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Deregulated expression of VHL mRNA variants in papillary thyroid cancer

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ABSTRACT

Recent findings demonstrated that a subset of papillary thyroid cancers (PTCs) is characterized by reduced expression of the von Hippel-Lindau (*VHL*) tumor suppressor gene, and that lowest levels associated with more aggressive PTCs. In the present study, the levels of the two VHL mRNA splicing variants, VHL-213 (V1) and VHL-172 (V2), were measured in a series of 96 PTC and corresponding normal matched tissues by means of quantitative RT-PCR. Variations in the mRNA levels were correlated with patients' clinicopathological parameters and disease-free interval (DFI). The analysis of VHL mRNA in tumor tissues, compared to normal matched tissues, revealed that its expression was either up- or down-regulated in the majority of PTC. In particular, V1 and V2 mRNA levels were altered, respectively, in 78 (81.3%) and 65 (67.7%) out of the 96 PTCs analyzed. A significant positive correlation between the two mRNA variants was observed ($p < 0.001$). Univariate analysis documented the lack of association between each variant and clinicopathological parameters such as age, tumor size, histology, TNM stage, lymph node metastases, and BRAF mutational status. However, a strong correlation was found between altered V1 or V2 mRNA levels and DFI. Multivariate regression analysis indicated higher V1 mRNA values, along with lymph node metastases at diagnosis, as independent prognostic factors predicting DFI. In conclusion, the data reported demonstrate that *VHL* gene expression is deregulated in the majority of PTC tissues. Of particular interest is the apparent protective role exerted by VHL transcripts against PTC recurrences.

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1. Introduction

The von Hippel-Lindau (*VHL*) gene, located on chromosome 3p25-p26, consists of 3 exons (Latif et al., 1993). Two different VHL mRNAs are generated because of differential splicing: the variant 1 (V1), including all three exons, and the variant 2 (V2), lacking exon 2 (Iliopoulus et al., 1998; Gnarra et al., 1994; Richards et al., 1996). From the V1 mRNA, two VHL protein (pVHL) isoforms are synthesized due to alternate initiation sites of translation; the $pVHL_{213}$, of about 30 kDa and containing 213 amino acids, and the $pVHL_{160}$, of about 19 kDa and containing 160 amino acids (Schoenfeld et al., 1998). The V2 mRNA variant encodes a protein of 172 amino acids

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lacking an amino-terminal pentameric acid repeat (β-domain), with a predicted molecular weight of about 19 kDa ($pVHL_{172}$) (Chesnel et al., 2015).

The *VHL* gene is widely expressed in human tissues and acts as a tumor suppressor gene (Los et al., 1996). Loss-of-function mutations cause the so-called VHL disease, an autosomal dominant disorder characterized by retinal angioma, cerebellar and spinal hemangioblastomas, clear-cell renal cell carcinoma (ccRCC), pheochromocytoma and pancreatic neuroendocrine tumor (Maher et al., 2011). Besides, a number of sporadic cancers, including ccRCC, are strongly associated with *VHL* reduced expression and/or loss-of-function mutations (Gnarra et al., 1994; Cassol and Mete, 2015). The best characterized pVHL function is ubiquitination followed by proteasome degradation of target proteins (Robinson and Ohh, 2014). In particular, the pVHL forms a multiprotein complex with elongins B and C, cullin 2 and Rbx-1, which functions as a ubiquitin-ligating enzyme (E3 ligase). Within the complex pVHL is responsible for recognition of substrates, among which the members of the hypoxia-inducible factor α (HIFα) family (Robinson and Ohh, 2014). In complex with HIFβ, HIFα act as transcription factors enhancing the expression of a variety of genes involved in the adaptive response to hypoxic condition and tumor progression as well, such as genes that promote neoangiogenesis (e.g. vascular endothelial growth factor, VEGF), energy metabolism (e.g. glucose transporter 1, GLUT1), erythropoiesis (e.g. erythropoietin, EPO), and cell survival (e.g. transforming growth factor-α, TGF-α) (Robinson and Ohh, 2014; Balamurugan, 2016). In addition, VHL has been shown to affect several hypoxia-independent cellular functions, including extracellular matrix formation, spindle microtubule stability, cilia formation, epithelial-to-mesenchymal (EMT) transition, cell proliferation, apoptosis and DNA damage response (Robinson and Ohh, 2014).

The pVHL region interacting with the elongins and Cul-2, named BC box, is coded by the third exon, thus all pVHL isoforms are able to form the E3 ligase complex (UniProtKB − P40337). However, the binding with HIFα occurs via the β-domain, which is missing in the $pVHL_{172}$ (Bonicalzi et al., 2001). Such domain is also essential for VHL interaction with the chaperonin-containing t-complex polypeptide 1 (CCT), a cytosolic molecular chaperone that assists in the folding of actin, tubulin and other cytosolic proteins. Even if the physiological role of $pVHL_{172}$ remains to be elucidated, it might be supposed that this isoform behaves, at least partially, as a VHL_{213} antagonist, and possibly is endowed with new functions.

