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ORIGINAL ARTICLE

Evaluation of neurotensin receptor 1 as potential biomarker for prostate cancer theranostic use

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Abstract

Introduction Despite recent developments in the diagnosis and treatment of prostate cancer, the advanced stages still have poor survival rates. This warrants further exploration of related molecular targets for patient screening, detection of metastatic disease, and treatment/treatment monitoring. Recent studies have indicated that neurotensin receptors (NTSRs) and their ligand neurotensin (NTS) critically affect the progression of prostate cancers. In this study, we evaluated the expression of neurotensin receptor1 (NTSR1) in patient tissues and performed NTSR1 PET imaging in a prostate cancer animal model.

Methods The NTSR1 expression was evaluated in 97 cases of prostate cancer and 100 cases of benign prostatic hyperplasia (BPH) of clinical patients by immunohistochemistry staining. The expression profile of PSMA and GRPR was also performed for comparison. The mRNA expression of NTSR1 in LnCap and PC-3 cells was measured by PCR. NTSR1 PET, and biodistribution studies were performed in PC-3 xenografts using ^{18}F-DEG-VS-NT.

Results NTSR1 showed high or moderate expression in 91.8% of prostate cancer tissue, compared with PSMA (86.7%) and GRPR (65.3%). All examined PSMA-negative tissues showed positive NTSR1 expression, suggesting the potential complementary role of NTSR1 targeted imaging or therapy. Only 8% of BPH shows strong or moderate expression of NTSR1, which is significantly lower than that in prostate cancer (91.8%). PCR results indicated LNCap (an androgen-dependent prostate cancer cell) showed negative NTSR1 expression while PC-3 demonstrated positive expression (an androgen-independent prostate cancer cell), which correlated well with previously reported western blot results. In a preclinical animal model, NTSR1 targeted PET probe ^{18}F-DEG-VS-NT demonstrated prominent tumor accumulation and low background.

Conclusion We have demonstrated that NTSR1 is a promising molecular marker for prostate cancer based on patient tissue staining. The NTSR targeted probe ^{18}F-DEG-VS-NT demonstrated high tumor to background contrast in animal models, which could be valuable in selecting patients for therapies targeting NTSR1 as well as monitoring therapeutic efficacy during treatment accordingly.

Keywords Neurotensin receptor · Prostate cancer · Positron emission tomography (PET) · ^{18}F · Prostate specific membrane antigen (PSMA)

Tingting He and Mengzhe Wang contributed equally to this work.

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Introduction

Prostate cancer is currently the most common cancer in males in Europe and the United States. According to the US National Cancer Institute, there were over 161 thousand newly reported cases of prostate cancer in US during 2017, which accounts for 19% of all new cancer cases [1]. Although prostate cancer patients have high 5-year survival rate when diagnosed at early stage, the survival rate dramatically drops to 29% once distant metastasis occurs [1]. This evidence shows the need for

new diagnostic and therapeutic tools to improve the management and prognosis in late-stage prostate cancer.

Recently, there is increasing evidence suggesting neurotensin receptors (NTSRs) and their ligand neurotensin (NTS) could play key roles in prostate cancer progression [2–5]. This prompted us to investigate its expression in prostate cancer patient tissues and validate the use of a NTSR1 targeted positron emission tomography (PET) probe to image its expression in animal models.

Indeed, high NTSR1 expression has been observed in various tumor tissues [6]. Previously, our group and others reported that NTSR1 could be used as a target for diagnostic imaging and therapy in pancreatic cancer and human colon adenocarcinoma [7, 8]. Nicholas et al. found that the NTSR1 antagonist SR48692 can not only inhibit the growth of prostate cancer xenografts, but also increase the sensitivity of tumor to radiotherapy, regardless of whether it is androgen-dependent or not [9]. Swift and coworkers have also reported that NTSR1 was positively expressed in prostate cancer cells but negatively in normal prostate tissue cells, suggesting that NTSR1 positive may be a sign of prostate deterioration [3]. Despite this promising report, the expression of NTSR in BPH and prostate intraepithelial neoplasia (PIN) remains unclear. In prostate cancer theranostics, prostate-specific membrane antigen (PSMA) and gastrin-releasing peptide receptor (GRPR) are two widely studied prostate cancer biomarkers [10–13]. It is also not clear whether NTSR1 targeting would have added value compared with PSMA and GRPR targeting. In order to answer these questions, in this study, we evaluate the NTSR1 expression in around 100 human prostate cancer and benign prostatic hyperplasia tissue samples by immunohistochemistry staining, and compare it with PSMA and GRPR expression. Furthermore, the expression of NTSR1 mRNA in human prostate cancer cell lines was tested by reverse transcription PCR. Small animal PET imaging and biodistribution study of neurotensin peptide-based PET tracer ^{18}F-DEG-VS-NT was performed to validate NTSR1 as a molecular imaging target for prostate cancers.

