The stem cell-specific intermediate filament nestin missense variation p.A1199P is associated with pancreatic cancer

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Abstract. The intermediate filament nestin is upregulated in stem/progenitor cells and cancers, and regulates cell proliferation, migration, invasion and stemness. The present study comparatively analyzed serial autopsies of Japanese patients (n=2,206; males, 1,225; females, 981; median, 80.7 years old; range, 33-104 years old) with malignant tumors of whole organs, with respect to the clinical information, and 5 single nucleotide polymorphisms of the nestin gene. p.A1199P associated with pancreatic cancer (odds ratio, 4.4; 95% confidence interval, 1.9-10.0, P=0.001) while it did not associate with malignant neoplasms in other organs. p.A1199P did not associate with precancerous lesions of the pancreas. Single nucleotide polymorphisms of nestin were not associated with sex, drinking, smoking, or body weight. In conclusion, the amino acid 1,199 of nestin is localized in the tail structure of the filament and polymerizes with other intermediate filament proteins. The present results suggest that missense variations of nestin affect pancreatic carcinogenesis in Japanese patients.

Introduction

New cancer cases are rising worldwide because of the growing aging population, and the increasing prevalence of risk factors including smoking, drinking, and obesity. Approximately 14.1 million new cancer cases and 8.2 million deaths occurred worldwide in 2012 (1). In Japan, the most common cause of death was malignant neoplasm (2). A substantial portion of cancer cases and deaths has declined by effective prevention methods, such as tobacco and alcohol control, vaccination, and the use of early detection tests. Inherited genetic mutations play a major role in determining the risk for cancers, and may provide useful information to determine the candidates for early detection tests (3).

Cytoskeletal components regulate cell migration, polarity, and morphology. A neuroepithelial stem cell marker, nestin (NES), is a cytoskeletal protein belonging to the group of class VI intermediate filament (IF) proteins (4,5). NES protein has head, coil, and tail structures. The tail structure of NES is known to interact with other IF proteins, including vimentin, desmin, α-internexin, and synemin, to form heterodimers (6). NES contributes to the disassembly of vimentin during mitosis (7) and to the inactivation of the proapoptotic cyclin-dependent kinase 5 (CDK5) (8). Mouse Cdk5 and Cdc2 induce phosphorylation at both threonine 316 (Thr316) and threonine 1495 (Thr1495) of NES protein (8,9), and phosphorylation of NES modulates mitosis-associated cytoplasmic reorganization during cell mitosis (10).

We have reported that expression of NES in various tumors such as pancreatic cancer (11,12), glioblastoma (13), lung cancer (14), malignant melanoma (15), and uterine cancer (16), regulates cell proliferation, migration, invasion and metastasis. NES regulates stemness in glioblastoma cells through the alteration of cyclin D1 and heat shock cognate 71 kDa protein (13). Phosphorylation of NES at Thr315 and/or Thr1299 regulates cell proliferation (9), and inhibition of both phosphorylation sites suppresses invasion and metastasis of human pancreatic cancer (17).

Data from previous studies (11,12,18-21) indicate that inhibition of either NES expression or phosphorylation may

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be a therapeutic target for several cancers (22). NES is not merely a cytoskeletal protein that serves as a progenitor cell marker, but also is a key regulator of cancer progression processes such as migration, invasion, and metastasis (5,23); therefore, we hypothesized that NES might play important roles in pathogenesis of various cancers. Multiple reports have shown that single nucleotide polymorphisms (SNPs) affect cancer predispositions. However, there have been no reports of a relationship between NES gene variations and cancer. Autopsy is a precious source to analyze various malignant tumors as well as of precursor lesions. In the present study, we comparatively analyzed serially autopsied patients with various malignant neoplasms, based on their clinical information and SNPs.

