Herpes and polyoma family viruses in thyroid cancer (Review)

DIMITRIS P. STAMATIOU[1,2*], STAVROS P. DERDAS[2*], ODYSSEAS L. ZORAS[1] and DEMETRIOS A. SPANDIDOS[2]

[1]Department of Surgical Oncology, University Hospital, University of Crete, Heraklion 71003;
[2]Laboratory of Clinical Virology, University of Crete, Medical School, Heraklion 71409, Greece

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Abstract. Thyroid cancer is considered the most common malignancy that affects the endocrine system. Generally, thyroid cancer derives from follicular epithelial cells, and thyroid cancer is divided into well-differentiated papillary (80% of cases) and follicular (15% of cases) carcinoma. Follicular thyroid cancer is further divided into the conventional and oncocytic (Hürthle cell) type, poorly differentiated carcinoma and anaplastic carcinoma. Both poorly differentiated and anaplastic carcinoma can arise either *de novo*, or secondarily from papillary and follicular thyroid cancer. The incidence of thyroid cancer has significantly increased for both males and females of all ages, particularly for females between 55-64 years of age, from 1999 through 2008. The increased rates refer to tumors of all stages, though they were mostly noted in localized disease. Recently, viruses have been implicated in the direct regulation of epithelial-mesenchymal transition (EMT) and the development of metastases. More specifically, Epstein-Barr virus (EBV) proteins may potentially lead to the development of metastasis through the regulation of the metastasis suppressor, Nm23, and the control of Twist expression. The significant enhancement of the metastatic potential, through the induction of angiogenesis and changes to the tumor microenvironment, subsequent to viral infection, has been documented, while EMT also contributes to cancer cell permissiveness to viruses. A number of viruses have been identified to be associated with carcinogenesis, and these include lymphotropic herpesviruses, namely EBV and Kaposi's sarcoma-associated herpesvirus [KSHV, also known as human herpesvirus type 8 (HHV8)]; two hepatitis viruses, hepatitis B virus (HBV) and hepatitis C virus (HCV); human papillomaviruses (HPVs); human T cell lymphoma virus (HTLV); and a new polyomavirus, Merkel cell polyomavirus identified in 2008. In this review, we examined the association between thyroid cancer and two oncogenic virus families, the herpes and polyoma family viruses, and we discuss their potential role as causative agents in thyroid carcinogenesis.

Correspondence to: Dr Stavros P. Derdas, Laboratory of Clinical Virology, University of Crete, Medical School, Heraklion 71409, Greece
E-mail: derdas@med.uoc.gr

*Contributed equally

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Contents

1. Introduction

Thyroid cancer is the most common malignancy of the endocrine system (1,2). The main types of thyroid cancer derive from follicular epithelial cells, including well-differentiated papillary (80% of cases) and follicular (15% of cases) carcinoma, the latter being further divided into conventional and oncocytic (Hürthle cell) type, poorly differentiated carcinoma and anaplastic carcinoma (2-4). Both poorly differentiated and anaplastic carcinoma can arise either *de novo*, or secondarily from papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) (2-4). Patients with early stage well-differentiated papillary or follicular carcinoma usually have an excellent prognosis, unlike those with either aggressive tumors or distant metastases, who have a 5-year survival rate of 40% (5). Unlike the 3 aforementioned types of thyroid cancer, medullary cancer, derives from the neural crest, more specifically, the parafollicular, or C cells (6). It accounts for 3-4% of all thyroid cancer cases (7,8), and its clinical course varies from indolent, to rather aggressive, with associated high mortality rates (6). Primary thyroid lymphoma represents a rare non-Hodgkin lymphoma (9), deriving predominantly from B-lymphocytes (10).

Thyroid cancer predisposition factors include a history of radiation exposure through either medical treatment, or fallout from nuclear accidents during childhood (11), thyroid nodules or goiter, as well as family history of thyroid cancer (11). From 1999 through 2008, the incidence of thyroid cancer has increased significantly for both individuals of both genders, and in particular for females aged between 55 and 64 years (12). These increased rates refer to tumors of all stages, although they have been moslty noted in localized disease (12). The reasons

for this increase have not yet been completely elucidated (12). However, medical scrutiny, through ultrasound imaging and the fine needle aspiration cytological confirmation of small lesions that may have otherwise gone undiagnosed, has been proposed to account for the rising incidence rates (13,14). The increased incidence of both small and large tumors across genders and multiple racial/ethnic groups suggests that the former factor does not solely drive this trend (12,15-17). Chronic lymphocytic thyroiditis constitutes the sole risk factor predisposing to primary thyroid lymphoma acquisition, being present in 50% of cases, and increasing the risk by 60-fold (18,19).