Materials and methods

General

All chemicals used were procured through commercially available sources. Rabbit anti-human NTSR1, mouse anti-human PSMA, and rabbit anti-human GRPR were purchased from Abcam.

Immunohistochemistry staining

Ninety-seven cases of primary carcinoma tissue samples and 100 cases of benign prostatic hyperplasia tissues were randomly collected from October 2013 in the department of pathology, Xiangya Hospital of Central South University. All HE staining results were graded by two experienced urological pathologists based on the criteria of the Gleason grading system [14]. TPSA were tested by technicians of the laboratory department of XiangYa hospital [15]. The immunohistochemistry staining was performed and evaluated according to a previously reported procedure [7]. Rabbit anti-human NTSR1 (1:500), mouse anti-human PSMA (1:400), or rabbit anti-human GRPR (1:400) primary antibodies were used as primary antibody for NTSR1, PSMA, and GRPR staining. After being washed with PBS, goat anti-rabbit IgG secondary antibody (1:1000) was used and incubated at 37 °C for 30 min for NTSR1 group. For PSMA and GRPR group, mouse- and rabbit-specific HRP/DAB detection IHC Kit was applied. Due to the availability of the tissue section, only 75 and 72 samples were collected for PSMA and GRPR staining respectively, while 97 samples were subjected to NTSR1 staining.

Reverse transcription PCR analysis of NTSR1 expression in prostate cancer cell lines

Total RNA was extracted from cells by the RNazol method according to the protocol suggested by the manufacturer. Then, 5 μg of total RNA were reverse transcribed and 5 μl of the resulting cDNA were used for subsequent NTR1 PCR amplification. Three pairs of specific primers were synthesized according to literature [16]. NTR1 forward 1: 5'-TCATCGCCTTTGTGGTCTGCT-3′, NTR1 reverse 1: 5'-TGGTTGCTGGACACGCTGTCG-3′; NTR1 forward 2: 5′-GAAGCCGCACCAAGAAGTTCATCA-3′, NTR1 reverse 2: 5'-TCAGCTTGTTGGCGATGATGGTGT-3′; NTR1 forward 3: 5′- CGAAGCCGCACCAAGAAGTT-3′, NTR1 reverse 3: 5′- AGGATGGGGTTGATGGTGGAG-3′. For glyceraldehyde-3-phosphate dehydrogenase (GAPDH), taken as a control for a housekeeping protein, forward 5'-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and reverse 5'-CATGTGGGCCATGAGGTCCACCAC-3′ were used. The PCR reactions were denatured at 94 °C for 1 min, followed by 30 cycles of 94 °C, 30 s; 57 °C, 30s, and 72 °C, 30s, then a final extension at 72 °C for 10 min. The PCR amplification mixtures were analyzed on 1% agarose gel. After GelRed staining, DNA bands were visualized with a ChemiDoc MP Imaging System (Bio-Rad).

Cells and animals

Human prostate cancer cell lines PC-3 and LNCaP were obtained from the Tissue Culture Facility of UNC Lineberger Comprehensive Cancer Center. Cell culture was performed according to a previously reported protocol [17]. For mice xenograft, PC-3 cells (5×10^6 resuspended in 100 μl PBS) were subcutaneously injected into the shoulder of male

BALB/c nude mice. PET imaging study was performed when the tumor size reached about 200–500 mm^3 (around 2–3 weeks after cell inoculation).

