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SSTR5 P335L monoclonal antibody differentiates pancreatic neuroendocrine tumor patients with different SSTR5 genotypes

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Abstract

Background—Somatostatin receptor type 5 (SSTR5) P335L is a hypofunctional single nucleotide polymorphism of SSTR5 with implications in tumor diagnostics and therapy. The purpose of this study is to determine whether a SSTR5 P335L-specific monoclonal antibody (mAb) could sufficiently differentiate pancreatic neuroendocrine tumor (PNT) patients with different SSTR5 genotypes.

Methods—Cellular proliferation rate, SSTR5 mRNA level and SSTR5 protein level were measured by performing MTS assay, qRT-PCR and western blotting and immunohistochemistry, respectively. SSTR5 genotype was determined with the TaqMan SNP Genotyping assay.

Results—1) SSTR5 analogue RPL-1980 inhibited cellular proliferation of CAPAN-1 cells more significantly than that of PANC-1 cells. 2) Only PANC-1 (TT) cells, but not CAPAN-1 (CC) cells expressed SSTR5 P335L. 3) In 29 Caucasian PNT patients, 38% had TT genotype for SSTR5 P335L, 24% had CC genotype for WT SSTR5, and 38% had CT genotype for both SSTR5 P335L and WT SSTR5. 4) Immunohistochemistry using SSTR5 P335L mAb detected immunostaining signals only from the PNT specimens with TT and CT genotypes, but not those with CC genotypes.

Conclusions—A SSTR5 P335L mAb that specifically recognizes SSTR5 P335L, but not WT SSTR5, could differentiate PNT patients with different SSTR5 genotypes, thus providing a potential tool for clinical diagnosis of PNT.

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INTRODUCTION

Somatostatin (SST) or somatotropin release inhibiting factor (SRIF) is a cyclic tetradecapeptide hormone and functions as a suppressor of growth hormone (GH) secretion and cell proliferation by binding to a group of specific G protein-coupled receptors, also called somatostatin receptors (SSTRs) [1]. Following SST binding, SSTRs undergo a series of initial events by which SSTRs mediate somatostatin signaling, including conformational changes, homo/heterodimerization, internalization, protein-protein interaction and activation of downstream signaling pathways [2,3].

Somatostatin receptor type 5 (SSTR5) is one of the five identified SSTRs that mediate the inhibitory effect of somatostatin on cellular functions, including the negative regulation of insulin expression/secretion and cell proliferation in islets of Langerhans [4], decreased pancreatic carcinogenesis [5–7], decreased islet angiogenesis [8] and increased apoptosis [9]. A number of single nucleotide polymorphisms (SNPs) have been identified in SSTR5, including 20 missense variations (A19T, P34S, G37R, A40T, L48M, A52V, W105R, P109S, V180M, R229K, R234C, R248C, L251S, V267I, R312C, A327V, T333M, P335L, R339K and G357R) [10]. Among them, SSTR5 P335L SNP results from a C to T change at the 1004th nucleotide of the human SSTR5 gene. It has been shown that SSTR5 P335L SNP is associated with neuropsychiatric diseases [11,12], pituitary adenomas [13] and pancreatic cancer [14,15]. Our recent studies also show that SSTR5 P335L is a hypofunctional SNP and, thus, could have a harmful effect on the normal functions of SSTR5 [15].

In the present study, we sought to investigate the genotype and allele distribution of the SSTR5 P335L SNP in PNT patients and test whether a SSTR5 P335L-specific monoclonal antibody could differentiate among PNT patients with different SSTR5 genotypes. We found that the SSTR5 P335L SNP exists in 57% of Caucasian PNT patients and that a mouse SSTR5 P335L mAb tested in this study provides a potential tool for clinical diagnosis of PNT since it detected immune signals only from the PNT specimens with TT and CT genotypes, but not those with CC genotypes.

MATERIALS AND METHODS

Sample collection and processing

Informed consent from 29 Caucasian patients with PNTs was obtained under an IRB-approved protocol. The genomic DNA was isolated from the flash-frozen tumor specimens using the Gentra Puregene kit (Qiagen, Valencia, CA) as per manufacturer instruction. SSTR5 Genotypes were determined with the TaqMan SNP Genotyping assay (Applied Biosystems, Foster City, CA). The reactions were prepared using 30 ng of gDNA, TaqMan universal master mix (Applied Biosystems), and a custom-designed SNP genotyping assay mix (Primers and TaqMan MGB probes) (Applied Biosystems) in a final volume of 6 μ l. Allele discrimination was accomplished by running end point detection using ABI Prism 7900HT Sequence Detection System, and SDS 2.3 software (Applied Biosystems).

