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Original article

Molecular alterations in sporadic pancreatic neuroendocrine microadenomas

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ABSTRACT

Background: Pancreatic neuroendocrine microadenomas (pNEMAs) are neuroendocrine tumors measuring <5 mm in diameter. They are considered the precursor of pancreatic neuroendocrine tumors (pNETs). The aim of this study was to investigate the immunohistochemical differences between pNEMA, pNET, and hyperplasia of pancreatic islet cells (HPIL) in patients with non-familial syndromes.
Methods: We evaluated 21 pNEMAs, 19 HPILs, and 21 non-functional pNETs (10 G1 and 11 G2 cases) in patients with non-familial syndromes. Immunohistochemistry for tumor-associated markers death domain-associated protein (DAXX), alpha thalassemia/mental retardation X-linked (ATRX), cytokeratin 19 (CK19), bcl-2, and CD99 was performed.
Results: DAXX was expressed in 95%, 71%, and 71% of HPIL, pNEMA, and pNET samples, respectively; the differences were not significant. ATRX expression in pNEMA and pNET was significantly lower than that in HPIL, whereas there was no significant difference between pNEMA and pNET (HPIL: 95%, pNEMA: 43%, and pNET: 52%). All HPIL and pNEMA cases were negative for bcl-2 and positive for CD99, whereas 29% of pNETs were positive for bcl-2 and 24% were negative for CD99. CK19 expression in HPIL was significantly lower than in pNEMA and pNET, although no significant difference was observed between pNEMA and pNET (HPIL: 5%, pNEMA: 57%, and pNET: 43%). Among G1 and G2 pNETs, CD99 was expressed in 50% of G1 pNETs but not in any G2 pNET cases.
Conclusion: Non-familial HPIL, pNEMA, and pNET patients exhibit distinct ATRX, CD99, CK19, and bcl-2 molecular profiles.

Introduction

Pancreatic neuroendocrine neoplasm (pNEN) is rare, accounting for 1–2% of all pancreatic neoplasms [1,2]. Although most pNENs are sporadic, 10–15% of the cases occur on the background of familial syndromes such as von Hippel-Lindau (VHL) syndrome, tuberous sclerosis complex (TSC), neurofibromatosis type 1 (NF1), and multiple endocrine neoplasia type 1 (MEN1) [3].

According to the World Health Organization (WHO) classification published in 2010, NEN of the digestive system is divided into neuroendocrine tumor (NET) G1, NET G2, and neuroendocrine carcinoma (NEC) [1]. This WHO classification is based on mitotic count and Ki-67 labeling index. NET can be equated with carcinoid, whereas NEC is subdivided into small- and large-cell type [1,4]. Pancreatic NETs (pNETs) are slow growing and relatively indolent, but once unresectable metastases occur, the disease could be fatal with a death rate per 100 person-years of 1.0% for pNET G1 and 5.1% for pNET G2 [5]. Several factors have been reported to predict the aggressiveness of pNETs, such as cytokeratin 19 (CK19), CD99, and

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bcl-2 [6–11]. However, it remains difficult to predict pNET tumor behavior. Pancreatic NECs (pNECs) are apparently more aggressive and have poorer prognoses than pNETs. Patients' overall survival ranges from one month to one year, despite some initial favorable responses to chemotherapy [1].

Several molecular alterations have been reported in pNEN; pNECs were reported to exhibit abnormal immunolabeling of p53, retinoblastoma protein (Rb), and p16, as well as bcl-2 overexpression and Kras mutation, while pNETs showed no abnormal p53, Rb, or p16 immunolabeling, no Kras mutation, and lower bcl-2 expression [11]. Furthermore, although DAXX (death domain-associated protein) and ATRX (alpha thalassemia/mental retardation X-linked) are reportedly intact in pNECs, their expression is lost in 43–45% of pNETs [11,12]. DAXX and ATRX are tumor suppressor genes encoding nuclear proteins that interact with one another. They are thought to function in chromatin remodeling within the telomeres and pericentromeric regions [13]. Mutations in these genes are tightly associated with the loss of their respective proteins' nuclear expression via immunohistochemistry and correlate with the alternative lengthening of telomere phenotype, a telomerase-independent telomere maintenance mechanism [13,14].

Pancreatic neuroendocrine microadenoma (pNEMA) is defined as a pNET measuring <5 mm in diameter [1]. It is regarded as the precursor to, or initial lesion of, pNET [15,16]. Multiple pNEMAs (microadenomatosis) are identified in >80% of MEN1 patients, and these pNEMAs usually show allelic deletion of the *MEN1* gene [15,16]. Sporadic pNEMA without familial syndromes is often incidentally discovered in surgically resected materials and autopsy cases. The incidence of sporadic pNEMA in unresected autopsy cases is 0.4–1.5% [1].