Recently, knowledge of the genetic alterations involved in thyroid cancer, has markedly increased (1,2). More than 70% of PTCs contain BRAF, a serine-threonine kinase of the RAF protein family, and RAS, an intracellular G protein that propagates signals from receptor tyrosine kinases and G coupled receptors, point mutations, as well as *RET/PTC*, fusion between the 3' portion of the cell membrane receptor tyrosine kinase encoded by the *RET* proto-oncogene and the 5' portion of other unrelated genes, and *TRK* rearrangements (1,2,20-23). The above mutually exclusive mutations may potentially activate the mitogen-activated protein kinase (MAPK) pathway (1). As regards FTCs, mutually exclusive *RAS* point mutations and *PAX8*/peroxisome proliferator-activated receptor γ (*PPARγ*) rearrangements-from t(2;3)(q13;p25) translocation resulting in fusion of the *PAX8* gene, coding for the thyroid-specific paired domain transcription factor (2), and the *PPARγ* gene (24) have been identified in 70-75% of cases (25). Poorly differentiated and anaplastic carcinomas contain mutations concerning phosphatidylinositol-3'-kinase (PI3K)/AKT, a serine threonine kinase that acts as a key effector of PI3K (26) signaling pathway, as well as mutations of *TP53* and *CTNNB1*, encoding for β-catenin, the mutation of which is identified in 25% and 65% of poorly differentiated and anaplastic carcinomas, respectively (26), genes rarely found in well-differentiated papillary and follicular cancers (27). Even though the majority of medullary cancers are sporadic, almost 20% are hereditary, arising from a germline mutation in the *RET* proto-oncogene (6). Hereditary cases may either present isolated, or as part of multiple endocrine neoplasia syndrome type 2 (MEN2) (6).

The metastasizing ability of thyroid cancer is attributed to the induction of an epithelial-mesenchymal transition (EMT), that involves the invading ability of epithelial cells into the surrounding tissues (28). Despite the fact that oncogenic mutations have been associated with the induction of EMT, secondary factors are required (29). A number of viruses have been implicated in the direct regulation of EMT and in the development of metastases (29). More specifically, Epstein-Barr virus (EBV) proteins may potentially lead to the development of metastasis through the regulation of the metastasis suppressor, *Nm23* (30), and the control of *Twist* expression (31). The significant enhancement of the metastatic potential, through the induction of angiogenesis and changes to the tumor microenvironment, subsequent to viral infection, has been documented (32), while EMT also contributes to cancer cell permissiveness to viruses (33).

Almost 20% of cancer cases have been linked to infectious factors, including viruses (34,35). To date, 7 human viruses, strongly associated with carcinogenesis, have been identified and characterized as tumorigenic (34). These are 2 lymphotropic herpesviruses, namely EBV and Kaposi's sarcoma-associated herpesvirus [KSHV, also known as human herpesvirus type 8 (HHV8)]; 2 hepatitis viruses, hepatitis B virus and hepatitis C virus; human papillomaviruses (HPVs); human T cell lymphoma virus (HTLV); and a new polyomavirus (PyV), Merkel cell PyV (MCPyV) identified in 2008 (36).

This review examines the association between thyroid cancer and 2 oncogenic virus families, the herpes and polyoma family viruses, and discusses their potential role as causative agents in thyroid carcinogenesis.

2. Polyomaviruses

Overview. Since 1971, 10 PyVs that infect humans have been identified, including JC virus (JCV) and BK virus (BKV) found over >40 years ago, as well as the recently isolated MCPyV, KI PyV (KIPyV), WU PyV (WUPyV), human PyV (HPyV)6, HPyV7, trichodysplasia spinulosa PyV (TSPyV), HPyV9 and MWPyV (37).

PyVs are obiquitous worldwide, with seroprevalence rates ranging from 35 to 90% (34,37). Apart from a productive life cycle with resultant cell lysis, often associated with minimal symptoms, PyVs can also establish a persistent infection; significant disease though, is limited to patients with immune system disfunction (34,37,38). More specifically, JCV is associated with progressive multifocal leukoencephalopathy in HIV-AIDS, autoimmune diseases treated with certain lymphocyte-specific antibodies and hematological diseases; BKV with hemorrhagic cystitis following allogeneic hematopoietic stem cell transplantation and post-kidney transplantation PyV-associated nephropathy (37). TSPyV is linked to trichodysplasia spinulosa, a skin disease found in immune-impaired transplant patients that is characterized by virus-induced lytic and proliferative tumor-like features (37). KIPyV and WUPyV have been isolated from the respiratory tract, HPyV6 and HPyV7 from the skin, HPyV9 from the serum and skin, and MWPyV from stools and skin; However, none of these viruses have been associated with specific human pathology (37). MCPyV is the only PyV with a robust correlation with human cancer, more specifically Merkel cell carcinoma, a rare skin tumor found in elderly, chronically immunosupressed patients (37).

All PyVs have a similar morphological and functional pattern (39), with their virions consisting of non-enveloped icosahedral particles 40-45 nm in diameter, and a genome of ~5 kb organized as circular double-stranded DNA wrapped around host cell-derived histones (37). This genome is divided into 3 functional parts: i) the non-coding control region (NCCR), which contains the origin of replication, the transcription start sites and promoter/enhancer elements, and regulates the expression of the early and late viral genes; ii) the early gene region, encoding the large T-antigen (LTag) and the small Tag which derives from a major transcript by alternative splicing and facilitate viral genome replication and transformation; and iii) the late gene region, encoding capsid proteins VP-1, VP-2 and VP-3, produced by alternative splicing of a primary transcript, and assembled in the nucleus to form the viral capsid (37).

Two possible outcomes may follow cell infection by PyV: i) in the case of a permissive host, viral entry results in viral DNA replication, followed by progeny virion production, and

Table I. Investigation of polyomaviruses in thyroid tumors.