Tissue culture and western blotting

CPAN-1 and PANC-1 cells were obtained from the American Type Culture Collection (Manassas, VA). Both CPAN-1 and PANC-1 cells were grown and maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. Expression of SSTR5 and SSTR5 P335L in CAPAN-1 and PANC-1 cells was determined by western blotting against a polyclonal anti-SSTR5 [16] (1:500) and a monoclonal anti-SSTR5 P335L [15] (2 μ g/ml) antibody, respectively, using enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences Corp, Piscataway, NJ) according to the manufacturer's protocols.

Quantitative reverse transcriptional PCR (qRT-PCR)

Total RNAs were prepared using TriZol reagent (Invitrogen) from CAPAN-1 and PANC-1 cells. The cDNA was prepared from the total RNA using qScript cDNA SuperMix (Quanta Biosciences, Maryland) according to the manufacturer's protocol. qRT-PCR was performed in 96-well plates with the Applied Biosystems. The mRNA levels of target genes in the samples were normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH). SSTR5 and GAPDH were measured in triplicate. The primers used are as follows: human SSTR5: 5'-TCTTCACCGTCAACATCGTCA-3' (forward) and 5'-CTCTGGCGGAAGTTGTCAG-3' (reverse) and GAPDH: 5'-ATGCCATCACTGCCACCCAGAACG-3' (forward) and 5'-GCCAGTGAGCTTCCC GTTCA-3' (reverse). PCR was performed on 1 µl aliquots from each cDNA reaction, using human SSTR5 and GAPDH primer sets for 45 cycles. The PCR products were subjected to electrophoresis on a 2% agarose gel.

Cell proliferation assay

CAPAN-1 and PANC-1 cells were seeded in 96-well plates (5×10^3 cells/well). After 24 h, the cells were treated with 10^{-5} M of RPL-1980 for 60 h before examination of the cell proliferation by performing MTS assay according to the manufacturer's protocols. Absorbance at 492 nm was recorded. Data were expressed as the mean $\pm$ standard deviation of triplicate values.

Immunohistochemistry

CAPAN-1 and PANC-1 cells were seeded in culture slides (5×10^4 /well). After 24 h, the cells were fixed in 4% (v/v) paraformaldehyde for 10 min, followed by treatment with methanol for 2 min. The slide chambers were overlaid with an polyclonal anti-SSTR5 (1:100) or monoclonal anti-SSTR5 P335L (15 µg/ml) antibody overnight at 4°C. Human PNT specimens were fixed in 4% (v/v) paraformaldehyde for 24 h and embedded in paraffin. Tissue sections were cut and slides were deparaffinized in xylene. Sections were rehydrated through graded alcohol. Slides were placed in a humidified chamber overlaid with anti-SSTR5 P335L mAb (15 µg/ml) overnight at 4°C. After washing with PBS, culture slides and sections were incubated with a FITC-conjugated anti-mouse IgG second antibody for 1 h at room temperature. Simultaneous fluorescence microscopy observation and photography were carried out using an Olympus IX70 microscope.

Statistical analysis

The unpaired Student *t* test was used for statistical analyses of cell proliferation rates with *p* < 0.05 indicating significance.

RESULTS

SSTR5 P335L mAb distinguishes PANC-1 cells from CAPAN-1 cells

The hypofunctional property of SSTR5 P335L SNP [15] and its association with human diseases [11–14] strongly suggest that SSTR5 P335L is a potential biomarker for these human diseases. Therefore, we generated an anti-SSTR5 P335L mAb that shows specificity to SSTR5 P335L, but not WT SSTR5, overexpressed in HEK293 cells [15]. To further confirm the specificity of this antibody, we sought to examine whether the SSTR5 P335L mAb could distinguish endogenous SSTR5 P335L from WT SSTR5 as well. Our previous studies have shown that human pancreatic cancer cells CAPAN-1 have homologous CC genotype expressing WT SSTR5 only, while PANC-1 cells have homologous TT genotype expressing SSTR5 P335L only [15]. Given that SSTR5 plays an essential role in mediating the antiproliferative function of somatostatin [2,17–19], we first examined the responses of