Patients and methods

Study population. Consecutive autopsy cases (N=2,206) were collected at the Tokyo Metropolitan Geriatric Hospital (Tokyo, Japan) between 1995 and 2012 (24). Participants with family relationships (n=26) were excluded from this study. There were 1,225 men and 981 women with a median age of 80.7 years (range, 33-104 years) and a median body mass index (BMI) of 17.4 kg/m^2 (range, 8.1-37.9). The patients were enrolled in the Internet Database of Japanese Single Nucleotide Polymorphisms for Geriatric Research (JG-SNP) (25). We collected information about smoking and drinking from the medical records. The most frequent causes of death were malignancies, infections, and cardiovascular diseases. Approximately 60% of patients had malignant tumors (26). Cancer-bearing subjects include those with any type of cancer, including pathologically verified surgical resected cancer as a past history and occult cancer found on autopsy. We reviewed all the pancreatic specimens from autopsies to determine the presence or absence of pancreatic cancers and pancreatic intraepithelial neoplasia (PanIN). PanIN was defined as microscopic, papillary or flat, non-invasive, epithelial lesions with diameters of 5 mm or less (27). PanIN lesions were classified as PanIN-1A, -1B, -2, or -3 according to previously described criteria (28,29). The present study was approved by the Tokyo Metropolitan Geriatric Hospital Ethics Committee (approval no. 15-02). This study was conducted in accordance with the principles embodied in the Declaration of Helsinki, 2013. Written informed consent was obtained prior to the autopsy from the family members of all participants involved in this study.

Genotyping and genotype calling. Genomic DNA was extracted from the renal cortex using a standard procedure as previously reported (24). All samples were analyzed with Illumina Infinium HumanExome BeadChip Version 1.1 (Illumina, San Diego, CA) by iScan (26). Genotype calling was performed 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. Validation of the polymorphisms was performed by direct sequencing, using the BigDyeTerminator v3.1 Cycle Sequencing kit on a 3130 Genetic Analyzer (both Applied Biosystems, Foster City, CA, USA) (26). The pathological assessment (YM and TA) and genotyping (MM and MNM) were performed in different institutions in a double-blind fashion to minimize bias. We could not provide the raw data of the present study because we are analyzing our data for use in future studies.

Statistical analysis. We performed Fisher's exact test to determine the association between the phenotypes and SNPs using SPSS version 22 (IBM Corp., Armonk, NY, USA). Power was analyzed using PASS 15.0.5. (NCSS, LLC., Kaysville, UT, USA). $P<0.05$ was considered to indicate a statistically significant difference. We also analyzed the odds ratio (OR) and 95% confidence interval (CI).

Results

The five SNPs we analyzed in the present study are shown in Table I. They are in exons and located in the tail structure of the NES protein, and four SNPs except for NES p.P1275L are rare variants. All SNPs are miss sense mutations. Two SNPs are possibly damaging. We analyzed the association between SNPs of NES and various cancers in major organs. NES p.A1199P did associate with pancreatic cancer (OR, 4.4; 95% CI, 1.9-10.0, P=0.001 by Fisher's exact test, Table II). Large cell lung carcinoma also showed association to NES p.A1199P (OR, 9.2; 95% CI, 0.9-90.9, P=0.02 by Fisher's exact test, Table II), but few patients harbored this change. The urinary tract malignancies showed an association with NES p.A1199P (P=0.053). Malignant neoplasms in other organs such as lung, colon, stomach, brain, and blood cancers did not associate with NES p.A1199P (Fisher's exact test, Tables II and III).

Other SNPs except for NES p.A1199P did not associate with pancreatic cancer (Table IV); therefore, we performed further analysis about NES p.A1199P. Alleles of NES p.A1199P were CC (n=2,127), GC (n=78) and GG (n=1); GC and GG alleles showed that amino acid number 1199 was changed from alanine to proline. It did not associate with sex, drinking, smoking, or BMI (Table V).