Virus investigated	Investigated thyroid tissue	Method	Authors/(Refs.)
SV40	PTC, MTC, NTT, AITD (Hashimoto's thyroiditis), DTG	PCR, Southern blotting, immunohistochemistry	Pacini *et al* (44)
SV40	PTC, ATC, MTC, NTT, AITD (Grave's thyroiditis)	PCR, RT-PCR, immunohistochemistry	Vivaldi *et al* (45)
SV40	PTC, Benign thyroid nodules (including Hashimoto's thyroiditis), NTT	PCR	Ozdarendeli *et al* (46)
BKV	PTC, MH, NTT	PCR	Stamatiou *et al* (47)

SV40, simian vacuolating virus 40; BKV, BK virus; AITD, autoimmune thyroid disease; PTC, papillary thyroid cancer; ATC, anaplastic thyroid cancer; MTC, medullary thyroid cancer; NTT, normal thyroid tissue; DTG, diffuce toxic goiter; MH, multinodular hyperplasia; RT-PCR, reverse transcription-polymerase chain reaction.

cell lysis; ii) in the case of a non-permissive host, an abortive infection or cell transformation, known as oncogenesis, ensues, characterized by the continued expression of the viral early genes (40-43). The starting point of this latter process, is the genetic and functional uncoupling of the early gene expression from the later steps of the viral life cycle, namely viral DNA replication, late gene expression, virion assembly and host cell lysis. Cell cycle control is subverted by the Tags through the inactivation of signal transduction pathways and the tumor suppressor proteins, pRB and p53, eventually leading to neoplastic transformation (37).

PyVs in thyroid cancer. To the best of our knowledge, few studies to date have investigated the association of PyVs with thyroid cancer (44-47), as shown in Table I. Three of these studies (44-46) detected sequences of simian vacuolating virus 40 (SV40), a virus that infected rhesus monkey kidney cells used for polio vaccine production (48), in thyroid gland specimens. Since SV40 has proven to be oncogenic in rodents (37,38,44) and has transforming capacity in human cells *in vitro* (44), the wide distribution of SV40 contaminated vaccines in the early 1960s, led to a concern for potential tumor induction in humans (37). A single study investigated the presence of BKV in post-operative thyroid specimens (47).

Most of our knowledge regarding the association of SV40 with thyroid cancer, is based on 3 studies examining the presence of SV40 DNA in thyroid specimens (44-46). The first study investigated the presence of SV40 sequences in 69 patients with PTCs using Southern blotting and polymerase chain reaction (PCR) (44). Seven normal peritumoral thyroid specimens, 4 blood specimens from patients with PTCs, 1 Hashimoto's thyroiditis, 5 toxic diffuse goiters, 3 medullary carcinomas and 9 breast carcinomas, were also studied. Southern blot analysis yielded positive results for 3 of the cases of PTC (4.3%). PCR amplification followed by sequence analysis, confirmed the presence of SV40 sequences, including the 203 bp fragment of the aminoterminus of LTag, the 294 bp fragment of the *VP1* gene, as well as the 483 bp entire regulatory region, integrated in the tumoral DNA of the aforementioned samples. All the other samples scored negative for SV40 (44). Immunohistochemistry (IHC), performed for the LTag, revealed positive cytoplasmic staining in the 3 SV40-positive cases, not found in the negative controls (44). Another study (45), enroling 109 patients (80 females/29 males), performed by the same group, investigated the presence of SV40 DNA in a larger variety of thyroid pathologies, including 29 specimens of papillary cancer, 20 myeloid cancers, 20 anaplastic cancers and 20 specimens of Graves' disease. Specimens of normal thyroid tissue, 10 adjacent to papillary cancer, 10 adjacent to anaplastic cancer, and 20 from patients affected by multinodular goiter, were also included. Additionally, 20 peripheral blood mononuclear cell samples from relatives of patients with sporadic myeloid cancer, were analyzed. PCR amplification of the 172 bp N-terminal SV40 *Tag* and filter hibridization confirmed the presence of viral sequences in a percentage ranging from 66% in papillary cancers (P=0.02), to 100% in anaplastic cancers (P=0.01), while 90% of medullary cancer samples were positive (P=0.01). SV40 sequences were detected in a similar percentage, ranging from 60 to 100%, in corresponding normal thyroid tissues next to the tumors, while the corresponding detection percentages in specimens taken from patients with multinodular goiter and Graves' disease, were 10 and 20%, respectively. A total of 25% of blood samples were positive for SV40 *Tag* DNA (45). Positive samples were further investigated for the presence of the 314 and 294 bp sequences of the SV40 regulatory and *VP1* regions, respectively (45). SV40 sequence specificity, was confirmed by DNA sequencing. Subsequent RT-PCR, that was performed in the 24 thyroid cancer specimens yielded positive results for SV40 DNA, revealing mRNAs specific for SV40 *Tag* in 69% (9/13) of PTCs, and 73% (8/11) of anaplastic carcinomas. None of the 30 samples were found to be SV40-negative, used as negative controls, was positive. IHC was finally performed in the samples found positive by RT-PCR, while the 30 SV40-negative thyroid tissues were used as controls. A total of 33% (3/9) of the PTCs and 100% (8/8) of the anaplastic carcinomas were immunoreactive, and showed mainly cytoplasmic staining. The third study included 99 patients (21 males/78 females) with thyroid nodules, 8 of whom (8.08%), were diagnosed with PTCs, while 91 (91.02%) had benign thyroid nodules, 8 of which, harboured Hashimoto's thyroiditis (46). Both nodular and normal thyroid tissue was obtained from each patient. All 198 specimens were investi-

gated for the presence of SV40, through the amplification of Tag coding sequences, using PCR (46). Sequences of SV40 were detected in 4 out of 99 nodules, 2 of which were PTCs, while the remaining were benign thyroid nodules. The presence of SV40 DNA in the thyroid nodules was confirmed by sequence analysis using the SV40P1 primer, and by cloning of the 243 bp PCR product into the Topo XL cloning vector (46).