CAPAN-1 cells and PANC-1 cells to RPL-1980, a SSTR5 analogue [15]. As shown in Fig. 1, RPL-1980 inhibited CAPAN-1 cell proliferation by 64%, while PANC-1 cell proliferation was inhibited only by 30% under the same conditions. Statistical analysis showed that the difference was significant, suggesting that the SSTR5 genotypes of CAPAN-1 and PANC-1 cells may contribute to their differing responses to RPL-1980.

Next, we examined whether SSTR5 or SSTR5 P335L were expressed in CAPAN-1 and PANC-1 cells. By performing qRT-PCR, we found that SSTR5 mRNA was expressed in both CAPAN-1 and PANC-1 cells at comparable levels (Fig. 2A), indicating that these cells did express SSTR5 proteins and were appropriate for studying the specificity of the SSTR5 P335L mAb to endogenous SSTR5 or SSTR5 P335L protein. By performing western blotting, we found that an anti-SSTR5 (N-terminal) antibody detected SSTR5 protein expression in both CAPAN-1 and PANC-1 cells (Fig. 2B, middle panel). However, the anti-SSTR5 P335L antibody detected SSTR5 protein expression only in PANC-1 cells, but not CAPAN-1 cells (Fig. 2B, top panel). Similarly, immunohistochemistry (IHC) analysis showed that both CAPAN-1 and PANC-1 cells were immunostained by an anti-SSTR5 antibody, while only PANC-1 cells, but not CAPAN-1 cells, were immunostained by the anti-SSTR5 P335L mAb (Fig. 2C). All results demonstrated that both CAPAN-1 and PANC-1 cells expressed SSTR5 proteins and that only PANC-1 cells, but not CAPAN-1 cells, expressed SSTR5 P335L protein. This is consistent with the genotyping results that CAPAN-1 cells are CC homologous and express WT SSTR5, and that PANC-1 cells are TT homologous and express SSTR5 P335L [15]. These results also clearly demonstrated that the SSTR5 P335L mAb was capable of specifically recognizing endogenous SSTR5 P335L, but not WT SSTR5.

SSTR5 P335L monoclonal antibody differentiates pancreatic neuroendocrine tumor patients with different SSTR5 genotypes

SSTR5 P335L SNP exists in neuropsychiatric diseases [11,12], pituitary adenomas [13] and pancreatic cancer [14,15]. In this study, we sought to investigate the genotype and allele distribution of the SSTR5 P335L SNP in human PNT by applying a TaqMan SNP Genotyping assay. As shown in Table 1, in a group of 29 Caucasian PNT patients, 38% (n=11) had TT genotype, which expresses SSTR5 P335L only; 24% (n=7) had CC genotype, which expresses WT SSTR5 only; and 38% (n=11) had CT genotype, which expresses both SSTR5 P335L and WT SSTR5. The frequency of T allele in PNT was 57%. These results indicated that SSTR5 P335L SNP widely exists in PNT.

To explore the diagnostic potential of the SSTR5 P335L mAb, we examined the expression of the SSTR5 P335L SNP in PNT by performing IHC using this SSTR5 P335L-specific antibody. Among the 29 Caucasians PNT specimens, only 22 were available for IHC analysis due to the limitation of the specimen sizes, including 6 TT genotypes (#3, 8, 14, 16, 25 and 27), 10 CT genotypes (#1, 5, 6, 7, 13, 17, 18, 19, 21 and 23) and 6 CC genotypes (#2, 9, 10, 12, 22 and 26). As shown in Table 2, all 6 PNT specimens with TT genotype were immunostained by the SSTR5 P335L antibody, although with different intensity of immunostaining signals. All PNT specimens, except one (#18), with CT genotype were also immunostained by the SSTR5 P335L antibody. However, among 6 PNT specimens with CC genotype, only two specimens were immunostained with mid-level immunostaining signals, the other four specimens were totally negative. Representative immunostaining results from each genotype of PNT patients were shown in Fig. 3, with highest level of immunostaining signal from #8 PNT patient with TT genotype, mid-level of immunostaining signals from #1 PNT patient with CT genotype and negative immunostaining signal from #2 PNT patient with CC genotype. We also observed that the immunostaining was not homogenous, but highly heterogeneous, indicating that SSTR5 P335L expression is tissue/cell-specific. Overall, the results of our IHC analysis were consistent with genotyping results of the PNT

patients (Table 1), indicating that the SSTR5 P335L mAb could potentially differentiate PNT patients with different SSTR5 genotypes.