We examined the association of NES p.A1199P with precancerous lesions, PanINs (Table VI). PanIN-1A, -1B, -2 and -3 did not associate with NES p.A1199P. In addition, there were no significance between NES p.A1199P and low grade PanIN (PpanIN-1 and -2), or PanIN and cancer. Presence of PanIN-3 and pancreatic cancer was associated with NES.p.A1199P (P=0.007, Table VI). All pancreatic cancers were invasive ductal adenocarcinomas. Pancreatic cancer cases with GC+GG of NES p.A1199P showed a tendency to be well differentiated as compared to CC (P=0.085, data not shown). Sex, age, and tumor stage had no association with NES p.A1199P.

Discussion

In the present study, we investigated the relationship between SNPs of NES and malignant neoplasm predispositions in autopsied Japanese patients. Our data suggests that NES p.A1199P associates with the occurrence of pancreatic cancer, though other malignant neoplasms did not show any

Table I. Single nucleotide polymorphisms of nestin.

Number	Alleles	In-exon	Mutation(s)	rs number	Prediction	Minor allele frequency
exm109872	[A/G]	EXON	Missense_P1275L	rs3748570	Benign	0.2413
exm109911	[T/C]	EXON	Missense_S1016N	rs2365718	Possibly damaging	0.0043
exm109937	[T/G]	EXON	Missense_L791I	rs77202633	Benign	0.0553
exm1719129	[G/C]	EXON	Missense_A1199P	rs78303930	Possibly damaging	0.0170
exm1719137	[A/G]	EXON	Missense_V876A	rs143673331	Benign	0.0028

Table II. Malignant tumors in major organs and NES p.A1199P.

Organ	Type	Tumor + (%)	Tumor - (%)	OR	95% CI	P-value
Lung	CC	253 (11.5)	1,871 (85.0)			
	GC+GG	7 (0.3)	71 (3.2)	0.729	0.332-1.603	0.430
Large cell carcinoma/lung	CC	3 (0.1)	2,121 (96.3)			
	GC+GG	1 (0)	77 (3.5)	9.174	0.944-90.0909	0.020[a]
Stomach	CC	239 (10.8)	1,887 (85.6)			
	GC+GG	7 (0.3)	72 (3.3)	0.767	0.349-1.686	0.509
Colorectum	CC	209 (9.5)	1,917 (86.9)			
	GC+GG	4 (0.2)	75 (3.4)	0.489	0.177-1.351	0.159
Pancreas	CC	47 (2.1)	2,078 (94.3)			
	GC+GG	7 (0.3)	71 (3.2)	4.367	1.905-10	0.001[b]
Liver	CC	68 (3.1)	2,058 (93.3)			
	GC+GG	0 (0)	79 (3.6)	N.D.	N.D.	0.106
Biliary tract	CC	61 (2.8)	2,065 (93.7)			
	GC+GG	2 (0.1)	77 (3.5)	0.880	0.211-4.219	0.860
Kidney	CC	32 (1.5)	2,094 (95.0)			
	GC+GG	1 (0)	78 (3.5)	0.839	0.113-6.211	0.863
Urinary tract	CC	41 (1.9)	2,085 (94.6)			
	GC+GG	4 (0.2)	75 (3.4)	2.710	0.947-7.752	0.053
Prostate	CC	197 (16.0)	987 (80.4)			
	GC+GG	7 (0.6)	37 (3.0)	0.948	0.416-2.155	0.898
Blood	CC	195 (8.9)	1,930 (87.6)			
	GC+GG	4 (0.2)	74 (3.4)	0.535	0.194-1.479	0.221
Brain	CC	2 (0.1)	2,122 (96.4)			
	GC+GG	0 (0)	78 (3.5)	N.D.	N.D.	0.786

[a]P<0.05; [b]P<0.001. OR, odds ratio of GC+GG to CC; CI, confidence interval; N.D., not determined.

association to SNPs of NES. Furthermore, NES p.A1199P did not associate with occurrence of PanIN, suggesting that only a small portion of PanINs are precancerous lesions (24). The high incidence rate of PanINs and our previous study (24) both support this conclusion.

