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Ileal neuroendocrine tumors show elevated activation of mammalian target of rapamycin complex

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ABSTRACT

Background: Neuroendocrine tumors (NETs) of the ileum are sporadic tumors derived from submucosal gastrointestinal stem cells. They often show clinical symptoms only after hepatic metastasation when curative therapy is limited or impossible. In this study, we analyzed the expression of the candidate genes mammalian target of rapamycin (mTOR), alpha thalassemia/mental retardation syndrome X-linked (ATRX), and death domain-associated protein (DAXX) to investigate the specific oncogenetics and potential therapeutic options for ileal NETs.

Methods: In a prospective database, all patients who underwent surgical removal of a NET of the ileum between 2001 and 2011 were specified. Expression analysis was performed for mTOR, ATRX, and DAXX by immunohistochemistry of paraffin-embedded tumor samples. To evaluate the results the immunoreactive score was applied. Normal tissue and tumor tissue were analyzed for the comparison of gene expression levels using quantitative-real-time polymerase chain reaction for ATRX and mTOR genes. Results were correlated under pathologic and clinical aspects.

Results: A total of 69 patients were admitted to the study. Positive cytosolic expression of the potential oncogene mTOR was immunohistochemically detected in 76.2% of the human probes. A loss of nuclear ATRX expression was detected in 13.0% of the samples. A nonexpression of the DAXX-protein in cell nuclei was not found (0%). Gene transcript levels did not show a significant alteration in ileal NETs in comparison with normal tissue.

Conclusions: mTOR is overexpressed in ileal NETs. Additionally, the loss of ATRX expression was registered, thus underlying a tumorigenic role in a subgroup of these tumors. To enable potential therapeutic application of mTOR inhibitors, further trials with larger study groups are needed.

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1. Introduction

Neuroendocrine tumors (NETs) are epithelial tumors originating in the neural crest. Although their predominant location of manifestation is the gastrointestinal tract, they are also found in the lung, adrenal glands, and neuronal tissue. Although NETs are still considered to be infrequent neoplasms, their overall incidence increased remarkably by approximately 300%–500% in recent years [1–3]. Twenty-five percent of all NETs are localized in the ileum. Despite their slow progression, they are often only diagnosed through lymph node and hepatic metastases. This mechanism impedes curative surgical resection and limits overall survival [4–6].

Only a few genes were identified, which are involved in tumorigenesis of NETs and most of them are found sporadically.

In medullary thyroid cancer, mutations of rearranged during transfection, a receptor tyrosine kinase, were detected [7]. Pancreatic NETs (panNETs) can be found in patients with genetic disorders, such as multiple endocrine neoplasia type 1 syndrome, von Hippel-Lindau syndrome, and neurofibromatosis type 1 [8–10]. The molecular etiology of ileal NETs is poorly understood but the existence of an inherited "familial ileal neuroendocrine carcinoma" has been suggested and seems to be associated with an aberration on chromosome 18 [11].

In addition to epigenetic mechanisms, Jiao *et al.* [12] recently identified gene alterations in alpha thalassemia/mental retardation syndrome X-linked (ATRX)/death domain-associated protein (DAXX) and mammalian target of rapamycin (mTOR) genes in panNETs. Physiologically, these proteins regulate apoptosis, cell growth, and chromatin remodeling. Their overexpression or loss of function caused by genetic mutations plays a role in the development of various types of cancers [13–15].

As a medical therapy, somatostatin analogs were assumed to be the only option to delay disease progression of ileal NETs [16]. In a placebo-controlled trial, the combination of somatostatin analogs with mTOR inhibitors showed an improved progression-free survival of patients with NET [17]. Bajetta *et al.* recently confirmed these results in a first-line setting for patients with NETs [18]. Consequently, a better understanding of the underlying tumor biology and molecular factors is required [19,20].

mTOR is an intracellular protein kinase, which is part of the Ras/PI3K/PTEN/Akt/mTOR signaling pathway. It regulates cellular response to nutrients and interacts through hormones for example, the insulin-like growth factor 1 [21]. Alterations in the mTOR pathway were already revealed in *in-vitro* models and panNETs [22,23]. Treatment of panNETs with mTOR inhibitors (e.g., everolimus) has shown a significant prolonged progression-free survival [24].

The ATRX gene is encoding for an ATPase/helicase domain, which belongs to the SWItch/Sucrose NonFermentable family of chromatin remodeling proteins. Mutations in the ATRX gene are associated with the alpha thalassemia/mental retardation syndrome, genome instability, and uncontrolled cell proliferation [25]. The correlation between mutations occurring at ATRX gene and tumorigenesis was already shown in promyelocytic leukemia [13].

DAXX encodes a multifunctional protein acting at different levels in the nuclear and cytosolic cell compartment. DAXX interacts with various proteins, such as the apoptotic Fas receptor and the centromere protein C. In the nucleus, DAXX works as a potent transcription repressor [26]. Because of its fundamental role in apoptosis, DAXX represents a relevant marker in cancer-related mutations [27].

Furthermore, recent findings demonstrate an interaction of ATRX and DAXX forming a chromatin remodeling complex [28,29]. The correlation between the loss of this chromatin remodeling complex and alternative lengthening of telomeres was shown recently in panNETs [30]. Loss of immunolabeling for DAXX or ATRX is associated with shorter overall survival of patients with panNETs in comparison with those who lack these mutations [31].

Thus, the thesis of this work is that mutations in ATRX, DAXX, and mTOR, which already were detected in panNETs, could also be found in ileal NETs. The aim of this study was to analyze these three genes to be able to detect patients who qualify for targeted therapy in the future.

2. Materials and methods

2.1. Patients

Primary midgut NET samples were obtained from 69 patients who underwent surgical removal at the Department of Surgery, University Hospital Medical Centre Giessen and Marburg, Marburg, Germany between 2001 and 2011. Most of the samples could be used for immunohistochemical staining. Sufficient amount of tumor tissue to isolate RNA was obtained from 22 patients.

Before surgical resection, a written informed consent was obtained from each patient. The study was approved by the local ethics committee at Philipps University Marburg, Marburg, Germany (approval code: AZ: 206/10). Specimens were fixed in phosphate-buffered formalin or snap-frozen immediately after surgical removal for further analysis.

2.2. Classification of the tumors

The classification of the tumors was conducted through the TNM-Classification according to European Neuroendocrine Tumor Society with grading and Ki-67 expression. Tumors were considered as G1 with Ki-67 $\leq$2%, G2 with Ki-67 3%–20%, and G3 with Ki-67 $\geq$20%.

2.3. Immunohistochemical staining

Paraffin-embedded tumor tissues were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol ending in phosphate-buffered saline. Heat antigen retrieval was performed in citrate buffer pH 7 for 20 min (mTOR, ATRX) or Tris-EDTA buffer (10 mM Tris/1 mM EDTA/0.05% Tween20) pH 9 for 30 min (DAXX), respectively. Samples were incubated overnight with primary antibodies against mTOR (ab32028; Abcam, Cambridge, United Kingdom; 1:1000), ATRX (HPA001906; Sigma–Aldrich, St Louis, MO, 1:1000), or DAXX (HPA008736; Sigma–Aldrich, 1:500). Signals were