A 95 bp sequence of the BKV *VP1* gene was investigated in frozen thyroid samples obtained from 30 patients with thyroid nodules (6 males/24 females) using PCR (47). A total of 14 out of the 30 patients (46.7%) suffered from PTC, while 16/30 (53.3%) had multinodular hyperplasia. Nodular, as well as adjacent normal tissues, were obtained from each patient. Taken as a whole, 18/30 (60.0%) nodular tissue samples harboured BKV DNA, while the corresponding percentage in adjacent normal tissue was 43,3% (13/30). More specificaly, the *VP1* sequence was detected in 8/14 malignant specimens (57.1%), compared to 6/14 (42.8%) of adjacent normal tissue. The corresponding percentages for multinodular hyperplasia were 62.5% (10/16) and 43.7% (7/16), respectively (47).

3. Herpesviruses

Overview. Eight identified viruses from the herpesviridae family infect humans, categorized in 3 subfamilies, namely α, β and γ. The α subfamily includes herpes simplex viruses (HSV) type 1 and 2, as well as varicella zoster virus (VZV or HHV-3). Cytomegalovirus (CMV or HHV-5), and roseola viruses (HHV) 6 and 7 constitute the β subfamily, while EBV (HHV-4) and KSHV/HHV8, belong to the γ subfamily (49,50). Human herpesviruses are large (100-200 nm), enveloped, that contain double stranded DNA, enclosed in an icosahedral protein capsid (49).

As with PyVs, herpesviruses are widespread infectious agents, and almost 100% of adults have been infected in their lifetime (49). Lifelong infection is established, as the viral genome latently persists in the host cell nucleus (50). Although all herpesviruses infect epithelial cells (49), thus gaining access to the host, long-term residency and latency is strictly dictated by the specific herpes subfamily (49,50), determined by the presence of cell-surface receptors, as well as intracellular conditions (49), α herpesviruses establish latency in neurons, β herpesviruses in macrophages and lymphocytes, and γ herpesviruses in lymphocytes alone (49,50).

HSV-1 is associated with blisters on the lips, known as herpes labialis, while HSV-2 causes genital blisters (49). Both viruses may be associated with either lesion though (49). HSV may additionaly reach the eyes causing keratitis, potentially leading to blindness if left untreated (51), or may attack the brain, with resultant encephalitis or meningitis (52). Primary infection with VZV causes chickenpox, associated with a vesicular itchy rash primarily affecting the trunk and the head, while later reactivation is known as herpes zoster or shingles, limited to a specific body area (49). HHVs-6 and -7, known as roseola viruses, form skin rashes referred to as exanthema subitum (53). Congenital CMV infection may lead to birth defects (54), while mononucleosis, characterized by fever, sore throat, fatigue, as well as swolen lymph nodes, is the main disease associated with CMV in adolescents, though it is more commonly caused by EBV (49). Both γ subfamily herpesviruses are tumorigenic agents (49). HHV-8 is mainly the etiologic agent associated with Kaposi's sarcoma, a cancer most frequently diagnosed in patients with AIDS (55). EBV is mainly associated with nasopharyngeal carcinoma, as well as with Burkitt, Hodgkin's, immune-suppression-related non-Hodgkin, and extranodal NK/T-cell lymphomas (56).

EBV represents the typical example of herpesviral strategy (49). Upon infection of a cell, the two ends of the initially linear viral genome, bind to each other, persisting in an episomal state, facilitating latency establishment (57,58), as opposed to lytic activation, which requires genome linearization (59). No virions are produced during the latent state, allowing for the expression of only a few viral genes, which affect B-lymphocyte growth mechanisms, causing their immortalization (60). Six nuclear antigens, namely Epstein-Barr nuclear antigen (EBNA)1, -2, -3A, -3B, -3C, as well as the protein EBNA-LP; 3 membrane proteins, EBV latent membrane protein (LMP)1, -2A, -2B; 2 small nuclear RNAs, EBV-encoded small RNA (EBER)1 and -2, as well as BART region trancripts, encoding most of EBV's microRNAs, are associated with this latent infection of immortalized B cells (61). Conversely, the key role for the transition from the latent to the lytic cycle, inducing viral replication, is played by the BZLF1 and BRLF1 proteins (62).

Herpesviruses in thyroid cancer. Similar to PyVs, the association of members of the herpes family with thyroid cancer is based on detection methods of the presence of viral genes and gene products in thyroid tumor tissue (29,47,63). The studies that investigate this association are presented in Table II. The majority of these studies refer to EBV (47,63-69), while 4 other members of the family have also been studied, although less extensively (29,69).

The detection of EBV in thyroid cancer has proven controversial (47,63-69). The EBV persisting capacity in B-lymphocytes and its contribution to lymphoma formation (56), prompted the initial investigation of EBV in thyroid lymphomas (63-65), while subsequent studies investigated the presence and contribution of EBV in other types of thyroid malignancies (66-69).

