1331
- Martini F, Iaccheri L, Lazzarin L, Corallini A, Gerosa M, Iuzzolino P, Barbanti-Brodano G, Tognon M 1996 Simian virus 40 early region and large T antigen in human brain tumors, peripheral blood cells and sperm fluids from healthy individuals. *Cancer Res* 56:4820–4825
- Huang H, Reis R, Yonekawa Y, Lopes JM, Kleihues P, Ohgaki H 1999 Identification in human brain tumors of DNA sequences specific for SV40 large T antigen. *Brain Pathol* 9:33–42
- Zhen HN, Zhang X, Bu XY, Zhang ZW, Huang WJ, Zhang P, Liang JW, Wang XL 1999 Expression of the simian virus 40 large tumor antigen (Tag) and formation of Tag-p53 and Tag-pRb complexes in human brain tumors. *Cancer* 86:2124–2132
- Martini F, Lazzarin L, Iaccheri L, Vignocchi B, Finocchiaro G, Magnani I, Serra M, Scotlandi K, Barbanti-Brodano G, Tognon M 2002 Different simian virus 40 genomic regions and sequences homologous to SV40 large T antigen in DNA of human brain and bone tumors and of leukocytes from blood donors. *Cancer* 94:1037–1048
- Carbone M, Pass HI, Rizzo P, Marinetti M, Di Muzio M, Mew DJ, Levine AS, Procopio A 1994 Simian virus 40-like DNA sequences in human pleural mesothelioma. *Oncogene* 9:1781–1790
- Pepper C, Jasani B, Navabi H, Wynford-Thomas D, Gibbs AR 1996 Simian virus 40 large T antigen (SV40LTAg) primer specific DNA amplification in human pleural mesothelioma tissue. *Thorax* 51:1074–1076
- Galateau-Sallé F, Bidet P, Iwatsubo Y, Gennetay E, Renier A, Letourneux M, Paireon JC, Moritz S, Brochard P, Jaurand MC, Freymuth F 1998 SV40-like DNA sequences in pleural mesothelioma, bronchopulmonary carcinoma, and non-malignant pulmonary diseases. *J Pathol* 184:252–257
- Pass HI, Donington JS, Wu P, Rizzo P, Nishimura M, Kennedy R, Carbone M 1998 Human mesotheliomas contain the simian virus-40 regulatory region and large tumor antigen DNA sequences. *J Thorac Cardiovasc Surg* 116:854–859
- Testa JR, Carbone M, Hirvonen A, Khalili K, Krynska B, Linnainmaa K, Pooley FD, Rizzo P, Rusch V, Xiao GH 1998 A multi-institutional study confirms the presence and expression of simian virus 40 in human malignant mesotheliomas. *Cancer Res* 58:4505–4509
- Carbone M, Rizzo P, Procopio A, Giuliano M, Pass HI, Gebhardt MC, Mangham C, Hansen M, Malkin DF, Bushart G, Pompetti F, Picci P, Levine AS, Bergsagel JD, Garcea RL 1996 SV40-like sequences in human bone tumors. *Oncogene* 13:527–535
- Lednický JA, Stewart AR, Jenkins JJIII, Butel JS 1997 SV40 DNA in human osteosarcomas shows sequence variability among T-antigen genes. *Int J Cancer* 72:791–800
- Mendoza SM, Konishi T, Miller CW 1998 Integration of SV40 in human osteosarcoma DNA. *Oncogene* 17:2457–2462
- Yamamoto H, Nakayama T, Murakami H, Hosaka T, Nakamata T, Tsuboyama T, Oka M, Nakamura T, Toguchida J 2000 High incidence of SV40-like sequences detection in tumour and peripheral blood cells of Japanese osteosarcoma patients. *Br J Cancer* 82:1677–1681
- Gamberi G, Benassi MS, Pompetti F, Ferrari C, Ragazzini P, Sollazzo MR, Molendini L, Merli M, Magagnoli G, Chiesa F, Gobbi AG, Powers A, Picci P 2000 Presence and expression of the simian virus-40 genome in human giant cell tumors of bone. *Genes Chromosomes Cancer* 28:23–30
- Woloschak M, Yu A, Post KD 1995 Detection of polyomaviral DNA sequences in normal and adenomatous human pituitary tissues using the polymerase chain reaction. *Cancer* 76:490–496
- Pacini F, Vivaldi A, Santoro M, Fedele M, Fusco A, Romei C, Basolo F, Pinchera A 1998 Simian virus 40-like DNA sequences in human papillary thyroid carcinomas. *Oncogene* 16:665–669