Morbidity and mortality of pancreatic cancers have been increasing worldwide (30,31). In Japan, pancreatic cancer is the fifth and fourth leading cause of cancer-related death in men and women, respectively (32). Risk factors for pancreatic cancer are tobacco use (33), heavy alcohol consumption, diabetes, obesity, pancreatitis, low 25-(OH) vitamin D levels, and aging (34,35). The vast majority of pancreatic cancers are thought to arise from PanINs; high-grade PanINs (carcinomas *in situ*) are considered as precursors of pancreatic cancer (29,36-38). Approximately 5-10% of patients with pancreatic cancer have family histories of pancreatic cancer (39,40). Recent studies for pancreatic ductal adenocarcinoma (PDAC) using Caucasian populations have identified associations with chromosome bands of ABO, KLF5, NR5A2, CLPTM1L-TERT (41,42); LINC-PINT, BRCAR1, PDX1, ZNRF3, PVT1 (43); LINC00673, SUGCT and TP63 (44). A recent study also showed that three SNPs in NR5A2, MYC and CLPTM1L-TERT represent independent risk factors of pancreatic cancer; NR5A2 expression in the pancreatic cancers was markedly decreased (45). In Japanese

Table III. Other malignant tumors and NES p.A1199P.

Organ	Type	Tumor + (%)	Tumor - (%)	OR	95% CI	P-value
Adenocarcinoma/lung	CC	115 (5.2)	2,009 (91.2)			
	GC+GG	2 (0.1)	76 (3.5)	0.460	0.112-1.894	0.270
Squamous cell carcinoma/lung	CC	77 (3.5)	2,047 (93.0)			
	GC+GG	3 (0.1)	75 (3.4)	1.064	0.328-4.785	0.918
Adenosquamous carcinoma lung	CC	8 (0.4)	2,116 (96.1)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.587
Small cell carcinoma lung	CC	55 (2.5)	2,069 (94.0)			
	GC+GG	2 (0.1)	76 (3.5)	0.990	0.237-4.132	0.989
Unclassified cancer lung	CC	11 (0.5)	2,113 (96.0)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.524
Mesothelioma	CC	1 (0.0)	2,124 (96.4)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.848
Esophageal cancer	CC	32 (1.5)	2,091 (95.0)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.275
Colon cancer	CC	163 (7.4)	1,963 (89.0)			
	GC+GG	3 (0.1)	76 (3.4)	0.475	0.148-1.524	0.201
Rectal cancer	CC	52 (2.4)	2,074 (94.1)			
	GC+GG	1 (0.0)	78 (3.5)	0.511	0.070-3.745	0.501
Small intestine cancer	CC	11 (0.5)	2,115 (95.9)			
	GC+GG	0 (0.0)	79 (3.6)	N.D.	N.D.	0.522
Lymphocytic leukemia	CC	18 (0.8)	2,107 (95.6)			
	GC+GG	1 (0.0)	77 (3.5)	1.520	0.200-11.494	0.683
Malignant lymphoma	CC	119 (5.4)	2,005 (91.1)			
	GC+GG	1 (0.0)	77 (3.5)	N.D.	N.D.	0.099
Myelodysplastic syndrome	CC	39 (1.8)	2,086 (94.7)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.227
Myelogenous leukemia	CC	104 (4.7)	2,021 (91.7)			
	GC+GG	2 (0.1)	76 (3.4)	0.512	0.124-2.110	0.345
Myeloma	CC	38 (1.7)	2,087 (94.7)			
	GC+GG	1 (0.0)	77 (3.5)	0.713	0.097-5.263	0.739
Breast cancer	CC	74 (3.4)	2,050 (93.1)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.094
Uterine cancer	CC	20 (2.1)	919 (94.4)			
	GC+GG	0 (0.0)	35 (3.6)	N.D.	N.D.	0.383
Ovarian cancer	CC	5 (0.5)	939 (95.9)			
	GC+GG	0 (0.0)	35 (3.6)	N.D.	N.D.	0.666
Thyroid cancer	CC	52 (2.4)	2,072 (94.1)			
	GC+GG	2 (0.1)	76 (3.5)	1.048	0.250-4.386	0.948
Sarcoma	CC	7 (0.3)	2,117 (96.1)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.612
Melanoma	CC	1 (0.0)	2,123 (96.4)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.848
Skin cancer	CC	9 (0.4)	2,112 (96.0)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.564
Head and neck cancer	CC	25 (1.1)	2,099 (95.3)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.335
Other tumor	CC	9 (0.4)	2,115 (99.2)			
	GC+GG	0 (0.0)	9 (0.4)	N.D.	N.D.	0.565
Unclassified tumor	CC	4 (0.2)	2,120 (96.3)			
	GC+GG	0 (0.0)	78 (3.5)	N.D.	N.D.	0.701