Some reports suggested that pVHL could play a role also in the progression of epithelial thyroid cancer (TC) (The Cancer Genome Atlas Research Network, 2014; Stanojevic et al., 2015; Hinze et al., 2000; Hunt et al., 2003). The latter represents the most common endocrine malignancy accounting for roughly 1% of all human cancers, and its incidence has been increasing over the last decades mainly due to the improved ability to diagnose malignant transformation in small non-palpable thyroid nodules (Jemal et al., 2009; Davies and Welch, 2006; Kinder, 2003; Patel and Shaha, 2006; Pasieka, 2003). More than 90% of TC are differentiated thyroid carcinomas (DTC), 70–80% of which are represented by the papillary (PTC), and the remaining by the follicular (FTC) histotype (Nikiforov et al., 2009). Although derived from the same cell type, the DTC show specific histological features, biological behavior and degree of differentiation because of different genetic alterations (The Cancer Genome Atlas Research Network, 2014; Nikiforov et al., 2009). Among the somatic activating mutations, those of genes involved in the mitogen activated protein kinase (MAPK) signaling pathway, e.g. Ras, BRAF and RET/PTC rearrangements, are held responsible for the majority of PTC (The Cancer Genome Atlas Research Network, 2014; Nikiforov et al., 2009). Treatment of patients includes total thyroidectomy followed, if necessary, by ^{131}I therapy. The prognosis is generally favorable, with a 10-year-survival rate of approximately 90%. Nevertheless, nearby 20% of patients have disease recurrences and tumor-related deaths (Eustatia-Rutten et al., 2006). The stratification and prognosis of patients depends on clinicopathological variables such as age, tumor size, histology, lymph nodal or distant metastases (Eustatia-Rutten et al., 2006; Gospodarowicz et al., 2001; Passler et al., 2004; Castagna et al., 2011). These parameters, however, are capable of providing only a rough prediction of the disease outcome placing patients with very different disease-specific progression and survival times within the same risk group. Similarly, they fail to predict the risk of cancer relapse. Therefore, the identification of new prognostic molecular biomarkers able to testify tumor aggressiveness is required (Handkiewicz-Junak et al., 2010; Baldini et al., 2012; Ulisse et al., 2011).

Although, to date, no loss-of-function mutations of the *VHL* gene in PTC tissues have been described, recent studies demonstrated that a subset of PTCs was characterized by low levels of VHL mRNA, which associated with more aggressive PTCs (The Cancer Genome Atlas Research Network, 2014; Stanojevic et al., 2015).

In the present work, we measured the expression of the *VHL* gene, at mRNA level, in a case-study of 96 PTC tissues compared with their normal matched counterparts, and we evaluated the correlation of the *VHL* expression changes with clinicopathological parameters and disease-free interval.

2. Patients and methods

2.1. Tissue samples, histology and patient's staging

Normal and matched tumor thyroid tissues were obtained from surgical specimens of 96 patients (19 males and 77 females, age range 11–83 yr, median 44 yr) who underwent total thyroidectomy for PTC at the Department of Surgical Sciences, "Sapienza" University of Rome (39 patients) or at the Department of Medicine, University of Padua (57 patients). All patients gave their informed consent, and the study was approved by the local ethical committee (Protocol No. 2615). Tissue samples were collected, frozen in liquid nitrogen and stored at −80 °C. Of the 96 PTC patients, 72 exhibited classical, 17 follicular, 3 tall cell and 4 oncocytic variants. The histological diagnoses were made independently by two different histopathologists according to the World Health Organization classification (Hedinger et al., 1989). At the time of surgery lymph node metastases were found in 39 patients. Following TNM staging, 62 patients were at stage I, 1 at stage II, 27 at stage III and 6 at stage IV. Approximately 40–50 days later, all the patients underwent radioiodine treatment followed by thyroid hormone replacement therapy. To ascertain their disease-free condition, 4–5 months later all the patients underwent neck ultrasound and serum Tg measurement. Recurrences were diagnosed by measurement of serum Tg levels either in basal conditions or following recombinant human TSH stimulation; FNA cytology and/or Tg determination in the FNA wash-out from lymph nodes; ^{131}I whole body scan; histological analysis following surgical resection of the lesion. The follow-up included 80 patients (mean 57.9 ± 36.0 months, range 5–141 months), 54 of whom were at TNM stage I. During the follow-up 17 recurrences were recorded.

2.2. Determination of $BRAF^{V600E}$ mutation

Genomic DNA was extracted from the frozen tumor tissues using the DNeasy Blood and Tissues kit (QIAGEN, Milan, Italy) according to the manufacturer's protocol. The BRAF status of exon 15 was assessed by both direct sequencing and mutant allele-specific PCR amplification for the T to A substitution at nucleotide 1799

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