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STEAP mRNA detection in serum of patients with solid tumours

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ABSTRACT

STEAP was identified by the strategy of suppression subtractive hybridizations in Los Angeles prostate cancer xenografts. It is expressed in prostate and other cancers, and not in most normal tissue; it can be used as a marker to evaluate biological samples from individuals suspected of having a disease associated with STEAP dysregulation, such as cancers, and may provide prognostic information useful in defining appropriate therapeutic options.

The aim of this study was to test the STEAP mRNA detection in the serum of patients with different malignant tumours by using Real-Time reverse transcription PCR. The results were compared with biological samples obtained by age-matched non-malignant donors.

Our data demonstrated that STEAP mRNA is detectable in serum of patients with different solid tumours whereas it is not amplifiable in non-malignant donors.

This marker revealed with the molecular method of quantitative PCR in serum, may be useful to discriminate normal and cancer patients.

1. Introduction

STEAP (six-transmembrane epithelial antigen of the prostate) was identified by the strategy of suppression subtractive hybridizations in LAPC (Los Angeles prostate cancer) xenografts [1]. The prototype member of the STEAP family, STEAP-1, appears to be a membrane protein expressed predominantly in prostate cells. Structurally, STEAP-1 is a 339 aminoacid protein characterized by a molecular topology of six transmembrane domains and intracellular N- and C-termini, suggesting it folds in a "serpentine" manner into three extracellular and two intracellular manner; this putative secondary structure may predict that it functions as a potential channel or transporter protein. STEAP is highly expressed at all steps of prostate cancer and does not seem to be modulated by hormones; it also expressed in multiple cancer while showing restricted expression in normal human tissue [1]. In fact, this protein over-expressed in prostatic cancer, is also present in numerous human cancer cell line from pancreas, colon, breast, testicular, cervical, bladder and ovarian carcinoma, acute lymphocytic leukaemia and Ewing sarcoma [2]. Since the STEAP gene family is predominantly expressed in epithelial tissues, it seems possible that the STEAP protein function as ion channels, transports proteins or gap-junction proteins in epithelial cell function. Ion channels have been implicated in proliferation and invasiveness of prostate cancer cells [3]. Both rat and human prostate cancer cells contain sub-population of cells with higher and lower expression levels of sodium channels. Higher levels of sodium channel expression correlate with more aggressive invasiveness in vitro [4]. Similarly, it has been shown that a specific blockade of sodium channels inhibits the invasiveness of PC-3 cells in vitro [5], while specific inhibition of potassium channels in LNCaP cells inhibited cell proliferation [6]. These reports suggest a role for ion channels in prostate cancer and also demonstrate that small molecules that inhibit ion channel function may interfere with prostate cancer proliferation. This large pattern of expression strongly suggests the use of this tumour antigen for diagnosis and in a broad-spectrum antitumour therapy.

The finding that STEAP is expressed in prostate and other cancers, and not in most normal tissue, provides evidence that this gene is associated with dysregulated cell growth

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and therefore identifies this gene and its products as targets that can be used to evaluate biological samples from individuals suspected of having a disease associated with STEAP dysregulation. For example, because STEAP is so highly expressed in prostate, and not in most normal tissues, and because its expression is associated with certain cancers, assays that evaluate the relative levels of STEAP mRNA transcripts or proteins in a biological sample may be used to diagnose a disease associated with STEAP dysregulation, such as cancer and may provide prognostic information useful in defining appropriate therapeutic options.

The idea that it should be possible to obtain circulating RNA from peripheral blood is intriguing because this is a easily available potential biomarker representing a non-invasive technique for diagnosing and monitoring tumours [7]; in patients with breast, hepatocellular, colorectal and other cancers, circulating RNA has been detected at high levels; in addition, it has been demonstrated that circulating RNA is derived from tumour tissues [8].

STEAP mRNA detection in the serum of tumour patients have not been investigated, probably because this marker was recently identified. The aim of this study was to test the STEAP mRNA detection in the serum of patients with different malignant tumours by using Real-Time reverse transcription PCR. Our data demonstrated that STEAP mRNA is detectable in serum of patients with different solid tumours whereas it is not amplifiable in non-malignant donors; however, in the light of this finding, this marker revealed with the molecular method of quantitative PCR in serum, may be useful for diagnosing tumours.

