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Heme Oxygenase-1 Promoter Polymorphism is a Predictor of Disease Relapse in Pancreatic Neuroendocrine Tumors

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Background. **Current available preoperative diagnostic workup is insufficient to differentiate between benign and malignant pancreatic neuroendocrine tumors (PNET). The aim of the present study was to evaluate the potential prognostic role of the promoter GTn repeat polymorphism (GTn) of the heme oxygenase-1 gene in PNET.**

Methods. **Tumor, metastasis, corresponding healthy tissue, and peripheral blood leukocyte DNA of 46 patients who underwent surgical resection for PNET were analyzed for GTn by PCR, capillary electrophoresis, and DNA-sequencing. The GTn was correlated to clinicopathologic parameters and clinical outcome.**

Results. **GTn was classified into short (<25) and long (≥25) alleles and three (SS, SL, and LL) genotypes were defined. There was no difference in GTn genotype among tumor, healthy tissue, metastasis, and peripheral blood leukocyte DNA. The SS and SL genotype displayed significantly more poor differentiated tumors ($P = 0.001$) and higher tumor recurrence rate ($P = 0.0001$) compared with LL patients. The LL genotype patients presented predominantly benign PNET ($P < 0.001$). The LL genotype had the longest disease-free ($P < 0.001$) and overall survival ($P = 0.006$). Besides the WHO classification the GTn was identified as a strong predictor of tumor recurrence (hazard ratio 3.1, 95% confidence interval 1.3–7.3) in PNET.**

Conclusion. **GTn differentiates between benign and malignant PNET and is a strong predictor of tumor recurrence.**

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Key Words: **pancreatic neuroendocrine tumors; polymorphism; heme oxygenase-1; prognostic marker; recurrence; survival.**

INTRODUCTION

Pancreatic neuroendocrine tumors (PNET) are rare tumors of the endocrine pancreas and make up to 3% of all pancreatic malignancies [1, 2]. The new classification of PNET as proposed by Capella *et al.*, and modified later on by Kloeppel and coworkers, provides a valuable tool for postoperative prognosis and management of PNET [3, 4]. Surgery is the only curative treatment for PNET. However, the type of surgery (organ-sparing *versus* oncologic) is dependent upon the biologic behavior of PNET. The WHO classification of PNET draws a cut-off line between benign and malignant lesions based on tumor differentiation only. Malignant lesions are further classified into well- and poorly-differentiated neuroendocrine carcinoma (NEC). From a clinician's point of view, preoperative assessment of the biologic behavior of PNET is crucial for customized therapy. There is an overall paucity of prognostic markers in PNET and no marker is available to differentiate between benign and malignant lesions before therapy initiation. Ideal prognostic marker should be easily to detectable, independent of tumor tissue availability and harbour genomic stability that remains unbiased by type of tumor therapy. Genetic variations in cancer patients might be important prognostic indicators of clinical outcome.

Heme oxygenase-1 (HO-1) is the rate limiting enzyme in heme degradation and represents a stress-responsive protein. HO-1 has been reported to play an important role in several diseases including malignant

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tumors [5]. However, tumor promoting and anti-tumor properties have been reported for HO-1 [6]. The HO-1 gene is located at chromosome 22 that is known to play a pivotal role in development and progression of several malignancies including pancreatic ductal adenocarcinoma [7–11]. The basal transcriptional activity of the heme oxygenase-1 gene (HMOX-1) is dependent upon GTn repeats in the promoter region of HMOX-1 [12]. The GTn polymorphism can easily be determined in tissue and blood leukocyte DNA. The GTn repeat polymorphism has been reported to be associated with melanoma, oral squamous cell carcinoma, lung and gastric cancer, as well as gastrointestinal stromal tumors previously [13–18].

The role of HMOX-1 GTn promoter polymorphism has not yet been analyzed in PNET. The aim of the present study was to correlate the GTn polymorphism with clinicopathologic parameters in PNET and to evaluate the potential role of GTn as a useful prognostic marker for allocation of patients in to individual risk profiles and customized therapy.

MATERIAL AND METHODS

This study was approved by the ethical committee of the chamber of physicians, Hamburg, Germany. Informed consent was obtained from all patients before including them in to a prospective data base. For this study, 46 patients who were surgically treated for PNET between 1995 and 2006 at our institution were analyzed retrospectively. All tumors were histologically proven PNET. Tumor size, mitotic count, differentiation, angioinvasion, and immunohistochemical staining of hormones, precursor amines, and peptides were evaluated to classify tumors according to the new WHO classification. Additional clinical data were obtained from the clinical records. Clinical follow-up data were obtained by studying the patient clinical charts and/or by contacting them on an outpatient basis or by phone call. All patients were recruited into the study after histologic reclassification according to the new WHO classification for PNET. Ki-67 was used to determine the proliferation index. Functional tumors were defined by positive hormone immunohistochemistry and presence of clinical symptoms associated with systemic secretion of the hormones as proposed by Capella [4].