Three studies examined the presence of EBV in thyroid lymphomas (63-65). EBV-related mRNA, as well as the associated proteins were investigated in 32 cases of thyroid lymphoma and 30 cases of Hashimoto's thyroiditis by *in situ* hybridization (ISH) and IHC on routinely processed tissue sections (63). Three cases of thyroid lymphoma demonstrated the presence of EBER. Gene products, BHLF1, LMP, and BZLF1 proteins were detected in 2 of the EBER-positive cases (63). The second study enrolled 30 patients with thyroid lymphoma and 28 with chronic lymphocytic thyroiditis (10 males/48 females) (64). Only 24 and 16 cases of thyroid lymphoma and chronic thyroiditis, respectively, were finally used, as the rest of the samples revealed poorly preserved DNA, based on the presence of the β-globin PCR band, and were thus excluded from further analysis (64). PCR amplification revealed positive EBV products in 1 case of chronic lymphocytic thyroiditis and 2 cases of thyroid lymphoma, while ISH yielded positive signals in the nucleus of tumor cells of only 1 of the lymhomas found positive by PCR, while LMP-1 was also expressed in the cytoplasm of lymphoma cells (64). The third and most

Table II. Investigation of herpesviruses in thyroid tumors.

Virus investigated	Investigated thyroid tissue	Method	Authors/(Refs.)
EBV	TL, AITD (Hashimoto's thyroiditis)	*In situ* hybridization, immunohistochemistry	Takahashi *et al* (63)
EBV	TL, CLTH	PCR, *in situ* hybridization	Tomita *et al* (64)
EBV	TL	*In situ* hybridization, immunohistochemistry	Lam *et al* (65)
EBV	PTC, undifferenciated carcinoma, SCC, AITD (Grave's disease), MH, NTT	PCR, RT-PCR, *in situ* hybridization, immunofluorescence	Shimakage *et al* (66)
EBV	PTC, MH, NTT	PCR	Stamatiou *et al* (47)
EBV	PTC	*In situ* hybridization	Kijima *et al* (67)
EBV	WT	PCR, *in situ* hybridization, immunohistochemistry	Ludvikova *et al* (68)
EBV, CMV, HSV1, HSV2, HHV8	Benign thyroid tumors (not specified)	PCR, Southern hybridization	Tsai *et al* (69)
HSV1, HSV2	AITD, FA, FTC, FVPC, PTC, ATC	PCR, immunohistochemical staining, western blot analysis	Jensen *et al* (29)

EBV, Epstein-Barr virus; CMV, cytomegalovirus; HSV, herpes simplex virus; HHV8, human herpesvirus type 8 (also known as Kaposi's sarcoma-associated herpesvirus (KSHV); AITD, autoimmune disease; FA, follicular adenoma; FTC, follicular thyroid carcinoma; PTC, papillary thyroid carcinoma; FVPC, follicular variant of papillary thyroid carcinoma; ATC, anaplastic cancer; TL, thyroid lymphoma; CLTH, chronic lymphocytic thyroiditis; SCC, squamous cell cancer; MH, multinodular hyperplasia; NTT, normal thyroid tissue; WT, warthin-like tumor; PCR, polymerase chain reaction.

recent study, analyzed the clinicopathological characteristics of primary and secondary thyroid lymphomas affecting the Hong Kong Chinese population over a period of 3 decades, and investigated the expression of EBV genes, using ISH and IHC (65). A total of 23 patients with primary disease, 15 of whom had Hashimoto's thyroiditis, as well as 9 patients with secondary disease were enroled. EBV mRNAs were detected in 1 primary and 1 secondary thyroid lymphoma (65).

EBV has been suggested to contribute to thyroid tumor progression, defined as the pathological dedifferentiation of tumor cells, leading to a more undifferentiated tumor type (66). To examine the potential involvement of EBV expression in the progression of thyroid cancer, 10 PTCs, normal tissue specimens at the peripheral region of 4 of the PTCs, 11 undiferentiated carcinomas, 1 thyroid squamous cell carcinoma (SCC), as well as negative controls including 2 thyroid nodular hyperplasia and 2 Graves' disease specimens, all taken from Japanese patients were used. Specimens were subjected to PCR, RT-PCR, mRNA ISH and indirect immunofluorescence staining. PCR was performed in 13 of the samples, including 9 PTCs, 3 undifferentiated carcinomas and 1 SCC, all of which showed amplified EBV DNA in the region of *Bam*HIW. ISH for the detection of EBV mRNAs expression was performed using *Bam*HIW, EBER1 and EBNA2 probes, while immunofluorescence for the detection of EBV proteins was performed using 4 monoclonal antibodies, namely anti-EBNA2, anti-LMP1, anti-BZLF1 and anti-CD21. Despite the fact that EBV infection, and mRNA and protein expression was detected in all carcinoma specimens, irrespective of the degree of histological differentiation, both mRNA and protein exression was much more prominent in the undifferentiated carcinomas. Both the normal thyroid tissue specimens, as well as the nodular hyperplasia and Graves' disease specimens used as controls, showed no, or very few signals during ISH with any of the probes (66).