- Martini F, Dolcetti R, Gloghini A, Iaccheri L, Carbone A, Boiocchi M, Tognon M 1998 Simian-virus-40 footprints in human lymphoproliferative disorders of HIV⁻ and HIV⁺ patients. *Int J Cancer* 78:669–674
- Rizzo P, Carbone M, Fisher SG, Matker C, Swinnen LJ, Powers A, Di Resta I, Alkan S, Pass HI, Fisher RI 1999 Simian virus 40 is present in most United States human mesotheliomas, but it is rarely present in non-Hodgkin's lymphoma. *Chest* 116:470–473
- David H, Mendoza S, Konishi T, Miller CW 2001 Simian virus 40 is present in human lymphomas and normal blood. *Cancer Lett* 162:57–64
- Vilchez RA, Madden CR, Kozinetz CA, Halvorson SJ, White ZS, Jorgensen JL, Finch CJ, Butel JS 2002 Association between simian virus 40 and non-Hodgkin lymphoma. *Lancet* 359:817–823
- Shivapurkar N, Harada K, Reddy J, Scheuermann RH, Xu Y, McKenna RW, Milchgrub S, Kroft SH, Feng Z, Gazdar AF 2002 Presence of simian virus 40 DNA sequences in human lymphomas. *Lancet* 359:851–852
- Jafar S, Rodriguez-Barradas M, Graham DY, Butel J 1998 Serological evidence of SV40 infections in HIV-infected and HIV-negative adults. *J Med Virol* 54:276–284
- Lednický JA, Arrington AS, Stewart AR, Dai XM, Wong C, Jafar S, Murphey-Corb M, Butel JS 1998 Natural isolates of simian virus 40 from immunocompromised monkeys display extensive genetic heterogeneity: new implications for polyomavirus disease. *J Virol* 72:3980–3990
- Lednický JA, Garcea RL, Bergsagel DJ, Butel JS 1995 Natural simian virus 40 strains are present in human choroid plexus and ependymoma tumors. *Virology* 212:710–717
- Shein HM, Enders JF 1962 Multiplication and cytopathogenicity of simian vacuolating virus 40 in cultures of human tissues. *Proc Soc Exp Biol Med* 109:495–500
- Bocchetta M, Di Resta I, Powers A, Fresco R, Tosolini A, Testa JR, Pass HI, Rizzo P, Carbone M 2000 Human mesothelial cells are unusually susceptible to simian virus 40-mediated transformation and asbestos cocarcinogenicity. *Proc Natl Acad Sci USA* 97:10214–10219
- O'Neill FJ, Carroll D 1981 Amplification of papovavirus defectives during serial low multiplicity infections. *Virology* 112:800–803
- Topp WC, Lane D, Pollack R 1980 Transformation by SV40 and polyomavirus. In: Toozé J, ed. *DNA tumor viruses*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 205–296
- Bryan T, M, Reddel RR 1994 SV40-induced immortalization of human cells. *Crit Rev Oncol* 5:331–357
- Barbanti-Brodano G, Martini F, De Mattei M, Lazzarin L, Corallini A, Tognon M 1998 BK and JC human polyomaviruses and simian virus 40: natural history of infection in humans, experimental oncogenicity and association with human tumors. *Adv Virus Res* 50:69–99
- Fanning E, Knippers R 1992 Structure and function of simian virus 40 large tumor antigen. *Annu Rev Biochem* 61:55–85
- Butel JS, Lednický JA 1999 Cell and molecular biology of simian virus 40: implications for human infections and disease. *J Natl Cancer Inst* 91:119–134
- Ray FA, Peabody DS, Cooper JL, Scott-Cram L, Kraemer PM 1990 SV40 T antigen alone drives karyotype instability that precedes neoplastic transformation of human diploid fibroblasts. *J Cell Biochem* 42:13–31
- Stewart N, Bacchetti S 1991 Expression of SV40 large-T antigen, but not small-t antigen, is required for the induction of chromosomal aberrations in transformed human cells. *Virology* 180:49–57

37. Woods C, LeFeuvre C, Stewart N, Bacchetti S 1994 Induction of genomic instability in SV40 transformed human cells: sufficiency of the N-terminal 147 amino acids of large T antigen and role of pRB and p53. *Oncogene* 9:2943–2950
