Relationship between pancreatic intraepithelial neoplasias, pancreatic ductal adenocarcinomas, and single nucleotide polymorphisms in autopsied elderly patients

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Relationship between pancreatic intraepithelial neoplasias, pancreatic ductal adenocarcinomas, and single nucleotide polymorphisms in autopsied elderly patients

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Running title: Pancreatic cancer lesions and SNPs

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Abstract

A growing body of evidence shows that the presence of single nucleotide polymorphisms (SNPs) influences individual predisposition to pancreatic cancer. In the present study, we comparatively analyzed serially autopsied, elderly Japanese patients (n = 2,205) with pancreatic intraepithelial neoplasias (PanINs) and pancreatic ductal adenocarcinomas (PDACs) on the basis of their pancreatic lesions, clinical information, and SNPs. The incidence of PanIN-1, -2, -3, and PDACs in these patients was 55%, 12%, 1.4%, and 2.4%, respectively. The occurrence of PanINs was associated with female sex, increasing age, and lower body mass index. We did not identify any common SNPs between PanINs and PDACs. There were no common SNPs associated with PanINs and PDACs between men and women. In previously reported pancreatic cancer-associated SNPs, rs3790844 (NR5A2) showed a significant correlation with PDAC in our cohort. Six SNPs (rs7016880, rs10096633, rs10503669, rs12678919, rs17482753, rs328) that were correlated with blood lipid levels were associated with the risk for PDACs. Our data suggest that different clinicopathological characteristics and predispositions may affect pancreatic carcinogenesis in elderly Japanese patients.

Key words: single nucleotide polymorphisms, pancreatic intraepithelial neoplasia, pancreatic ductal adenocarcinoma
Introduction

Morbidity and mortality due to pancreatic cancers have been increasing worldwide (Warshaw et al., 1992, Bidoli et al., 2012). In Japan, pancreatic cancer represents the fifth and the fourth leading cause of cancer-related deaths in men and women, respectively. Tobacco use (MacLeod et al., 2006), heavy alcohol consumption, diabetes, obesity, pancreatitis, low 25-(OH) vitamin D levels due to low exposure to solar radiation, and aging are known to be risk factors for pancreatic cancer (Pandol et al., 2012, Matsuda et al., 2016). Studies on autopsied patients, surgically resected tissue specimens, and recent molecular studies have shown that pancreatic cancer does not arise de novo; it progresses through a multistep process involving either intraepithelial proliferation or through a dysplasia-cancer sequence (Mukada et al., 1982, Hruban et al., 2001, Kanda et al., 2012).

The vast majority of pancreatic cancers are thought to arise from pancreatic intraepithelial neoplasias (PanINs); high-grade PanINs (carcinomas in situ) are believed to be immediate precursors of pancreatic ductal adenocarcinomas (PDACs), which are the most common type of pancreatic cancers (Basturk et al., 2015). Mutations in KRAS, CDKN2A, TP53, and SMAD4 are the main driver mutations in PDACs. These mutations accumulate and drive neoplastic transformation and tumor progression (Bardeesy et al., 2002, Kore, 2010).

Inherited genetic factors also play important roles in determining the risk for PDAC (Klein, 2012). PDAC is fundamentally a genetic disease caused by both inherited and somatic mutations. It is estimated that 5–10% of patients with PDAC have family histories of PDAC (Hruban et al., 2010). Recent genome-wide association studies for PDAC using Caucasian populations have identified associations with
chromosome 9p34.2 (ABO), 13q22.1 (KLF5), 1q32 (NR5A2), 5p15.33 (CLPTM1L-TERT), (Amundadottir et al., 2009, Petersen et al., 2010), 7q32.3 (LINC-PINT), 16q23.1 (BRCAR1), 13q12.2 (PDX1), 22q12.1 (ZNRF3), 8q24.21 (PVT1) (Wolpin et al., 2014), 17q25.1 (LINC00673), 7p13 (SUGCT) and 3q29 (TP63) (Nature).