2. Material and methods

2.1. Patients

We selected 50 patients, consecutively hospitalized in our Institute. Patients have previous diagnosis of solid tumour or were diagnosed during the hospitalization. Patients included in the study were untreated, surgical treated or they stopped chemotherapy at least six months before the enrollment. Results from these patients were compared with 29 non-malignant age-matched donors, hospitalized in our Institution for cardiovascular or metabolic diseases. All the subjects had given written informed consent. Characteristics of the study population are showed in Table 1.

2.2. Serum preparation

Centrifugation of collected blood and harvesting serum samples were done by using three steps of centrifugation (800 *g* with 0.45 μm filtration, 1000*g*, and 1500*g*) to decrease lymphocyte to a minimum as previously described [9]. The samples were then used to RNA extraction or stored at −80 °C.

2.3. RNA extraction

Total RNA was extracted from 1 ml of serum using the QIAamp UltraSens Virus kit (Quiagen) with DNAse I treatment. The RNA from LNCaP culture for standard curve was extracted from a 75-cm^2 flask using the RNAeasy minikit (Quiagen) with DNAse I treatment. The amount of extracted RNA was quantified by measuring the absorbance at 260 nm. The purity of the RNA was checked by measuring the ratio of the absorbance at 260 and 280 nm, where a ratio ranging from 1.8 to 2.0 was taken to be pure.

2.4. Reverse transcription

First-strand cDNA was generated from 1 μg of RNA using the High-Capacity cDNA Archive Kit, with random hexamers, (Applied Biosystems PE) according to the manufacturer's protocol. RT product was aliquoted in equal volumes and stored at −80 °C

2.5. RT Real-Time quantification of STEAP mRNA

PCR was performed in a total volume of 50 μl containing 1× Taqman Universal PCR Master mix, no AmpErase UNG and 5 μl of cDNA; pre-designed, Gene-specific primers and probe sets for each gene (STEAP; Hs00185180_m1) (Beta 2 microglobulin (B2 M); Hs99999907) were obtained from Assay-on-Demand Gene Expression Products (Applied Biosystems). The real-time amplifications included 10 min at 95 °C (AmpliTaq Gold activation), followed by 50 cycles at 95 °C for 15 s and at 60 °C for 1 min. PCR efficiencies were calculated with a relative standard curve, derived from a four cDNA dilution series in triplicate and gave regression coefficients greater than 0.98 and efficiencies greater than 90%. To normalize the STEAP mRNA expression from sample to sample in RNA input, quality and reverse transcriptase efficiency, we amplified the housekeeping gene B2M. The B2M endogenous/internal control gene was abundant and remained constant, in proportion to total RNA, among the samples. The STEAP and B2M ratio represented the normalized STEAP (the STEAP/B2M ratio). The amount of STEAP cDNA in each sample, obtained as average of triplicates, was determined from the standard curve and the results are expressed as pg equivalent to STEAP RNA obtained from LNCaP and simplified as STEAP RNA pg/ml.

2.6. Standard curves

The relative standard curves were obtained using the STEAP and B2 M gene primers and probes in singleplex, amplified with 10, 20, 40 and 80 pg of total RNA from LNCaP. Each sample was run in triplicate. The curves obtained for each cell line showed a linear relationship between RNA concentration and the C_t value of PCR real-time for both STEAP gene and B2M gene.

We selected the ΔRn in the exponential phase of amplification plots to determine the C_t values and to obtain the linearity of calibration curves.

2.7. Calculation of performance

Results of STEAP RT Real-Time PCR in serum were plotted against disease status (present or absent) and sensitiv-