38. Carbone M, Rizzo P, Grimley P, M, Procopio A, Mew DJ, Shridhar V, de Bartolomeis A, Esposito V, Giuliano MT, Steinberg SM, Levine AS, Giordano A, Pass HI 1997 Simian virus-40 large-T antigen binds p53 in human mesotheliomas. *Nat Med* 3:908–912
39. De Luca A, Baldi A, Esposito V, Howard CM, Bagella L, Rizzo P, Pass HI, Giordano GG, Baldi F, Carbone M, Giordano A 1997 The retinoblastoma gene family pRb/p105, p107, pRb2/p130 and simian virus-40 large T-antigen in human mesotheliomas. *Nat Med* 3:913–916
40. Waheed I, Guo ZS, Chen GA, Weiser TS, Nguyen DM, Schrupp DS 1999 Antisense to SV40 early gene region induces growth arrest and apoptosis in T-antigen-positive human pleural mesothelioma cells. *Cancer Res* 59:6068–6073
41. Geissler E, Konzer P, Scherneck S, Zimmerman W 1985 Sera collected before introduction of contaminated polio vaccine contain antibodies against SV40. *Acta Virol* 29:420–423
42. Schlumberger M, Pacini F 1999 Thyroid tumors. *Paris: Nucleon*; 33–42
43. Wright PA, Lemoine NR, Mayall ES, Wyllie FS, Huges D, Williams ED, Wynford Thomas D 1991 Papillary and follicular thyroid carcinomas show a different pattern of ras gene mutation. *Oncogene* 6:471–473
44. Kroll TG, Sarrof P, Pecciarini L, Chen CJ, Mueller E, Spiegelman BM, Fletcher JA 2000 Pax8-PPAR γ 1 fusion oncogene in human thyroid carcinoma. *Science* 289:1357–1360
45. Grieco M, Santoro M, Berlingieri MT, Melillo RM, Donghi R, Bongarzone I, Pierotti MA, Della Porta G, Fusco A, Vecchio G 1990 PTC is a novel rearranged form of the RET proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinoma. *Cell* 60:557–563
46. Nikiforov YE, Rowland JM, Bove KE, Monforte-Munoz H, Fagin JA 1997 Distinct pattern of *ret* oncogene rearrangements in morphological variants of radiation-induced and sporadic thyroid papillary carcinomas in children. *Cancer Res* 57:1690–1694
47. Elisei R, Romei C, Vorontsova T, Cosci B, Veremeychik V, Kuchinskaya E, Basolo F, Demidchik EP, Miccoli P, Pinchera A, Pacini F 2001 RET/PTC rearrangements in thyroid nodules: studies in irradiated and not irradiated, malignant and benign thyroid lesions in children and adults. *J Clin Endocrinol Metab* 86:3211–3216
48. Suarez HG 1998 Genetic alterations in human epithelial tumors. *Clin Endocrinol (Oxf)* 48:531–546
49. Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pierotti S, Belfiore A, Costantino A, Vigneri R, Pierotti MA, Comoglio PM 1992 Overexpression of the c-Met/HGF receptor gene in human thyroid carcinoma. *Oncogene* 7:2549–2553
50. Fagin JA, Matsuo K, Karmakar A, Chen DL, Tang SH, Koeffler HP 1993 High prevalence of mutations of the p53 gene in poorly differentiated human thyroid carcinoma. *J Clin Invest* 91:179–184
51. Eng C 1999 Ret proto-oncogene in the development of human cancer. *J Clin Oncol* 17:380–393
52. Romei C, Elisei R, Pinchera A, Ceccherini I, Molinaro E, Mancusi F, Martino E, Romeo G, Pacini F 1996 Somatic mutations of the *ret* protooncogene in sporadic medullary thyroid carcinoma are not restricted to exon 16 and are associated with tumor recurrence. *J Clin Endocrinol Metab* 81:1619–1622
53. Davies TF 1996 The pathogenesis of Graves disease. In: Braverman LE, Utiger RD, eds. *Werner and Ingbar's the thyroid: a fundamental and clinical text*, 7th Ed. Philadelphia: Lippincott-Raven; 525–536
54. Jasani B, Cristaudo A, Emri SA, Gazdar AF, Gibbs A, Krynska B, Miller C, Mutti L, Radu C, Tognon M, Procopio A 2001 Association of SV40 with human tumors. *Semin Cancer Biol* 11:49–61
55. Tognon M, Martini F, Iaccheri L, Cultrera R, Contini C 2001 Investigation of the simian polyomavirus SV40 as a potential causative agent of human neurological disorders in AIDS patients. *J Med Microbiol* 50:165–172