Recent study also showed that three SNPs on chr1q32.1 (NR5A2), shr8q24.21 (MYC) and chr5p15.33 (CLPTM1L-TERT) represent independent risk variants of pancreatic cancer and NR5A2 expression in the pancreatic cancers markedly decreased (Zhang). In Japanese populations, the single nucleotide polymorphisms (SNPs) located on chromosomes 6p25.3, 12p11.21, 7q36.2, and 1q32 (NR5A2) have shown a significant association with PDAC (Low et al., 2010, Ueno et al., 2015).

The prognosis of PDAC remains poor, with an overall 5-year survival rate of only 6%, partly due to difficulties in diagnosing carcinomas at early stages or in identifying high-risk precursor lesions (Ahrendt et al., 2002, Siegel et al., 2014). Thus, identifying patients with a predisposition to PDAC and early identification of PDAC, preferably in its pre-invasive stage, especially in high-grade PanINs, is of paramount importance (LeBlanc et al., 2014). Autopsy samples are important tools in the analysis of various grades of PanINs and different stages of progression of PDACs. In the present study, we comparatively analyzed serially autopsied, elderly patients with PanINs and PDACs, including information about their pancreatic lesions, clinical information, and SNPs.

**Materials and Methods**

**Study Population**

We studied 2,205 autopsy cases, which were collected at the Tokyo
Metropolitan Geriatric Hospital between 1995 and 2012. There were 1,221 men and 984 women with a mean age of 80.7 ± 8.8 years (range, 33–104 years) and a mean body mass index (BMI) of 17.4 ± 3.7 kg/m² (range, 8.1–37.9). The autopsied patients were enrolled in the Internet Database of Japanese Single Nucleotide Polymorphisms for Geriatric Research (JG-SNP) (Sawabe et al., 2004). Most of the cause of death was malignancies, infections, cardiovascular diseases. Approximately 60% of patients had malignant tumors (Yamada et al., 2015). The presence or absence of PanINs and PDACs was determined via an examination after autopsy.

After gross examination, a 3-μm-thick section was cut from each paraffin-embedded tissue block, and the sections were stained with hematoxylin and eosin. PanINs were defined as microscopic, papillary or flat, non-invasive, epithelial lesions with diameters of 5 mm or less (Longnecker et al., 2005). Papillary, non-invasive epithelial lesions that were larger than 1 cm in diameter were diagnosed as intraductal papillary mucinous neoplasms. PanIN lesions were classified as PanIN-1, -2, or -3 on the basis of their structural and cellular atypia (Figure 1), in accordance with previously described criteria (Hruban et al., 2001, Hruban et al., 2010). All pathological diagnoses were performed by gastrointestinal pathologists (Y.M., T.A.). We collected information about smoking and drinking habit from medical records. Classification of smoking habits in patients included both current and past smoking history. Similarly, classification of alcohol consumption in patients included both current and past consumption.

**Genotyping and Genotype Calling**

Genomic DNA was extracted from the renal cortex using a standard
procedure. All samples were genotyped with Illumina Infinium HumanExome BeadChip Version 1.1 (Illumina, San Diego, CA) by iScan, in accordance with Illumina protocols at the Center for Molecular Biology and Genetics/Core-Lab, Graduate School of Regional Innovation Studies, Mie University, Mie, Japan (Yamada et al., 2015). Genotype calling was performed for all samples as a single project using the Genotyping Module (version 1.9) of the GenomeStudio data analysis software package. Initial genotype clustering was performed using the default Illumina cluster file (HumanExome 12v1-1_A.egt) and the manifest file (HumanExome-12v1-1_A.bmp), using the GenTrain2 clustering algorithm. We considered a per sample call rate of >98% as eligible, and 15 samples were excluded using this criterion. Validation of the polymorphisms was performed by direct sequencing, using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on a 3130 Genetic Analyzer (Applied Biosystems). The pathological assessment and genotyping were performed in different institutions in a double-blind fashion to minimize bias.