A similarly high EBV detection percentage in thyroid nodules consisting of PTCs, and multinodular hyperplasia specimens, as well as adjacent normal thyroid tissues, was recently reported in a previously mentioned study (47). The investigation included the detection of 161, 168 and 118 bp sequences of the *LMP1*, *EBNA2* and *EBER1* genes, respectively. As regards *LMP1*, the sequence was detected in 50% (15/30) of the nodules, and in 46.7% (14/30) of the adjacent normal tissues. More specifically, 57.1% (8/14) of the PTCs and 43.8% (7/16) of multinodular hyperplasia specimens harboured the sequence, while the percentages of the adjacent normal tissues were 35.7% (5/14) and 56.2% (9/16), respectively. Much higher percentages considering *EBNA2* sequence detection, were found in the study, although similar between nodular (90%-27/30) and adjacent normal (90-27/30) tissue. The vast majority of both malignant (92.9%, 13/14) as well as multinodular hyperplasia (87.5%, 14/16) specimens were *EBNA2*-positive, while the corresponding percentages for the adjacent normal tissues were 85.7% (12/14) and 93.8% (15/16), respectively. Sequence analysis confirmed the results. Interestingly, *LMP1* sequence frequency in nodular and adjacent normal thyroid tissue presented significant differ-

ences compared to *EBNA2* sequence frequency (P=0.0015 for nodular tissue, and P=0.0006 for normal tissue). None of the samples contained the *EBER1* sequence (47).

In contrast to the studies mentioned above, negative results regarding the association between thyroid tumors and EBV have been reported (67-69). None of the 45 PTCs resected from patients in the southern part of Kyushu, Japan, and subjected to EBER1 ISH tested positive for EBV, despite the fact that a few infiltrating lymhoid cells detected in 1 (2.2%) of the specimens, revealed EBER1 positivity (67). Additionaly, no significant positive signal for EBV was revealed by PCR, ISH for EBER, and IHC, performed in 12 cases (11 females/1 male) of oncocytic PTC with lymphoid stroma (Warthin-like tumor) (68). Positive PCR results of 1 sample, were not confirmed from repeat PCR, or ISH, thus the sample was considered negative for EBV (68). Finally, all 16 benign thyroid tumor samples taken from 34 to 56 year-old female patients from Taiwan, used as controls in a study associating non-familial breast cancer with viral factors, scored negative for EBV using PCR and Southern hybridization (69).

The hypothesis of HSV1 and HSV2 association with thyroid tumors, was investigated analyzing the detection of viral DNA in both benign and malignant thyroid lesions, as well as the expression of nectin-1, a herpesvirus entry mediator, in thyroid tissues and cancer cell lines (29). Thyroid cancer cell susceptibility to HSV, and its associated molecular mechanisms, were explored *in vitro* (29). Thyroid samples from 109 patients (44 benign/65 malignant), including 43 PTCs, 16 FTCs, 6 anaplastic carcinomas, 30 follicular adenomas and 14 autoimmune thyroid disease specimens were examined (29). The presence of thyroid oncogene mutations was also sought in 73 thyroid tumors. Initialy, HSV DNA was amplified with PCR, followed by sequencing. Subsequent investigation included the detection of HSV proteins by immunohistochemical staining, and the detection of nectin-1 by in-cell western blot analysis. Collectively, HSV DNA was detected in 43/109 (39.4%) of the samples. Considering benign lesions, HSV1 DNA was detected in 11/44 (25%), while HSV2 DNA was detected less frequently (1/44-2%). HSV DNA was much more frequently detected in malignant lesions (31/65, 47.7%; P=0.0454), although HSV1 sequences had the same prevalence in benign and malignant lesions. PTCs and lymph node metastases harboured mostly HSV2 DNA, while HSV1 DNA was detected predominantly in FTCs. Oncogenic mutations were revealed in 70% (21/30) of the HSV-positive tumors, compared with 27.2% (12/44) of the HSV-negative tumors. Immunoreactivity was detected in 21/25 (84%) of the HSV-positive samples investigated, restricted to epithelial cells, unlike stromal fibroblasts, endothelial cells and infiltrating lymphocytes, which were negative. As regards nectin-1, its expression has been shown to be increased in thyroid tumors, particularly papillary cancers compared to normal specimens, as well as in all thyroid cancer cell lines, and correlated with cancer cell susceptibility to HSV infection (29).

Unlike the results of the above-mentioned study, neither HSV1 nor HSV2 were detected in any of the 16 benign thyroid tumor samples, in the previously mentioned study reported by Tsai *et al* (69). The results were also negative for HHV8, while CMV was related to thyroid tumors (P<0.05), and its DNA was detected in 4/16 (25%) of the specimens.

4. Conclusions and future perspectives

Data from the above-mentioned studies have revealed that thyroid cancer specimens harboured viral DNA and/or gene products, from both the polyoma (44-47) and herpes (47, 63-66) families. High sensitivity in DNA and RNA detection even in small biopsy samples has been achieved using PCR (40). Using a PCR-based assay to detect viral sequences though, needs extreme care, since the large number of PCR cycles to increase its sensitivity, renders it susceptible to false-positive results due to laboratory contamination (40). Moreover, a PCR-based assay, used to investigate the presence of a viral sequence in a tumor biopsy sample, may detect these sequences in normal cells contained in the sample, and thus makes the tumor appear positive for the investigated virus (40). ISH (63-66), IHC (29,44,45,63,65,66) and Southern blotting (44,69), used by studies reviewed in this review article, as well as *in situ* PCR, are less susceptible to contamination, while *in situ* methods discriminate between various cellular locations of the virus in tissue sections, avoiding false-positive results from adjacent infected normal cells (40). Furthermore, DNA sequencing analysis, performed by several studies that were included in this review (29,44-47), confirmed the presence of the viral sequences detected, aiding in the accuracy of the individual studies.

