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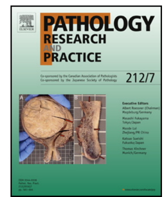

Original article

ApolipoproteinL1 is expressed in papillary thyroid carcinomas

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ABSTRACT

The apolipoprotein L (apoL) family has not yet been ascribed any definite patho-physiological function although the conserved BH3 protein domain suggests a role in programmed cell death. As repression of the regular apoptotic program is considered a hallmark of tumor progression, we investigated apoL expression in cancer. We show that the levels of one member of the family, apolipoprotein L1 (apoL1) is higher in papillary thyroid carcinoma compared to normal tissue. A combination of qRTPCR, immunohistochemistry and in situ hybridization allowed us to ascribe this increase to endogenous overexpression in carcinoma cells. Whether apoL1 plays an instrumental role in refraining cell death is the subject of ongoing molecular biology experiments.

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1. Introduction

Thyroid cancer is the most frequent endocrine cancer. The number of reported cases increases steadily (for instance, they increased by 6.6% in the USA between 2000 and 2010). Benign adenomas account for 90% of cases. Malignant tumors, apart from medullary cancer (a small% of thyroid cancers) or very rare cases such as Hürthle cell thyroid cancer or thyroid sarcomas, are mainly of 3 types: differentiated follicular and papillary carcinomas (FTC and PTC), and very aggressive dedifferentiated anaplastic carcinomas (ATC). PTCs account for 80% of the malignant thyroid carcinomas [1] whilst ATCs account for only 5%. However, ATCs account for 50% of mortality. Adenomas can evolve into differentiated carcinomas that can themselves evolve in ATC [2,3]. Carcinomas (FTC, PTC and ATC) all originate from thyroid follicular cells. PTC, the most frequent well-differentiated thyroid cancer is associated with features that are not necessary present all together in the same lesion: they include distinguishable neoplastic papillae lined by one or several layers of cells with the presence of psammoma bodies and distinctive nuclear signatures such as nuclei overlapping, ground glass appearance, longitudinal nuclear groove or cytoplasmic invagination in the nucleus [4,5].

Oncogenic mutations most commonly associated with PTCs involve *BRAF*^{V600E} mutation with enhanced serine-threonine kinase activity (45% of cases) or RET-PTC rearrangement (30% of cases), as well as activating mutations of RAS. These mutations all activate the RAS/RAF/MEK/ERK signal transduction pathway [6–8].

Several high throughput analyses of gene expression in PTCs [9,10] pointed to the deregulation of well-known tumor marker genes [11,12]. They also indicated down-regulation of anti-apoptotic genes including Bcl2 [10,13] and of most genes involved in thyroid hormone synthesis consistent with cell dedifferentiation [14] or upregulation of the beclin-1 gene, a key regulator of autophagy [15]. Genes coding for enzymes involved in energy metabolism were also deregulated [16]. Finally, as in many cancers, many genes coding for proteins involved in inflammation were expressed, suggesting an associated pro-inflammatory state.

Only few biological markers have been identified to date as useful to the diagnostic and prognostic analysis of PTC.

The apolipoprotein L (apoL) family involves several dozens of diverging members (all mammalian species included –for example, 6, 13 and 5 members in humans, mice and rats respectively) [17]. The physio-pathological function of apoL family members remains elusive except for human apolipoprotein L1 (apoL1) endowed with microbicidal (trypanolytic) activity. However, apoL1 sequence analysis uncovered resemblances between apoLs and proteins of

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the apoptotic/anti-apoptotic Bcl-2 family, as a multi-helical ionic pore-forming domain and a Bcl-2 homology 3 (BH3) signature [17]. Accordingly, recent reports correlated ectopic overexpression of some apoL members (apoL6, apoL1) with pro-apoptotic or pro-autophagic activities in cancer cell lines [18–20], while induction of another member (apoL2) correlated with resistance to apoptosis [21].

As one of the hallmarks of cancer is interference with the regular apoptotic process and since apoLs share features with proteins involved in programmed cell death (PCD), we studied apoL expression in cancer. In the current study, we focus on apoL1 expression in thyroid cancer. We show that apoL1 is overexpressed in PTC both at the mRNA and protein levels.

2. Materials and methods

2.1. Patients, RNA and tissue samples

Ten papillary thyroid tumor and patient-matched non-tumoral thyroid RNA samples harvested for previous studies [14] were used for the analysis of apoL1 gene expression by Real-time RT-PCR. Formalin Fixed Paraffin embedded tissue (FFPE) from 28 cases of papillary thyroid carcinoma as well as their normal tissue counterparts were obtained from the IPG's (Institute of Pathology and Genetics, Gosselies, Belgium) tumor Biobank. All cases that were selected were histologically proven to be PTCs and had not undergone radiotherapy or chemotherapy prior to surgical excision. Data regarding the patients included in this study are provided in Table A.1. This research was approved by the local ethics committee of the ISPPC (Intercommunale de Santé Publique du Pays de Charleroi) under the protocol number B325201213975.