DISCUSSION

SSTR5 P335L is a germline, non-synonymous SNP resulting from a C to T change at the 1004th nucleotide of human SSTR5 and widely exists in normal human populations, [20]. SSTR5 P335L has been implicated in a number of human diseases such as neuropsychiatric diseases [11,12], pituitary adenomas [13] and pancreatic cancer [14,15]. Our recent studies demonstrate that SSTR5 P335L is a hypofunctional SNP since it enhances cell proliferation, insulin secretion and PDX-1 expression in contrast to WT SSTR5 [15]. Therefore, SSTR5 P335L is a potential biomarker with diagnostic and therapeutic implications in human diseases. It is necessary, therefore, to develop efficient approaches to determine whether genetic variants exist in SSTRs. We have generated a mouse SSTR5-P335L monoclonal antibody that recognized only SSTR5 P335L, but not WT SSTR5, overexpression in HEK293 cells [15]. In the current study, we further characterized this antibody and found that it could sufficiently differentiate among pancreatic cancer cells and pancreatic neuroendocrine tumor patients with different SSTR5 genotypes. Therefore, the development of a SSTR5 P335L-specific monoclonal antibody provides a potential tool for clinical diagnosis of human diseases. This information also may help guide the choice of therapeutic treatments for those SSTR5 P335L, SNP-positive PNT patients.

Somatostatin is an endogenous antiproliferative agent, a characteristic that makes it a promising antitumor agent in a variety of experimental tumor models [2,17–19]. However, the highly significant antiproliferative effect of somatostatin analogues becomes much less convincing when the laboratory data are translated to clinical trials. For example, somatostatin radionuclide therapy (SRT) is effective against pancreatic neuroendocrine tumors (PNT) in only 20% of patients, and 20% and 50% of patients have no or limited response, respectively. The underlying molecular basis for the lack of therapeutic effect of somatostatin remains unknown. The antiproliferative effect of somatostatin is mediated by a group of G protein-coupled receptor including SSTR2 and SSTR5 [21]. It is, therefore, reasonable to hypothesize that lack of expression of SSTRs, or expression of a mutant SSTR by which the signal of the somatostatin analogue is not transmitted into the cell, may contribute to the lack of therapeutic effect of somatostatin. A point mutation of SSTR2 exists in human small cell lung cancer [22]. The hypofunctional SSTR5 P335L SNP has been found to be associated with neuropsychiatric diseases [11,12], pituitary adenomas [13] and pancreatic cancer [14,15]. In this study, we found that CAPAN-1 cells and PANC-1 cells differentially responded to RPL-1980, a SSTR5 analogue. RPL-1980 inhibited CAPAN-1 cell proliferation more significantly than PANC-1 cell proliferation (64% vs. 30%). Given that CAPAN-1 cells (CC genotype) express WT SSTR5 and PANC-1 cells (TT genotype) express SSTR5 P335L, it is possible that the expression of hypofunctional SSTR5 P335L SNP might contribute to the resistant response of PANC-1 cells to RPL-1980 in comparison to CAPAN-1 cells.

We also examined the genotype and allele distribution of SSTR5 P335L in a group of 29 Caucasian patients with PNT. Our results showed that TT genotype, which expresses SSTR5 P335L only, and CT genotype, which expresses both WT SSTR5 and SSTR5 P335L, widely exist in the patients with PNTs. The total T allele frequency of SSTR5 between PNT patients and the healthy control cohort [15] was comparable (57% vs. 58%). However, due to the small sample size of this study (n=29), we cannot yet conclude that the SSTR5 P335L SNP is associated with the incidence of PNT. Moreover, according to the SNP NCBI database and the HapMap data on European, African, and Asiatic population [20] and our recent studies [15], the distribution of SSTR5 P335L among Caucasian, African, Hispanic

and Asian groups is significantly different. Therefore, it is necessary to increase the sample sizes to determine whether the presence of SSTR5 P335L SNP is functionally associated with the incidence of PNTs and if the association is race-dependent.