OR, odds ratio of GC+GG to CC; CI, confidence interval; N.D., not determined.

Table IV. SNPs of nestin and pancreatic cancer.

SNP	Reference no.	OR	95% CI	P-value
P1275L	rs3748570	0.630	0.366-1.085	0.116
S1016N	rs2365718	1.047	0.058-18.790	1.000
L791I	rs77202633	0.738	0.289-1.885	0.670
A1199P	rs78303930	4.367	1.905-10	0.001[a]
V876A	rs143673331	1.951	0.100-38.174	1.000

[a]P<0.001. CI, confidence interval; SNP, single nucleotide polymorphism; OR, odds ratio.

Table V. Patients and *NES* p.A1199P.

Comparison	CC	GC+GG	OR	95% CI	P-value
Sex (%)					
Male	1,182 (53.6)	43 (1.9)			
Female	945 (42.8)	36 (1.6)	0.955	0.608-1.499	0.841
Drinking habit (%)					
Drinker	674 (34.0)	27 (1.4)			
Non-drinker	1,240 (62.6)	41 (2.1)	1.212	0.739-1.988	0.447
Smoking habit (%)					
Smoker	1,023 (50.4)	38 (1.9)			
Non-smoker	936 (46.1)	33 (1.6)	1.054	0.655-1.695	0.829
BMI (%)					
BMI ≥25	948 (43.4)	36 (1.6)			
BMI <25	1,159 (53.0)	43 (2.0)	1.024	0.652-1.608	0.919

OR, odds ratio of GC+GG to CC; NES, nestin; CI, confidence interval; BMI, body mass index.

Table VI. Pancreatic intraepithelial neoplasia and NES p.A1199P.

Variable	Type	+ (%)	- (%)	OR	95% CI	P-value
PanIN-1A	CC	1,137(51.6)	990 (44.9)			
	GC+GG	42 (1.9)	36 (1.6)	1.016	0.646-1.597	0.646
PanIN-1B	CC	911 (41.3)	1,216 (55.1)			
	GC+GG	31 (1.4)	47 (2.1)	0.880	0.555-1.397	0.595
PanIN-2	CC	255 (11.6)	1,870 (84.9)			
	GC+GG	7 (0.3)	71 (3.2)	0.723	0.329-1.590	0.673
PanIN-3	CC	29 (1.3)	2,096 (95.1)			
	GC+GG	2 (0.1)	76 (3.4)	1.901	0.446-8.130	0.672
Low grade PanIN	CC	1,174 (53.2)	953 (43.2)			
	GC+GG	43 (2.0)	35 (1.6)	0.997	0.633-1.570	0.666
PanIN and cancer	CC	1,189 (53.9)	938 (42.5)			
	GC+GG	44 (2.0)	34 (1.5)	1.020	0.647-1.610	0.672
PanIN-3 and cancer	CC	72 (3.3)	2,054 (93.2)			
	GC+GG	8 (0.4)	70 (3.2)	3.215	1.493-6.944	0.007[a]

[a]P<0.01. Cancer indicates pancreatic invasive ductal carcinoma. Low grade PanIN includes PanIN-1 and -2. NES, nestin; PanIN, pancreatic intraepithelial neoplasia; OR, odds ratio of GC+GG to CC; CI, confidence interval.