2.2. Real-time RT-PCR validation of microarray data

In order to perform adequate gene expression normalization in PTC, we first studied different control candidates: RPL27 (Ribosomal protein L27), RPL13, SRP14 and GAPDH. RPL27 was identified as a gene steadily expressed and non-regulated between normal and cancer tissue. It was therefore further used to normalize apoL1 expression while comparing RNA originating from papillary thyroid tumor to normal tissue.

ApoL1 and RPL27 mRNA expression were quantified by RT-PCR. cDNA was synthesized using the Reverse transcriptase core kit (Eurogentec, Belgium) according to the manufacturer's instructions. Real-time PCR reactions were performed in 96-well plates using the SYBR Green PCR Master Mix (Absolute qPCR syber green ROX mix, Thermo Scientific) with each 20 μL reaction containing 50 ng cDNA and 0.3 μM sense and antisense primers. The amplification was performed on a Steponeplus Real-time PCR system (Applied Biosystems, life technologies) under the following conditions: 95 °C for 15 min, 40 cycles at 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. A preliminary analysis demonstrated linear and similar amplification efficiencies with similar melting curves. Relative quantification was determined by normalizing the crossing threshold (CT) of apoL1 with the CT of RPL27 (loading control) using the $2^{-\Delta\Delta CT}$ method. Sequence-specific primer sets designed for apoL1 and for RPL27 quantification are given in supplementary Table A.2.

2.3. Immunohistochemistry experiments

Formalin-fixed, paraffin-embedded human thyroid sections of 5-μm thickness on superfrost^{TM} plus adhesion slides (Thermo Scientific) were dried overnight, dewaxed with Xylene and rehydrated in graded ethanol series. Antigen retrieval was performed at 97 °C in citrate buffer, pH = 6.0 (Target retrieval solution, Dako) for 20 min and slides were then cooled to 25 °C. Endogenous peroxidase activity was blocked in 3% H_2O_2 solution. Non-specific sites were blocked in pre-blocking solution for 20 min at room temperature (Protein Block serum-free solution, Dako). Incubation with apoL1 primary antibody (1:500 dilution) (HPA018885, Sigma) was performed overnight, at 4 °C in a humidified chamber. After three washes with TBS–Tween, sections were incubated with HRP conjugated anti-rabbit secondary antibodies for 30 min at room temperature. Immunoreactivity was revealed using 3′3′-diaminobenzidine (DAB) (liquid DAB+ chromogen solution, Dako) as a chromogen. Slides were counterstained with haematoxylin blue, prior to mounting with DPX permanent mount.

2.4. RNA In situ hybridization (ISH) assay

ISH for apoL1 mRNA was performed using the RNAscope 2.0 FFPE assay kit (Advanced cell diagnostics) according to the manufacturer's instructions. Briefly, sections were deparaffinized with Xylene and dehydrated with ethanol 100%. Endogenous peroxidase activity was blocked with pre-treatment 1 solution provided by the kit and protease digestion was performed using pre-treatment 2 solution. These steps were followed by hybridization with a mixture containing probes targeting *apoL1 mRNAs*. mRNA coding for the housekeeping RNA polymerase II (POLR2A) was used as a positive control and the bacterial gene *DapB* as a negative control. A cascade of signal amplifier and enhancer then preceded the Hematoxylin counterstaining. This analysis was performed on 15 patient-matched pairs of normal and tumoral tissues. In one pair, the ISH signals were counted in all cells present in tumor and normal zone with a light microscope using an ×40 objective.

2.5. Statistical analysis

Sigmaplot 13.0 was used for the analysis. Data was evaluated by Student's *t*-tests. P values of <0.05 (∗) were considered significant.

3. Results

3.1. ApoL1 mRNA expression in PTC samples

A previous microarray analysis [9] coupled with multidimensional scaling (MDS) analyzed overall gene expression in 48 PTCs. It uncovered 451 commonly up-regulated and 226 commonly down-regulated RNAs and indicated that 43% of the genes commonly deregulated in PTC were similarly regulated in anaplastic thyroid carcinoma (ATC). Reanalyzing the results focusing only on apoLs indicated that apoL1 was upregulated from 2 to 32 fold compared with a reference pool of 23 normal, non-neoplastic thyroid tissues from the contra-lateral lobe with respect to the thyroid carcinoma, an increase observed in 44 individual patients out of 48 analyzed (Fig. 1 and Table A.3). This upregulation was also found in 9 out of 10 ATC analyzed in the same study (from 2 to 8 fold compared to the normal pool of RNAs). In order to validate the results of this high throughput study comparing tumor samples to controls from unmatched patients, we analyzed 10 pairs of normal thyroid tissue and papillary carcinoma both originating from the same patient. As shown in Fig. 1, qRT-PCR analysis indicated that apoL1 was upregulated in all the samples, from 2 to 18 times with a mean of 5.4. On the other hand other apoL family members showed either no significant change in their expression level (apoL2, apoL3, apoL4 and apoL5) or only a slight increase (from 2 to 8 times, mean: 2.23) in the case of apoL6 (data not shown).

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