In summary, our study showed that SSTR5 P335L SNP widely exists in patients with PNTs. A SSTR5 P335L-specific mAb that specifically recognizes SSTR5 P335L, but not WT SSTR5, could sufficiently differentiate pancreatic cancer cells and PNT patients with different SSTR5 genotypes, thus providing a potential tool for clinical diagnosis of human cancers.

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The abbreviations used are

PNT	pancreatic neuroendocrine tumor
SSTR5	somatostatin receptor subtype 5
SST	somatostatin
mAb	monoclonal antibody
IHC	immunohistochemistry

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Fig. 1.

CAPAN-1 and PANC-1 cells differentially respond to RPL-1980 in cell proliferation. CAPAN-1 and PANC-1 cells were seeded in 96-well plates (5×10^3 cells/well). After 24 h, the cells were treated with 10^{-5} M of RPL-1980 for 60 h before examining the cell proliferation by performing MTS assay. Absorbance at 492 nm was recorded. Data were expressed as the mean $\pm$ standard deviation of triplicate values. $*P < 0.05$.

A. **B.**

Fig. 2.

Anti-SSTR5 P335L mAb only recognizes SSTR5 P335L expressed in PANC-1 cells, but not WT SSTR5 expressed in CAPAN-1 cells. (A) Total RNA was extracted from CAPAN-1 and PANC-1 cells and the mRNA levels for SSTR-5 were analyzed by real-time polymerase chain reaction (RT-PCR), and normalized to that of the house-keeping gene, GAPDH. All data shown are the mean $\pm$ standard deviation of three separate experiments. (B) 50 μ g of whole cell lysates from CAPAN-1 and PANC-1 cells were subjected to SDS-PAGE, followed by western blotting with a monoclonal anti-SSTR5 P335L (upper panel), anti-SSTR5 (middle panel) and anti- β -actin (bottom panel) antibody, respectively. (C) CAPAN-1 and PANC-1 cells (5×10^4) were seeded on eight Chamber Polystyrene Vessel Tissue Culture Treated Glass Slides. Twenty-four (24) h later, immunohistochemical staining was performed using a monoclonal anti-SSTR5 L335 antibody (616 D2F2, 20 μ g/ml) and a polyclonal anti-SSTR5 antibody (1:100). Fluorescence was developed using FITC-conjugated secondary antibody. Simultaneous fluorescence microscopy observation and photography were carried out using an Olympus Ix70 microscope (200 $\times$).

Fig. 3.

Representative micrographs showing that SSTR5 P335L-specific mAb differentiates PNT patients with different SSTR5 genotypes. Human PNT specimens with different SSTR5 genotypes were fixed in 4% (v/v) paraformaldehyde and embedded in paraffin. After deparaffinization in xylene and rehydration through graded alcohol, tissue sections were incubated with an anti-SSTR5 P335L mAb (15 μ g/ml) or an anti-Ki67 antibody (1:300) overnight at 4°C. Fluorescence was developed using FITC-conjugated secondary antibody. Simultaneous fluorescence microscopy observation and photography were carried out using an Olympus IX70 microscope (200 $\times$).

Table 1

Genotype and allele distribution of the SSTR5 P335L SNP in Caucasian pancreatic neuroendocrine tumor (PNT)

Genotype and allele	PNT Patient (n=29)
CC	24% (7)
CT	38% (11)
TT	38% (11)
Total T	57%

Table 2

IHC analysis of PNT with different SSTR5 genotypes by anti-SSTR5 P335L mAb

Genotype	PNT	IHC intensity
TT	#3	+++
TT	#8	++++++
TT	#14	++++++
TT	#16	++
TT	#25	++++
TT	#27	++
CT	#1	+++
CT	#5	++
CT	#6	++
CT	#7	+
CT	#13	++
CT	#17	++++
CT	#18	–
CT	#19	+++
CT	#21	+++
CT	#23	+++
CC	#2	–
CC	#9	++
CC	#10	+++
CC	#12	–
CC	#22	–
CC	#26	–