56. Tsai SC, Pasumarthi KB, Pajak L, Franklin M, Patton B, Wang H, Henzel WJ, Stults JT, Field LJ 2000 Simian virus 40 large T antigen binds a novel Bcl-2 homology domain 3-containing proapoptosis protein in the cytoplasm. *J Biol Chem* 275:3239–3246
57. Basolo F, Pollina L, Fontanini G, Fiore L, Pacini F, Baldanzi A 1997 Apoptosis and proliferation in thyroid carcinoma: correlation with bcl-2 and p53 protein expression. *Br J Cancer* 75:537–541
58. Zuo F, JE Mertz JE 1995 Simian virus 40 late gene expression is regulated by members of the steroid/thyroid hormone receptor superfamily. *Proc Natl Acad Sci USA* 92:8586–8590
59. Zuo F, Kraus RJ, Gulick T, Moore DD, Mertz JE 1997 Direct modulation of simian virus 40 late gene expression by thyroid hormone and its receptor. *J Virol* 71:427–436
60. Farrell ML, Mertz JE 2002 Cell type-specific replication of simian virus 40 conferred by hormone response elements in the late promoter. *J Virol* 76:6762–6770
61. Schmutzler C, Brtko J, Winzer R, Jakobs TC, Meissner-Weigl J, Simon D, Goretzki PE, Kohrle J 1998 Functional retinoid and thyroid hormone receptors in human thyroid-carcinoma cell lines and tissues. *Int J Cancer* 76:368–376
62. Puzianowska-Kuznicka M, Krystyniak A, Madej A, Cheng SY, Nauman J 2002 Functionally impaired TR mutants are present in thyroid papillary cancer. *J Clin Endocrinol Metab* 87:1120–1128
63. Hahn WC, Counter CM, Lundberg AS, Beijersbergen RL, Brooks MW, Weinberg RA 1999 Creation of human tumour cells with defined genetic elements. *Nature* 400:464–468
64. Rich JN, Chuanhai G, McLendon Re, Bigner DD, Wang XF, Counter CM 2001 A genetically tractable model of human glioma formation. *Cancer Res* 61:3556–3560
65. Cacciotti P, Strizzi L, Vianale G, Iaccheri L, Libener R, Porta C, Tognon M, Gaudino G, Mutti L 2002 The presence of simian-virus 40 sequences in mesothelioma and mesothelial cells is associated with high levels of vascular endothelial growth factor (VEGF). *Am J Respir Cell Mol Biol* 26:189–193
66. Catalano A, Romano M, Martinotti S, Procopio A 2002 Enhanced expression of vascular endothelial growth factor (VEGF) plays a critical role in the tumor progression potential induced by simian virus 40 large T antigen *Oncogene* 21:2896–2900
67. Cacciotti P, Libener R, Betta P, Martini F, Porta C, Procopio A, Strizzi L, Penengo L, Tognon M, Mutti L, Gaudino G 2001 SV40 replication in human mesothelial cells induces HGF/Met receptor activation: a model for viral-related carcinogenesis of human malignant mesothelioma. *Proc Natl Acad Sci USA* 98:12032–12037
68. Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM 1991 Expression of the Met/HGF receptor in normal and neoplastic human tissues. *Oncogene* 6:1997–2003
69. Soh EY, Duh QY, Sobhi SA, Young DM, Epstein HD, Wong MG, Garcia YK, Min YD, Grossman RF, Siperstein AE, Clark OH 1997 Vascular endothelial growth factor expression is higher in differentiated thyroid cancer than in normal or benign thyroid. *J Clin Endocrinol Metab* 82:3741–3747
70. Lennard CM, Patel A, Wilson J, Reinhardt B, Tuman C, Fenton C, Blair E, Francis GL, Tuttle RM 2001 Intensity of vascular endothelial growth factor expression is associated with increased risk of recurrence and decreased disease-free survival in papillary thyroid cancer. *Surgery* 129:552–558
71. Fiers W, Contreras R, Haegemann G, Rogiers R, Van de Voorde A, Van Heuverswyn H, Van Herreweghe J, Volckaert G, Ysebaert M 1978 Complete nucleotide sequence of SV40 DNA. *Nature* 273:113–120