Radiochemistry

^{18}F-DEG-VS-NT was synthesized using our previously reported method [18].

Small animal PET imaging and biodistribution study

Approximately 3.7 MBq of the ^{18}F-DEG-VS-NT was intravenously injected into PC-3 tumor bearing mice ($n = 3$) 3 h before static PET imaging. Animals were sacrificed for the biodistribution study immediately after the PET imaging. Tissues of interest were collected, and both weight and radioactivity of each sample were measured. The data were converted to percentage of injected dose per gram (%ID/g).

Data analysis

Quantitative data are shown in the form of mean ± SD. *P* value is calculated to compare mean values with Student's *t* test, and less than 0.05 is considered to be statistically significant. The Kruskal–Wallis test was used to analyze the correlation between biomarker staining results and Gleason score, using SPSS 18.0.

Results

Immunohistochemistry staining

To evaluate the NTSR1 expression in prostate cancer and benign tissues, 97 cases of prostate cancer and 100 cases of benign prostatic hyperplasia tissue sections were subjected to NTSR1 immunohistochemistry staining. It was found that 91.8% of the prostate cancers were strongly or moderately NTSR1-positive, while only 8% of the BPH showed strongly or moderately positive results based on the criteria discussed above. There was a significant difference between prostate cancer and BPH ($P < 0.0001$) (Table 1). Figure 1a and b shows the representative NTSR1 immunohistochemistry staining images of prostate cancer and BPH tissue sections.

We also compared the PSMA and GRPR expression in prostate cancer tissues with that of NTSR1. The total positive rate of PSMA was 93.3% (strong, moderate and weak), while the total positive rate of GRPR was 79.2%. There was no significant difference between the positive rates for NTSR1 and PSMA expression; however, the positive rate for both NTSR1 and PSMA was significantly higher than that of GRPR (Table 2). Representative images of NTSR1, PSMA, and GRPR immunohistochemistry staining are shown in Fig. 1c-e. The correlation between the three biomarkers and the Gleason score was also compared, and the result is shown in Table 3. The expression of PSMA had positive correlation with Gleason score ($P = 0.022$), while GRPR expression showed negative correlation with Gleason score ($P = 0.044$). However, the NTSR1 expression showed no correlation with Gleason score ($P = 0.422$), which indicated that NTSR1 is a suitable biomarker to diagnose prostate cancer with any level of Gleason score, but its application as a prognostic marker needs to be further evaluated.

PSMA negative sample shows NTSR1 positive result

Because PSMA has demonstrated promising theranostic roles in prostate cancer recently, it is important to determine whether the NTSR1 target has added value in prostate cancer management. It is noteworthy that all five of our samples which were PSMA-negative were also highly or moderately NTSR1-positive (Table 4, Fig. 2). These samples have Gleason scores from 5 to 7 with TPSA ranging from 2.2 to 61, which suggests that NTSR1 may have an advantage in prostate cancer management when PSMA targeting fails.

NTSR1 expression in prostate cancer cell lines

After obtaining encouraging results from patient tissue staining, we then evaluated the NTSR1 expression in human prostate cancer cell lines LnCap and PC-3 by measuring the relative expression of NTSR1 mRNA. According to the reverse transcription PCR results shown in Fig. 3, LnCap cells were NTSR1-negative while PC-3 cells were

Table 1 NTSR1 expression in prostate cancer and BPH

	N	Expression level				X^2	*P* value
		Strongly positive	Moderately positive	Weakly positive	Negative		
						100.730	< 0.0001
Prostate cancer	97	77	12	3	5		
BPH	100	1	7	24	68		