PyVs in thyroid cancer. SV40 was detected in groups of specimens, including thyroid malignancies, PTC (44-46), medullary carcinoma (45), anaplastic carcinoma (45), benign thyroid lesions-Graves' disease (45), benign thyroid nodules (46), as well as normal thyroid tissues, adjacent to papillary and anaplastic cancers (45) and from patients with multinodular goiter (45). On the contrary, groups of specimens from both malignant thyroid disease, medullary cancer (44), benign lesions, toxic diffuse goiter, Hashimoto thyroiditis (44), as well as normal thyroid tissue, adjacent to PTCs (44,46), adjacent to benign thyroid nodules (46), from the same studies, all scored negative for SV40.

A significant difference in the SV40 detection rate in similar histotype specimen groups was observed in 2 of the studies (44,45), which was attributed to the higher sensitivity of PCR (45), compared to that of Southern blotting hybridization (44). As regards the study by Vivaldi *et al* (45), the SV40 detection rate in thyroid tumor samples was interestingly high, varying according to the degree of tumor differentiation, and this pattern also applied to the corresponding adjacent normal thyroid tissues, although there was statistical significance in the prevalence of SV40 Tag N-terminal coding sequences in each tumor specimen vs. the adjacent normal tissue. The detection rates were much lower for benign thyroid lesions and normal thyroid tissue adjacent to benign thyroid disease (45). The high prevalence of SV40 in both neoplastic and normal tissues, led the authors to hypothesize that viral infection potential spreads from neoplastic to adjacent normal tissue (45). The presence of SV40 in blood cells of healthy individuals, indicates that viral transfer to the thyroid gland may be thus achieved, and provides clues as to the long-term viral persistence in these cells in a latent state (45). Despite the use of the highly sensitive PCR, the prevalence of SV40 in PTC and benign nodules was relatively small in the third study (46). All normal thyroid

tissues were negative, a feature that was attributed to the remote location of the specimens relative to the tumor (46), in contrast to the immediate proximity of the normal tissues relative to the tumor in the study by Vivaldi *et al* (45).

Although the presence of SV40 in tumor samples does not prove its causative role in the development of thyroid cancer, several findings indicate its potential role in thyroid carcinogenesis (44-46). Indeed, SV40 *Tag* has been shown to interfere with thyroid cell growth and differentiation, as evidenced by studies on transgenic mice, in which JC *Tag* under the transcriptional control of SV40 promoter, caused thyroid cell hyperplasia (70). Moreover, thyroid dedifferentiation and follicular cell proliferation, leading to the development of hyperplasia and adenocarcinomas, was achieved by genating transgenic mice that carried the SV40 *Tag* gene under the transcriptional control of the thyroid-specific thyroglobulin gene promoter (71). Furthermore, the insertion of the SV40 *Tag* gene in cultures of normal follicular cells, caused their escape from early mortality and loss of thyroid differentiated functions (72). Thyroid hormone receptor-α1 (TRα1), together with retinoid X receptor-α (RXRα) have been found to regulate the SV40 late promoter, and their regulators are hypothesized to block the transcription of the viral late genes until the onset of viral replication (73,74). With a cell-specific mechanism, early genes, including *Tag*, are poorly transcribed in the presence of late gene overexpression, the amount of *Tag* molecules, viral DNA copies, and virions being thus reduced (75). TRα1 and RXRα are active in follicular thyroid cells (76), while TRα1 is found inactive due to gene mutations in approximately 60% of PTCs (77). In such cases, low levels of SV40 DNA replication, *Tag* expression, as well as virion production in thyroid cancer cells may occur, as the viral late promoter is left uninhibited by inactive TRα1 and RXRα (45). All of the above data indicate that the transforming activity of SV40 may affect the thyroid gland during persistent infection.

The presence of BKV in thyroid cancer specimens, was investigated by only one study (47). Viral sequences were commonly found in both nodular, as well as in adjacent normal tissues. Thus, it was suggested that BKV 'infection' was a very early event, occurring apparently within normal tissue. No assumptions considering the oncogenic potential of the virus in thyroid cancer development were made, and a coexistence or 'endemicity' pattern, rather than a causal effect was instead suggested (47). Exposure of the thyroid gland to the virus, is probably achieved in the context of the viremic phase of infection by BKV (78).

Herpesviruses in thyroid cancer. The suggestion of the causal role of EBV in the development of B cell lymphoma, particularly in immunocompromized patients, formed the background of the 3 studies that investigated the role of the virus in thyroid lymphoma development in the Japanese (63,64) and Hong Kong Chinese (65) population, respectively. Although it was concluded that EBV may play a role in a subset of thyroid lymphomas (65), and may specifically participate in the transformation of Hashimoto's thyroiditis, an entity known to play an important role in thyroid lymphoma development, to thyroid lymphoma (63), the activation of the virus in thyroid lymphoma is not common (64). The role of EBV in the development of thyroid lymphoma is further complicated by reports considering the incidence rates of EBV contact (64,79). No obvious difference in the frequency of EBV exposure among individuals with thyroid lymphoma, Hashimoto's thyroiditis and simple goiter were found in Kuma Hospital (64), while higher EBV antibody titers were recorded in patients with thyroid lymphoma compared to those with Hashimoto's thyroiditis, whereas the latter frequently had higher titers compared to normal subjects (79). This finding, underscored the potential role of EBV in thyroid lymphoma development, either as a pathogenetic factor, or as a consequence of the disease (79). Investigation of proviral DNA in the nuclei of lymphoma cells, was suggested to prove the direct role of EBV infection in thyroid lymphoma development (79).