Analysis

For statistical analysis, cases without PanINs and PDACs were used as controls. We performed chi square, Fisher, Cochran-Armitage, Dominant-Fisher analyses to determine the association between the phenotypes and SNPs. P value of Dominant-Fisher analysis was used to pick up relevant SNPs. The analyses were adjusted for age, sex, BMI, smoking and drinking using SPSS version 22 (IBM Corp., New York, NY), and a comparison was performed between patients with or without PanINs and PDACs identified at autopsy. In the present study, there was no result which reach a genome-wide threshold (P<5×10⁻⁸). P<0.05 is considered as statistically significant.
**Ethics Statement**

This study was approved by the Tokyo Metropolitan Geriatric Hospital Ethics Committee (approval no. 230405). Written informed consent was obtained before autopsy from family members of all participants involved in this study.

**Results**

The demographics of the patients included in the study are summarized in Tables 1 and 2. Of the total 2,205 patients, 55% had PanIN-1, 12% PanIN-2, 1.4% PanIN-3, and 2.4% PDAC. Women showed a higher incidence of PanIN-1 and -2 and PDACs (Table 1, $P < 0.05$, Figure 2). The incidence of PanINs increased with age (Figure 2). The patients with PanIN-1, -2, and -3 were older than the patients without these lesions (Table 1, $P < 0.05$). Furthermore, contrary to previous reports, patients with PanIN-1 had lower BMIs than patients without PanIN-1 (Table 1, $P < 0.05$). Smokers had a lower incidence of PanINs-1 and -2, and alcohol consumers showed a lower incidence of PanINs-1 (Table 2, $P < 0.05$); however, no statistically significant changes were noted even when this association was analyzed separately for men and women.

We examined the association of SNPs with low-grade PanINs, including PanINs-1 and -2. Our results (Supplemental Table 1) showed that SNPs were related to the risk for low-grade PanINs, with $P < 5 \times 10^{-2}$, in all patients, including both men and women. We then examined the association of SNPs with PDACs, including invasive cancers and carcinomas in situ/PanIN-3 (Supplemental Table 2). We found that 329 SNPs were related to the risk for low-grade PanINs, and 343 SNPs were related to the risk for PDACs, at $P < 0.05$. No SNPs were common between men and women, both in
cases of low-grade PanINs and PDACs, respectively.

The SNPs that were previously identified via genome-wide association studies to be associated with the risk for developing PDAC were chromosome 9p34.1 (ABO), 15q14, 13q22.1, 5p15.33 (CLPTM1L-TERT) (Amundadottir et al., 2009, Petersen et al., 2010), 1q32 (NR5A2)(Ueno et al., 2015), 7q32.3 (LINC-PINT), 16q23.1 (BRCA1), 13q12.2 (PDX1), 22q12.1 (ZNRF3), 8q24.21 (PVT1) (Wolpin et al., 2014), 6p25.3, 12p11.21, and 7q36.2 (Low et al., 2010). In our study, only rs3790844 (NR5A2) (Petersen et al., 2010, Ueno et al., 2015) showed a statistically significant association with the risk for PDAC (P = 0.00962, OR = 2.19, Supplemental Table 2).

From among the SNPs associated with occurrence of either low-grade PanINs or PDAC, we extracted SNPs that were common between these two groups. Six SNPs were common between these two groups (Supplemental Table 3); however, the P values for most of them were > $10^{-4}$. Only WAPAL correlated with the risk for PanIN in women (P < $10^{-4}$).

We did not find SNPs that correlated with the risk for developing low-grade PanINs and PDACs, at a genome-wide threshold (P<5x10-8). Table 3 and Supplemental Table 4 showed SNPs correlated with PanINs and PDACs at P < $10^{-4}$. There have been no reports correlating these SNPs with the risk for developing PDAC. Among those 23 SNPs, 12 variants were non-synonymous. Six SNPs previously reported to be related to blood lipids (rs7016880 (Johansen et al., 2010), rs10096633 (Kamatani et al., 2010), rs10503669, rs12678919 (Willer et al., 2008), rs17482753 (Saxena et al., 2007), rs328 (Ariza et al., 2010)) were found to be associated with the risk for developing PDACs in men (Supplemental Table 4). There were no common SNPs between PanINs and PDACs.
Discussion

In the present study, we investigated the relationship between SNPs associated with clinicopathological characteristics of pancreatic precancerous lesions and their predispositions in autopsied elderly Japanese patients. Our data suggested that the incidence of low-grade PanINs increases with advancing age, and this might be associated with the occurrence of PDACs in the elderly. We did not identify any SNPs that were common between PanINs and PDACs, suggesting that most PanINs are independent of PDACs and that only a small portion of PanINs are precancerous lesions. The high incidence of PanINs supports this conclusion.