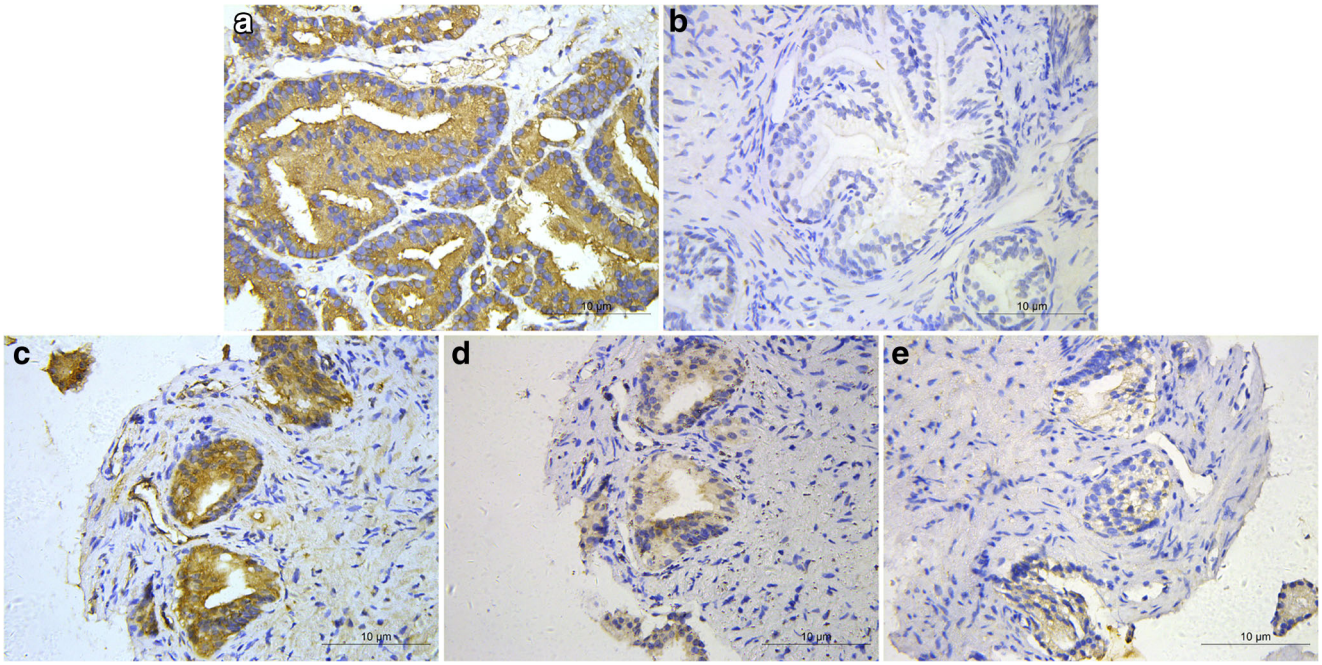

Fig. 1 Representative immunohistochemistry staining in **a** prostate cancer showed positive NTSR1 expression and **b** benign prostatic hyperplasia showed negative NTSR1 expression. Representative immunohistochemistry staining of **c** NTSR1, **d** PSMA and **e** GRPR in prostate cancer tissue

NTSR1-positive. We used three pair primers which amplified different fragments of NTSR1 mRNA, and all three sets of primers gave same results.

Small animal PET imaging and biodistribution study

>The NTSR expression in PC-3 prostate cancer was also evaluated using ^{18}F-DEG-VS-NT. As shown in Fig. 4, good tumor uptake (1.3 ± 0.1 %ID/g) and clear tumor to background ratio can be visualized at 3 h post injection. Biodistribution also confirmed the high uptake and low background of ^{18}F-DEG-VS-NT in PC-3 tumor model. The tumor to muscle, liver, and kidney ratios were 19.4 ± 5.5, 15.6 ± 4.1 and 3.0 ± 0.3 respectively. In addition, NTSR1 expression was evaluated in PC-3 tumor tissues by immunohistochemistry staining (Fig. 5), and strong signal can be observed in cytoplasm. This further confirmed the high NTSR1 expression level in prostate cancer tumors. As a negative control, PET imaging and biodistribution study were also performed using LnCap model. The NTSR1-targeted probe demonstrated minimal tumor accumulation in both experiments (Fig. S1).