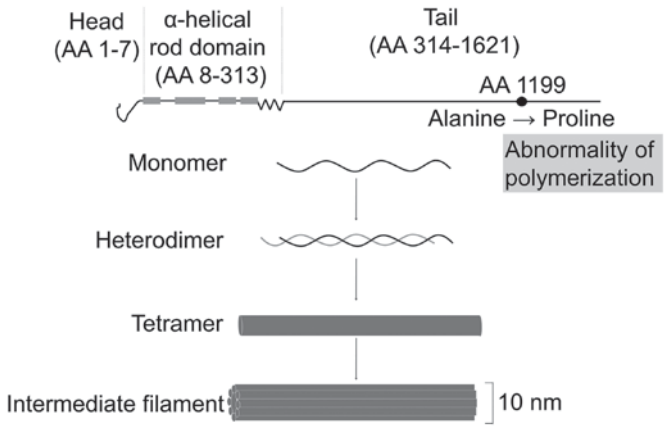

Figure 1. Structure of Nestin protein. Nestin protein has a head, α-helical rod domain, and tail lesion. It forms heterodimers with vimentin and internexin, and these heterodimers combine to form intermediate filaments. The nestin single nucleotide polymorphism (NES p.A1199P) is located in the tail region. AA, amino acid.

populations, SNPs of NR5A2 have shown a significant association with PDAC (46,47). Previously, we have reported that six SNPs (rs7016880, rs10096633, rs10503669, rs12678919, rs17482753, and rs328) that associated with blood lipid levels were associated with the risk for pancreatic cancer in the same cohort (24).

In the present study, we focused on SNPs of NES in autopsied patients, because NES plays important roles in many processes in various organ neoplasms as well as tissue regeneration. A previous report has shown that SNPs of NES (rs11582300 and rs3748570) were associated with early-onset coronary heart diseases in Irish people (48). The present study is the first report to clarify the relationship between SNPs of NES and various malignancies. Amino acid 1199 of NES is conserved in various mammals including primates and pigs, and is located in the tail lesion of NES (Fig. 1). The tail lesion polymerizes with other IF proteins, and regulates cell morphology, migration, and mitosis. In the present study, we did not find any association between clinicopathological characteristics of pancreatic cancer patients and NES p.A1199P. Pancreatic exocrine progenitor cells of mice express NES protein (49), and pancreatic cancer might originate from pancreatic exocrine progenitor cells. These data suggest that NES p.A1199P might influence carcinogenesis steps in the pancreas. We need biological studies and a larger cohort study to clarify molecular mechanisms of NES p.A1199P.

The present study has several limitations. The average age of our patients is much higher than that observed in most patients with pancreatic cancer as previously reported (24). Japan is experiencing a 'super-aging' society. PDAC is projected to surpass breast, prostate, and colorectal cancers to become the second leading cause of cancer-related deaths by 2030 in the U.S (50). In this context, it is definitely important to identify the characteristics of age-related pancreatic carcinogenesis. Furthermore, the power of statistical analysis in the present study was 48.6% between presence of pancreatic cancer and NES p.A1199P. We need further analysis using large scale different cohort.

In conclusion, we found that missense variations of NES appear to affect pancreatic carcinogenesis in Japanese patients by an undetermined mechanism.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YM, MT and TA conceived and directed the project, analyzed the data and wrote the manuscript. MS, SM, MM, MNM and TI analyzed data and supervised. All authors have read and approved the manuscript.

Ethics approval and consent to participate

The present study was approved by the Tokyo Metropolitan Geriatric Hospital Ethics Committee (approval no. 15-02). This study was conducted in accordance with the principles embodied in the Declaration of Helsinki, 2013. Written informed consent was obtained prior to the autopsy from the family members of all participants involved in this study.

Patient consent for publication

Written informed consent was obtained prior to the autopsy from the family members of all participants involved in this study.

Competing interests

The authors declare that they have no competing interests.

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