Human Pathology (2015) **46**, 50–57

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Human PATHOLOGY

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Original contribution

The PDCD4/miR-21 pathway in medullary thyroid carcinoma☆,☆☆,★

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Received 26 May 2014; revised 5 September 2014; accepted 15 September 2014

Keywords:
PDCD4;
MiR-21;
Medullary thyroid cancer;
Immunohistochemistry;
Akt

Summary Programmed cell death 4 (PDCD4) is a tumor suppressor gene involved in tumorogenesis. MicroRNA-21 (miR-21) specifically targets PDCD4, and recent studies suggest that PDCD4 is also regulated by Akt (antiapoptotic regulator within phosphatidylinositol 3-kinase). Medullary thyroid carcinoma (MTC) is a rare neuroendocrine cancer, and disease stage at diagnosis represents the main prognostic indicator. A consecutive series of 64 MTCs was considered. REarranged during Transfection (*RET*) and rat sarcoma (*RAS*) mutation status was assessed by direct sequencing. Quantitative real-time polymerase chain reaction was used to quantify mature hsa-miR-21. PDCD4 and Ki-67 immunostaining was performed with an automated platform. Immunoblot analysis of PI3K/Akt pathway was done on thyroid tissues. MTCs were consistently associated with miR-21 up-regulation ($P < .0016$) and featured significant PDCD4 nuclear down-regulation. An inverse correlation emerged between miR-21 overexpression and PDCD4 down-regulation ($P = .0013$). At enrollment, high miR-21 levels were associated with high calcitonin levels ($P = .0003$), lymph node metastases ($P = .001$), and advanced stages ($P = .0003$). At the end of follow-up, high miR-21 levels were associated with biochemically persistent disease ($P = .0076$). At enrollment, instead, PDCD4 nuclear down-regulation was associated with high calcitonin levels ($P = .04$), more advanced stages of disease ($P < .01$), and persistent disease after the follow-up ($P = .02$). p-Akt was more expressed in *RAS*-mutated MTC than in nonmutated cancers and normal tissue. This study showed, in MTCs, that miR-21 regulates PDCD4 expression and also that the miR-21/PDCD4 pathway correlates with

☆ Funding/Support: This work was supported by a MIUR grant ("Progetto di Ricerca di Ateneo"—CPDA118051; assigned to C.M.).
☆☆ Authors' contributions: All authors of this research paper participated directly in the planning and execution of the study, and in the analysis of the results.
★ Competing interests: The authors have no competing interests to declare.
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http://dx.doi.org/10.1016/j.humpath.2014.09.006

clinicopathological variables and prognosis. Further studies should investigate the role of miR-21 as a prognostic biomarker and the feasibility of using PDCD4-restoring strategies as a therapeutic approach to MTC.

1. Introduction

Programmed cell death 4 (PDCD4) is a tumor suppressor gene involved in apoptosis, cell transformation, invasion, and tumor progression. PDCD4 exerts its activity by interacting with the eukaryotic translation initiation factors 4A (eIF4A) and 4G (eIF4G) [1–4]. PDCD4 protein expression is consistently down-regulated in human cancers and cancer cell lines [5–7]. Recent investigations on the role of PDCD4 in inflammatory disease showed that it influences the production of proinflammatory signals by interacting with activator protein 1 (AP-1), nuclear factor κ–like light-chain enhancer of activated B cells and eIF4A, and it has been associated with the inflammatory microenvironment surrounding tumor cells [8–11].

Several mechanisms are involved in PDCD4 dysregulation in human cancer. Among others, the oncogenic microRNA miR-21 (hsa-miR-21) has been shown to specifically target the PDCD4 3′ untranslated region (3′ UTR), which negatively regulates PDCD4 expression [5–7]. The mitogen-activated protein kinase pathway and the phosphatidylinositol 3-kinase PI3k/protein kinase B (Akt) cascade also cooperate in PDCD4 proteasome-dependent protein degradation [12].

Medullary thyroid carcinoma (MTC) is a rare neuroendocrine cancer originating from parafollicular, calcitonin (Ct)-producing C cells. It accounts for 5% to 10% of all thyroid carcinomas with a global 10-year survival rate of about 65% to 70%. About 75% of MTCs are sporadic, whereas the remainder is hereditary, due to germinal mutations that activate the REarranged during Transfection (*RET*) proto-oncogene [13]. Various clinical, pathological, and genetic variables have been proposed as markers of prognosis, including age at diagnosis, extent of nodal disease, distant metastasis, pathological stage, and mutational damage in tumor suppressor genes [14]. Distinctive germinal *RET* mutations in the inherited forms and somatic *RET* mutations in sporadic cases represent the most important molecular markers for an adequate prognostic stratification of MTC patients [15,16]. It has been demonstrated that a combined analysis of somatic *RET* and Ki-67 is useful for identifying patients with a more aggressive cancer, and their joint assessment could ameliorate the initial risk stratification of patients with sporadic MTC and thus be of prognostic relevance [17].

The role of microRNA in the pathogenesis and prognosis of MTC has recently been investigated [18–21]. Only one study investigated the PDCD4/miR-21 pathway in a small series of MTCs, finding it significantly dysregulated [22].

To expand on the currently available information, the aims of the present study on a large series of familial and sporadic MTC were as follows: (1) to confirm that miR-21 modulates PDCD4 expression in MTC, (2) to see if the miR-21/PDCD4 pathway correlates with patient outcome, and (3) to investigate the relationship between the Akt/PI3K pathway and PDCD4 expression in MTC.

2. Materials and methods

2.1. Patients

The cases considered were retrospectively selected from the electronic archives of the Surgical Pathology & Cytopathology Unit at Padua University. All patients involved in this study gave their informed written consent, and the institute's ethical regulations on research on human tissues were followed.

The study concerned a consecutive series of 64 patients with MTC (56 sporadic and 8 familial; 28 men and 36 women; median age, 59 years; range, 5-81 years) collected from 2006 to 2010 with a median follow-up of 48 months (range, 12-226 months); 4 nontumor thyroid tissue samples removed from the contralateral lobe of patients with papillary thyroid carcinoma were also included in the series.

Ct serum levels at diagnosis were available for the most part of patients who were considered biochemically cured if they had Ct levels less than 10 pg/mL 1 year after primary surgery and/or at the latest follow-up.

DNA was extracted, for all patients, both from frozen tissues after surgery and from whole blood using the DNeasy Blood and Tissue kit (Quiagen, Milano, Italy), according to the manufacturer's protocol, to define its mutational status and also if the mutations were germline or sporadic. Analyses were performed for *RET* (NM_020975.4; exons 5, 8, 10, 11, 13, 14, 15), *N-RAS* (NM_002524.3; exons 2 and 3), *K-RAS* (NM_033360.2; exons 2 and 3), and *H-RAS* (NM_005343.2; exons 2 and 3) mutations by direct sequencing (bidirectionally, as standard practice in positive samples), as described elsewhere [17,23].

2.2. MiR-21 quantitative real-time polymerase chain reaction

Tissue cores were deparaffinized with xylene at 50°C for 3 minutes. Total RNA extraction was done using the RecoverAll kit (Ambion, Austin, TX) according to the manufacturer's instructions. The NCode miRNAquantitative