Discussion

>Due to the worldwide high occurrence rate of prostate cancer and poor survival rate at late stage, there is an urgent need to look for specific molecular targets for prostate cancer diagnosis and therapy. The prostate-specific antigen (PSA) test is one of the widely chosen options for prostate cancer screening. However, it is common for clinical BPH patients to have higher PSA than normal level. Indeed, it could be debatable whether the patient has prostate cancer

Table 2 NTSR1, PSMA, and GRPR expression in prostate cancer

Markers	*N*	Expression				X^2	*P* value
		Strongly positive	Moderately positive	Weakly positive	Negative		
						12.555	0.002
NTSR1	97	77	12	3	5		
PSMA	75	59	6	5	5		
GRPR	72	31	16	10	15		

Table 3 Correlation between NTSR1, PSMA, GRPR, and Gleason score

	Gleason score			X^2	*P* value
	Maximum value	Minimum value	Median		
NTSR1				1.725	0.422
Strongly	9	4	7		
Moderate	7	6	6.5		
Weakly	8	6	7		
Negative	8	7	7		
PSMA				7.669	0.022
Strongly	9	5	7		
Moderate	7	5	6.5		
Weakly	7	4	6		
Negative	8	4	6		
GRPR				8.119	0.044
Strongly	8	4	6		
Moderate	7	6	7		
Weakly	9	4	7		
Negative	9	5	8		

or not when the PSA value falls between 4 to 10 ng/ml [19, 20]. Therefore, in this study, we first evaluated the NTSR1 expression in both prostate cancer and BPH tissues. Prostate cancer showed 91.8% of strong or moderate expression of NTSR1, while BPH only had 8% of strong or moderate positive rate, indicating NTSR1 may be a promising target for prostate cancer diagnosis.

>To further explore the potential application of NTSR1 targeted imaging and therapy, it is important to compare this molecular marker with other prostate cancer related markers. PSMA and GRPR are widely studied prostate cancer biomarkers in clinical research [21–24]. In our study, the expression level of PSMA and GRPR in prostate cancer was evaluated by immunohistochemistry staining and compared with NTSR1 expression.

There is no doubt that PSMA targeted imaging and therapy have shown exciting results in prostate cancer patient management. However, a number of studies have suggested PSMA could have low expression in tumor tissues with low Gleason scores [25, 26]. A recent study also proved that prostate cancer patients with a Gleason score of 6–7 could have low uptake of ^{68}Ga-PSMA-11, and suggested that ^{68}Ga-PSMA-11 is only preferred for patients with Gleason score above 7 [25]. Furthermore, there are also clinical studies suggested that the PSMA expression in some bone metastases from prostate cancer could be low [27, 28]. As shown in our patient tissue staining, PSMA indeed has rather high positive rate in most of the prostate cancer tissues. However, five prostate cancer tissues have negative PSMA expression, with Gleason score ranging from 5 to 7. It is interesting to see that four of them have high NTSR1 expression and the fifth has moderate NTSR1 expression, suggesting the complementary role between NTSR1 and PSMA. In fact, the PSMA-negative PC-3 cell line (isolated from prostate cancer bone metastases) also demonstrated high NTSR1 expression [29–31]. We also compared the NTSR1 and PSMA expression with the 3–5 year follow-up outcomes of 19 patients. For those patients who were in good condition and showed no recurrence or metastasis after endocrine therapy, the NTSR1 expression level fell into strong, moderate, weak, and negative categories rather evenly. The same result was observed in the case of PSMA expression level. However, for patients who either had passed away or showed bone metastasis, all of them showed strong NTSR1 expression while one of them only had moderate PSMA expression level (nonetheless, the sample size is too small to draw any meaningful information in this aspect). Although our sample size is still limited, we did demonstrate that NTSR1 may serve as a complementary biomarker when PSMA

Table 4 Clinical data of five PSMA negative samples which shows NTSR1 positive

Age	Gleason score	NTSR1	PSMA	TPSA
67	2 + 3 = 5	Strongly positive	negative	61
56	3 + 3 = 6	Strongly positive	negative	7.48
69	3 + 4 = 7	Strongly positive	negative	46.1
70	3 + 3 = 6	Moderate positive	negative	2.2
73	4 + 3 = 7	Strongly positive	negative	30.01