For GTn polymorphism analysis, DNA was extracted from 46 snap-frozen tumors, corresponding healthy tissue, metastasis, and from peripheral blood leukocytes using a standard extraction protocol (Qiagen, Hilden, Germany). Before extraction, seven consecutive slides (two flanking slide of 7 μm and five core slides of 15 μm) were cryosectioned and stained with hematoxylin and eosin. A pathologist marked the tumor afterwards.

Sequencing and Analysis of GTn Polymorphism

For amplification of the 5'-flanking region with the poly(GT)n sequence by polymerase chain reaction (PCR), we used a sense primer OXY-F (5′-AGAGCCTGCAGCTTCTCAGA-3′) and a fluorescent Ig-labeled anti-sense primer OXY-RL* (5′-ACAAAGTCTGGCCATAG GAC-3′) complying with the previously published sequence.

PCR for the fragment-length analysis was performed in a 25 μL reaction mix consisting of 0.25 μL extracted DNA, 2.5 μL 10× PCR Buffer IV (ABgene, Epsom, UK), 4 μL deoxyribonucleotide triphosphate (1.25 mm), OXY-F/OXY-RL primer, and 1.25 μL Taq-polymerase. PCR conditions consisted of repeated cycles at 95, 55, and 72°C for 30 s to 2 min. Electrophoresis of PCR product was processed on a 2.5% agarose gel. The sizes of the amplified fragments were determined by capillary electrophoresis with an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using a composition of 40 μL formamide (Hi-Di), 0.2 μL Genescan-500-ROX Standard as well as 0.1–2.0 μL of PCR product (selected quantity was dependent on the band quality determined by electrophoresis) denaturized at 94°C for 2 min.

GTn repeats were calculated according to size marker homozygous DNA. For this purpose, sequencing reaction was processed in 20 μL mix containing 3.0 μL extracted DNA, 2.0 μL buffer (ABI 5× sequencing buffer; Applied Biosystems), 4.0 μL ABI BigDye dideoxyterminator sequencing mix ver. 1.1 (Applied Biosystems), and OXsFs primer (5′-GGGTTGCTAAGTTCCTGATG-3′). Synthesis cycles were repeated at 95, 50, and 60°C for 30 s to 4 min. The product was then analyzed by automatic sequencer ABI3110. Size markers were two homozygous samples with 23 and 30 GTn repeats, respectively. GTn repeats <25 were classified as short (SGTn) and ≥25 as long (LGTn) repeats. This classification is in accordance with the reported classification of GTn length polymorphism by other groups [13–15]. Based on the combination of the short and long alleles, SS, SL, and LL genotypes were defined.

Statistical Analysis

For statistical analysis SPSS for Windows (SPSS Inc., Chicago, IL) was used. The χ^2 test was used to correlate clinicopathologic parameters with GTn genotypes. Survival curves of the patients were plotted using the Kaplan-Meier method and analyzed using the log-rank test.

Results are presented as median survival in months with 95% confidence interval (95% CI) and number of patients at risk. Mean values are presented and specifically indicated in case if the median survival was not reached. The overall survival (OS) was computed as the time period from the date of surgery to either the date of death or last follow-up, whichever occurred first. The disease-free survival (DFS) was defined as the time period from the date of surgery to the date of recurrence, last follow-up, or date of death, whichever occurred first. Patients alive without recurrence at the last follow-up date were censored. Cox regression hazard model was used for univariate and multivariate analyses to assess the prognostic value of GTn polymorphism and other covariates on recurrence. Results are presented as hazard ratio (HR) and 95% CI. Significant statements refer to P values of two-tailed tests that were <0.05.

RESULTS

Patients

Forty-six patients with PNET who underwent surgical resection were included in the study. Patient characteristics, i.e., gender, tumor type, and tumor recurrence are depicted in Table 1. The median age of the study population was 52 y (range 27–73). Thirty-five (76.1%) patients had nonfunctional tumors and 11 (23.9%) patients presented with active functional tumors. Out of these 11 patients, four had an insulinoma, two a gastrinoma, and five a carcinoid syndrome. Four patients had a multiple endocrine neoplasia type I syndrome associated PNET and two had an association with Zollinger-Ellison syndrome. Histologically, 38 (82.6%) patients had grade I, two (4.3%) and 6 (13%) patients had grade II and III tumors, respectively.

According to the current WHO classification, 11 (23.9%) patients presented with a benign PNET.