Recently, de Wilde et al. reported that loss of DAXX and/or ATRX expression was observed in 6% of pNETs measuring ≥0.5 cm, but the expression was intact in pNEMAs of patients with MEN1 syndrome [13]. These results indicated that loss of DAXX and/or ATRX expression was a late event during pNET tumor development in patients with MEN1 syndrome. However, the alteration of DAXX and/or ATRX in sporadic pNEMAs with non-familial syndromes is unclear.

Therefore, in the present study, we evaluated the immunohistochemical expression of DAXX and ATRX in sporadic pNEMA, pNET, and hyperplasia of the islets of Langerhans (HPIL). We also assessed the expression of factors that are reportedly associated with pNET aggressiveness and prognosis, including CK19, CD99, and bcl-2.

Methods

Cases

We evaluated 21 pNEMAs identified at Tokai University Hospital between 1991 and 2014. They were obtained from 19 patients (9 men and 10 women). As control samples, 19 HPILs and 21 non-functional, sporadic pNETs that were identified between 2009 and 2014 were also evaluated. Patients did not undergo genetic testing for molecules such as VHL, TSC, NF1, and MEN1 as they were not suspected of having familial syndromes; those who were suspected to have familial syndromes were not included in this study. Samples were fixed in formalin, embedded in paraffin, and cut into 4-μm-thick sections. They were then stained with hematoxylin-eosin (HE) according to the standard procedures. We defined pNEMA as follows: 1) <5 mm in diameter, 2) well-demarcated, round nodule with or without fibrous capsule, 3) mainly composed of homogenous cells, and 4) nearly or completely negative for insulin. HPIL was defined as a well- or ill-demarcated, enlarged islet of Langerhans that retained the morphology of its normal counterpart and exhibited heterogeneous expression of insulin. pNET was defined as a tumor of WHO grade G1 or G2 measuring ≥5 mm in diameter. This study was approved by the Research Ethics Committee of Tokai University School of Medicine (No. 14R687).

Immunohistochemistry

Immunohistochemistry was performed on 4-μm thick sections of formalin-fixed, paraffin-embedded tissues using the following primary antibodies: insulin (polyclonal, dilution 1:100, Dako, Glostrup, Denmark), bcl-2 (clone 124, dilution 1:60, Dako), CK19 (clone Ks19.1, dilution 1:100, Progen Biotechnik, Heidelberg, Germany), CD99 (clone 12E7, dilution 1:100, Dako), Ki-67 (clone MIB-1, dilution 1:200, Dako), ATRX (polyclonal, dilution 1:400, Sigma–Aldrich, St. Louis, Missouri, United States), and DAXX (polyclonal, dilution 1:100, Sigma–Aldrich). All immunohistochemistry, except for insulin, was performed using the BondMax system (Leica Microsystems, Newcastle upon Tyne, United Kingdom) according to the manufacturer's instructions. Antigen retrieval was performed by treatment with Bond Epitope Retrieval Solution 2 (Leica) for 20 min for DAXX, ATRX, bcl-2, and Ki-67; Bond Epitope Retrieval Solution 1 (Leica) for 20 min for CD99; and Enzyme 1 of the Bond Enzyme Pretreatment Kit (Leica) for 5 min for CK19. For insulin detection, the sections were deparaffinized and placed in a solution of 0.3% hydrogen peroxide/methanol for 30 min. After washing with phosphate buffered solution, the sections were incubated with primary antibody against insulin for 60 min at room temperature. Sections were then exposed to Histofine Simple Stain MAX-PO(R) (Nichirei, Tokyo, Japan) secondary antibody detection reagent for 40 min at room temperature; 3,3'-diaminobenzidine was used as the chromogen. Appropriate positive and negative tissue control samples were used. Immunolabeling for DAXX and ATRX was considered positive if at least 5% of cells exhibited nuclear labeling. Similarly, bcl-2 and CK19 were considered positive by cytoplasmic labeling, while CD99 was so considered by membranous and/or cytoplasmic labeling.

Statistical analysis

All statistical analyses were performed using SPSS version 21 (IBM Japan, Tokyo, Japan). Fisher's exact test or Pearson's chi-squared test was used to analyze the relationship between clinicopathological features and immunohistochemical results. A p value of <0.05 was considered significant.

Results

Clinicopathological features

All pNEMAs and HPILs were incidentally found either during pancreatic resection for other diseases or in pancreases obtained via autopsy. Twenty one pNEMAs were obtained from 19 patients (nine men and 10 women). Of these, 18 patients had a single pNEMA; one had three pNEMAs. These patients were originally diagnosed with various diseases, including 10 with pancreatic ductal adenocarcinoma, two with pancreatic mucinous cystic neoplasm, two with pancreatic intraductal papillary mucinous neoplasm, one with pancreatic acinar cell carcinoma, one with chronic pancreatitis, and one with extrahepatic bile duct adenocarcinoma. Three of the included patients were autopsy cases. The average age of pNEMA patients was 67.2 years (range: 44–81 years). The mean pNEMA size was 1859.5 μm (range: 450–4300 μm). Nineteen HPILs were obtained from the resected

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