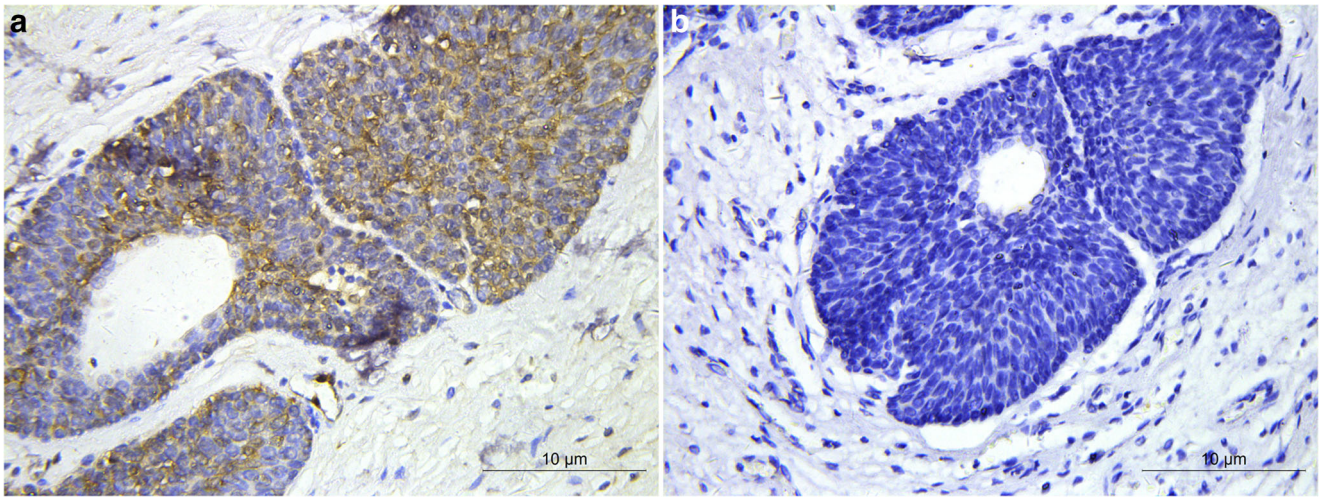

Fig. 2 Representative immunohistochemistry staining in **a** prostate cancer showed positive NTSR1 expression and **b** the same sample showed negative PSMA expression

targeting fails. Additional study is needed to further support the conclusion.

GRPR is another well-studied molecular target for prostate cancer. Previously, Rybalov et al. showed that the GRPR was 100% expressed in prostate cancer [32]. However, the number of samples in that study was only 17, and all of them were recurrent lesions. Based on the tissue bank stained in our study, NTSR1 positive rate was 94.8% in prostate cancer (including strong, moderate, and weak), which was significantly higher than that of GRPR. In fact, some previous studies showed that GRPR expression is low in high Gleason-rated prostate cancer and is only highly expressed in 40% of prostate metastases [33, 34]. Gleason score is currently widely used in prostate adenocarcinoma histological grading, and is closely related to the biological behavior and prognosis of prostate cancer [35]. Since it is an important index for prostate cancer treatment, we also compared the correlation between it and the three biomarkers: NTSR1, PSMA, and GRPR. NTSR1 showed no correlation with the Gleason score. However, PSMA had positive and GRPR had negative correlation with Gleason score, both of which results are consistent with previous studies. Due to the non-correlation between Gleason score and NTSR1 expression, NTSR1 may be used for prostate cancer diagnosis, but its application in prognosis could be limited and needs to be further explored. We should point out that all the staining results were based on primary tumor tissues rather than metastasis. More detailed information with regard to the expression profile of these three biomarkers in prostate cancer metastasis will be evaluated in our follow-up studies.