A previous Japanese genome-wide association study showed that rs3790844 and rs3790843 in the NR5A2 gene were associated with PDAC risk (Ueno et al., 2015). NR5A2 regulates cholesterol, fatty acids, and bile acid homeostasis (Lee et al., 2008). In addition, 6 SNPs that are related to blood lipids are associated with the risk for developing PDAC in men. This result is consistent with the findings from previous epidemiological reports, which showed that a high calorie diet is a risk factor for PDACs. Serum triglyceride levels and the key enzyme lipoprotein lipase, which catalyzes the hydrolysis of plasma triglycerides, may play important roles in carcinogenesis. The human LPL gene is located on chromosome 8p22 and is regulated by hormonal and inflammatory stimuli, such as insulin, glucocorticoids, or adrenaline.

Alterations in genomic DNA such as point mutations, deletions, and amplifications of epigenetic changes can influence LPL gene expression. An LPL Ser447Ter polymorphism is associated with prostate cancer risk (Narita et al., 2004). The loss of heterozygosity in the chromosome 8p22 region has been reported to be
associated with the development of colorectal, hepatic, and prostate cancers (Lu et al., 2006). Our study is the first to demonstrate the relationship between LPL polymorphisms and PDACs in men.

Unlike in previous reports, in this study, the occurrence of PanINs was correlated with low BMI and female sex (Rebours et al., 2015). This discrepancy may be due to the differences in ethnicity in the previous reports or the older mean age of our study cohort, which was associated with lower BMI. Several lines of evidence have shown that smoking and alcohol consumption are important risk factors for PDACs (Pandol et al., 2012); however, we did not observe an association of PanINs or PDACs with these two factors. These results suggest that pancreatic carcinogenesis is multifocal in nature, and factors other than smoking and alcohol consumption may have been more significant in our study cohort.

Our study has several limitations. Most importantly, the average age of our patients (80.7 years) is much higher than that observed in most patients with PDACs. However, our data suggested that different clinicopathological characteristics and predispositions might affect pancreatic carcinogenesis in elderly Japanese patients. Japan is dealing with an increasingly geriatric population. In the United States of America, PDAC is projected to surpass breast, prostate, and colorectal cancers to become the second leading cause of cancer-related deaths by 2030 (Rahib et al., 2014). In this context, it is definitely important to identify the characteristics of age-related pancreatic carcinogenesis. Furthermore, in the present study, there was no result which reach a genome-wide threshold (P<5x10^-8). Genotyping has been done with an exome array; the SNPs do not cover uniformly the whole genome. This could also explain why the majority of the previous data were not replicated in the present study. Further
analysis will be needed to clarify the relationships between PanIN and SNPs.

In conclusion, we identified clinicopathological characteristics of PanINs and PDACs in elderly Japanese patients. The PanINs and PDACs seen in our cohort showed characteristics that were different from those in previously reported studies, suggesting that different mechanisms operate in age-related pancreatic carcinogenesis.

Disclosure/conflict of interest
The authors declare no conflicts of interest exist for this report.

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Figure legends

Figure 1. Representative images of pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinomas. (A) Pancreatic intraepithelial neoplasias (PanIN)-1, (B) PanIN-2, (C) PanIN-3 or carcinomas in situ, and (D) pancreatic ductal adenocarcinoma. The tissue sections were stained with hematoxylin and eosin. Original magnification of all images was 400 ×.

Figure 2. The incidence of pancreatic intraepithelial neoplasias and pancreatic ductal adenocarcinomas in men and women. Upper graphs indicate percentage of patients with each lesion in autopsied cases. Lower graphs indicate absolute number of patients with each lesion. PanIN, pancreatic intraepithelial neoplasia; PDAC, pancreatic ductal adenocarcinoma.