The presence of EBV in other histotypes of thyroid cancer remains controversial. Various studies have revealed either the absence of the virus from all cases of PTC (67), presence in the majority of samples of PTC (47), and presence in 100% of both papillary and undifferentiated carcinomas, although EBV mRNA and protein expression was much more prominent in undifferentiated carcinoma samples, a fact that led to the assumption of EBV contribution to tumor dedifferentiation (66). Inconsistency between PCR and the less sensitive ISH method characterized the results of the investigation of Warthin-like tumor for EBV (68). These were attributed to a potential falsely positive PCR, resulting from the presence of circulating lymphocytes that carried EBV in the tumor sample, since one in 10^6 circulating B lymphocytes may carry EBV (68). The difference between the strongly positive results of Shimakage *et al* (66), and the negative results of Kijima *et al* (67) and Ludvíková *et al* (68), both of whom performed ISH using the EBER1 probe, was attributed to the different sensitivity of the probes used (66). In accordance to the results of Shimakage *et al* (66), considering the presence of EBV in PTC samples, Stamatiou *et al* (47) also tested adjacent normal thyroid samples using PCR, which showed comparatively high positivity for EBV. Interestingly, the detection rate of the 3 EBV genes investigated, differed significantly (47). The results were verified with DNA sequencing and a potential viral spread from normal to cancerous tissue was supposed (47).

Tumor B cell inflamatory infiltrates may play the role of viral reservoirs, as regards the infection of thyroid epithelial cells by EBV, a lymphotropic virus (68). The proven fusion of infected B cells with epithelial cells (80), as well as the observation that the *in vitro* infection of epithelial cell lines by EBV was only possible during coculture with EBV-infected B cells, but impossible using cell-free EBV (81), offer a potential mechanism of viral transfer between cell types.

Furthermore, it should be noted that despite the fact that studies investigating the presence of EBNA in oropharyngeal epithelial cells obtained from throat washings of healthy adult volunteers, recorded no differences regarding EBV positivity between Far Eastern populations, Osaka, Japan (27%), and Caucasians, United States (22%) (82), reports associating EBV with tumors, depend on regional and/or ethnic background, occuring in Asians but not in Caucasians (83). Since the majority of the above-mentioned studies refer to Far Eastern populations (63-67), locality features should be considered and larger studies with random patient selection performed.

HSV DNA and protein was detected in thyroid cancer specimens with high frequency, and their presence related to the activation of virus-inducible signaling in thyroid cells (29). Furthermore, thyroid cancer cell lines were permissive to HSV infection, while inhibition of mitogenic signaling, decreased their susceptibility to the virus (29). Activation of nuclear factor (NF)-κB and p-AKT signaling, usually associated with oncogene mutations, was shown to occur due to HSV infection, while the activation of the RAS/MEK/MAPK pathway, which characterizes thyroid cancer, contributes to active HSV replication in thyroid cancer cells (29). Increased nectin-1 expression in thyroid tumors relative to normal thyroid tissue, its expression being further increased during tumor progression, may provide an explanation for the increased frequency of HSV in thyroid tumors, its level correlating with HSV infection susceptibility in cancer cell lines (29). The specific propensity of FTCs and PTCs toward HSV1 and HSV2, respectively, analogous to the specific tropism of different neurons in the same ganglion to HSV1 and HSV2, may underlie the tumor-specific patterns of signal transduction (84).

Considering the presence of CMV in benign thyroid tumors, further studies need to be performed, to clarify its association with human thyroid tissues.

On the whole, after reviewing the studies investigating the presence of both viral DNA and gene products of the herpes and polyomaviridae families in thyroid cancer tissues, there seems to be an association of both virus families with thyroid malignancies. Caution should be undertaken when interpreting these virological data and in conclusion making. Since members of both viral families are ubiquitous among humans, the role of these viruses as causal factors in thyroid tumorigenesis cannot be proven by the mere detection of viral sequences in tumor samples, as the viruses may be attracted to the tumor by the specific milieu of growth factors and cytokines of the tumor environment. This also applies to the presence of specific virus-directed antibodies, that may just represent the trace of passage of a virus and its clearance from the body. On the other hand, the absence of viral markers is not sufficient to exclude the viral contribution in cancer development, since the triggering infection may have taken place many years previously, and viral interaction with the specific genetic background of the host may have quiescently played a role in host disease development. As regards their potential contribution to the thyroid oncogenic process, other genetic and environmental co-factors should definitely play a role in disease development, apart from the life-term persistent infection associated with members of both viral families reviewed herein, since the incidence of the neoplastic disorders is far smaller than the prevalence of the investigated viruses in the population, as is the case with EBV infection (85).

Since the fulfillment of Koch's four postulates, the formal criteria for microbial disease causation, has proven difficult for any of the oncogenic viruses discovered to date (56), the oncogenicity of herpes and PyVs in thyroid cancer remains controversial. Genomic integration, instead of mere detection of viral genomic sequences or proteins, proposed as a means of clarification of the association between viruses and cancer (56), may potentially provide an answer. Definitely, more convincing evidence is required, and the present review is just the beginning of a long research journey in the clarification of whether these viruses are responsible for thyroid cancer development, or just represent innocent bystanders.

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