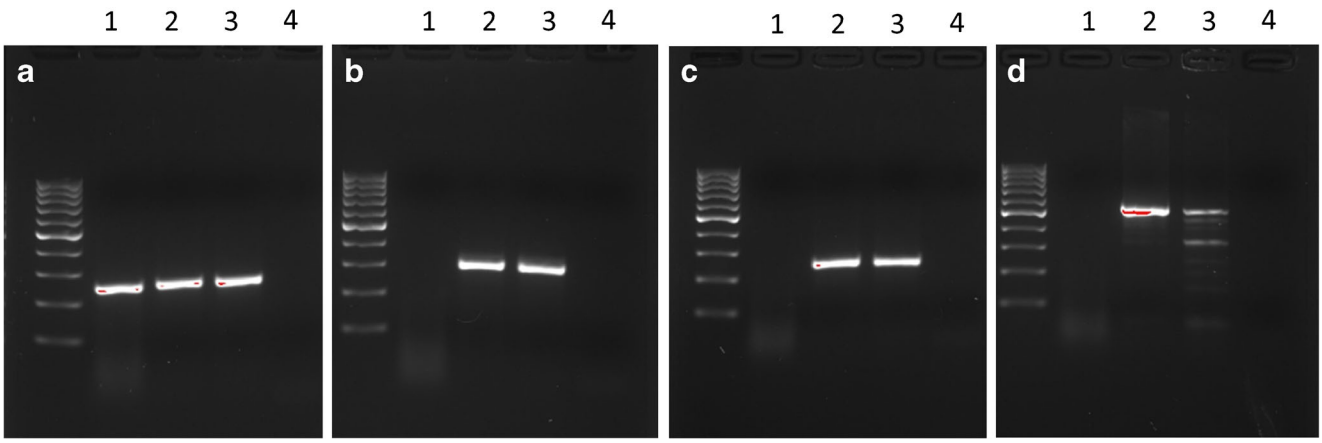

Fig. 3 RT-PCR analysis of NTR1 mRNA in LnCap and PC-3 cell lines. *Lane 1*: LNCaP cells; *lane 2*: H1299 human non-small cell lung cancer cells; *lane 3*: PC-3 cells, *lane 4*: no cDNA template control. **a** is the β-actin as internal reference while **b**, **c**, and **d** show the PCR amplification results using NTR1 primers set 1, 2, and 3 respectively. The expected PCR product size was 307 bp, 278 bp and 312 bp, respectively. (Note, H1299 cells were not relevant to this study)

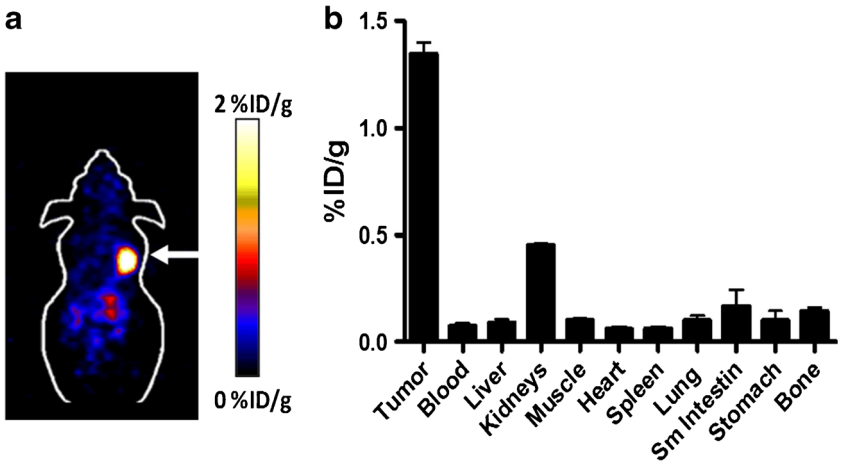

Fig. 4 **a** Representative microPET image of mice bearing PC-3 tumor 3 h after intravenously injection of ^{18}F-DEG-VS-NT. The *arrow* indicates the tumor site. **b** Biodistribution of ^{18}F-DEG-VS-NT in PC-3 tumor-bearing mice at 3 h post injection

With the encouraging tissue-staining results on hand, we then explored whether NTSR1 PET could be used for prostate cancer imaging. The NTSR1 mRNA expression was first evaluated in two prostate cancer cell lines: LnCap and PC-3. Reverse transcription PCR results showed that mRNA expression level was very low or negative in LnCap cells (androgen-dependent) but positive in PC-3 cells (androgen-independent). This result also correlated well with previous western blot study results, suggesting PC-3 could be used as an NTSR1 positive tumor model for a PET imaging study. It is noteworthy that the gene expression data do not equate with protein expression and are not quantitative. Though it is not adequate to justify the NTSR1 expression in prostate cancer, it is a good start to establish an animal model and test NTSR1 PET imaging in prostate cancer. The expression of NTSR1 in more patient-derived prostate cancer cells will be evaluated in future studies.

Using the reported PET imaging agent, ^{18}F-DEG-VS-NT, a small animal PET study was performed in PC-3 tumor model mice. As shown in Fig. 4, the NTSR1-positive PC3 tumor demonstrated prominent tumor uptake and very low background in all major tissues and organs including blood, muscle, liver, and kidneys. The relative high tumor uptake and the very low background in normal tissues may allow the detection of very small tumor metastases via PET imaging technique due to the high contrast. However, the absolute tumor uptake value was low; therefore, future probe design will be focused on further improving absolute tumor uptake.

Conclusion

NTSR1 demonstrated high occurrence rate in prostate cancer and low incident rate in BPH based on the immunohistochemistry staining of a clinical tissue bank. Compared with the expression profile of PSMA, initial IHC staining indicated that NTSR1 could serve as an alternative diagnostic or therapeutic target when PSMA has limited expression level in prostate cancer. Using the NTSR1 positive PC-3 tumor model, ^{18}F-DEG-VS-NT (an NTSR1-targeted PET imaging probe) demonstrated prominent tumor uptake and high tumor-to-background contrast. In summary, NTSR1 could be a promising biomarker in prostate cancer research.

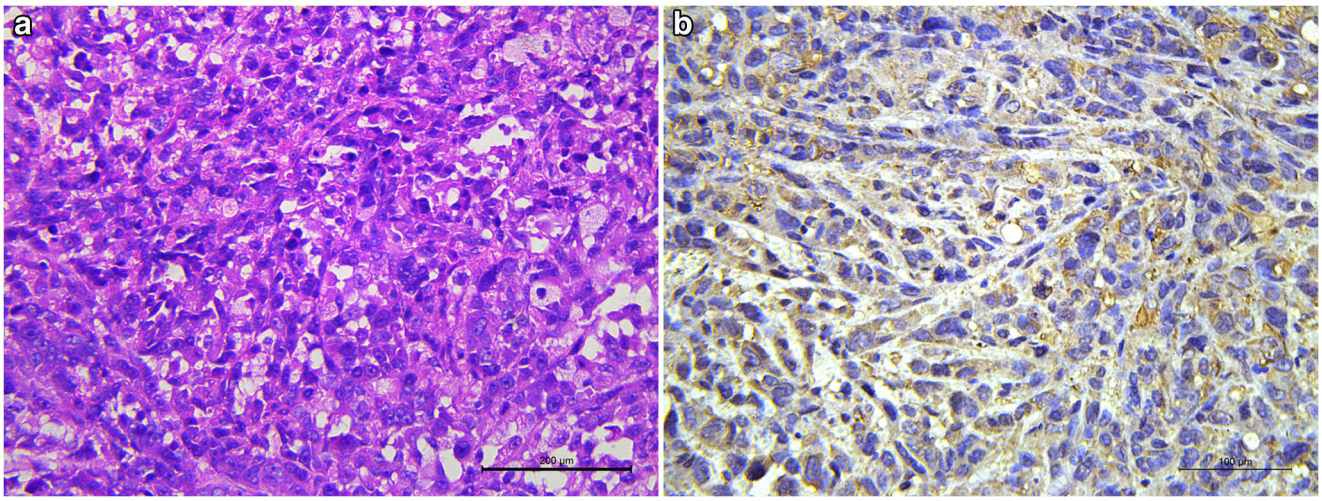

Fig. 5 **a** HE and **b** NTSR1 staining in PC-3 xenograft

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants and tissue samples were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The protocol was approved by the Human Research Ethics Committee of Xiangya Hospital, Central South University. Informed consent was obtained from all individual participants included in the study. All procedures performed in studies involving animals were in accordance with the ethical standards of UNC-CH, and the protocol was approved by the UNC Institutional Animal Care and Use Committee.

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