Table 1
Characteristics of the study population

Patient No.	Age	Sex	Type	TNM	Treatment
1	64	F	Pancreas	T3N0M0	–
2	77	F	Pancreas	T4N1M1	–
3	83	F	Pancreas	T2N0M0	–
4	77	M	Pancreas	T4N1M1	–
5	77	M	Pancreas	T2N0M0	–
6	60	M	Pancreas	T2N0M0	–
7	65	F	Pancreas	T2N1M1	–
8	72	F	Pancreas	T3N2M2	–
9	68	M	Pancreas	T2N0M0	–
10	79	M	Bladder	T3N1M0	–
11	67	F	Bladder	T3N1M0	S
12	68	M	Bladder	T2N1M1	–
13	88	M	Breast	T2N9M1	S
14	59	F	Breast	T1N1M1	S + CT
15	62	F	Breast	T1N1M1	–
16	60	F	Breast	T1N1M1	–
17	61	F	Breast	T1N1M1	–
18	63	F	Breast	T1N1M1	–
19	62	F	Breast	T1N1M1	–
20	57	F	Breast	T2N1M1	–
21	76	M	Prostate	T3N1M1	–
22	79	M	Prostate	T3N1M1	CT
23	65	M	Prostate	T2N1M0	S
24	83	M	Prostate	T1N0M0	–
25	72	M	Prostate	T2N1M1	–
26	73	M	Prostate	T1N1M1	–
27	70	M	Prostate	T1N1M1	–
28	68	M	Prostate	T1N1M1	–
29	71	M	Prostate	T1N1M1	–
30	73	M	Prostate	T1N1M1	–
31	74	M	Prostate	T1N1M1	–
32	71	M	Prostate	T2N1M1	–
33	68	M	Prostate	T1N1M1	–
34	65	M	Prostate	T1N1M1	–
35	69	M	Prostate	T1N1M0	–
36	71	M	Prostate	T1N1M1	–
37	70	M	Prostate	T1N1M1	–
38	83	M	Colon-rectum	T3N1M1	S
39	88	F	Colon-rectum	T4N1M1	–
40	84	M	Colon-rectum	T4N1M1	S
41	73	M	Colon-rectum	T3N1M1	S + CT
42	84	M	Colon-rectum	T3N1M1	S
43	84	M	Colon-rectum	T4N1M1	S
44	64	M	Colon-rectum	T4N1M1	–
45	63	M	Lung	T4N1M1	–
46	70	M	Lung	T4N1M0	CT
47	76	M	Lung	T4N1M0	–
48	85	M	Lung	T1N1M1	–
49	79	F	Stomach	T1N1M1	–
50	65	F	Stomach	T4N1M1	S
Donors					
Number	Mean age (years)	Sex (M/F)			
29	70.1 ± 17.7	19/10			

CT, chemotheraphy; S, surgery.

ity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with 95% confidence interval were calculated using standard formula.

2.8. Statistical analysis

Results were expressed as means ± SD. Differences among groups were assessed by one way analysis of variance (ANOVA) using SPSS for Windows version 16.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Standard curve from LNCaP

In each quantitative assay, a strong linear relation was demonstrated between pg STEAP RNA and PCR cycles using LNCaP RNA for concentration ($r^2 > 0.99$) and the efficiency of the reaction was always over 90%, as assessed by the slope of standard curve for target gene (Fig. 1).

3.2. STEAP RNA quantification

We obtained good quality of RNA from serum of patients and non-malignant donors as evaluated by ratio of the absorbance at 260 and 280 nm (ranging from 1.8 to 2.0). In addition, by RT Real-Time PCR, beta-2-microglobulin was positive in all samples ($C_t < 30$).

To quantify the STEAP RNA in serum of patients we performed the Real-Time PCR by quantitative relative standard curve and we obtained the amplification corresponding to STEAP RNA in serum from patients and non-malignant donors (Fig. 2).

The STEAP RNA in serum was positive in 92% of patients (C_t<40) whereas two non-malignant donors presented low levels of STEAP RNA amplifiable in serum (6.8%) ($C_t > 40$); on the contrary, in remaining non-malignant donors, RNA was not amplifiable for STEAP.

3.3. Performance of the assay

Sensitivity, specificity positive predictive value and negative predictive value of this molecular test were calculated from data presented in Table 2. The calculated performance with 95% Confidence Interval is shown in Table 3.

There were no difference in age, histological type, clinical stage, and presence of metastases among tumour patients. However, a negative correlation between treatment and STEAP levels was observed even if it did not reach the statistical significance (Table 4).

4. Discussion

Biochemical analysis and secondary-structure prediction suggest that STEAP is a cell-surface molecule with six transmembrane domains; cell surface molecules with six transmembrane domains are often ion channels [10] that have been implicated in the proliferation and invasiveness of prostate cancer cells [3]. It is known that cells expressing Na^+ channels correlate positively with their ability to invade basement membrane matrix in vitro. In addition, in vitro studies suggest that the presence of Na^+ channels in prostate carcinoma may act as a marker of metastatic ability [4].

STEAP may affect intercellular communications also indirectly by altering intracellular ion concentrations, which in turn regulate gap or adherence junction activity [11]. Nevertheless, cellular communications facilitate the intercellular exchange of small molecular weight solutes, such as nutrients, metabolites, electrolytes, and second messengers from distant blood vessels, thus supporting tumour growth. Many evidences from various models implicate cell-communication in the ability of tumour cells to invade and metastasize [12]. Structural domains of this protein may suggest its involvement in tumour cell

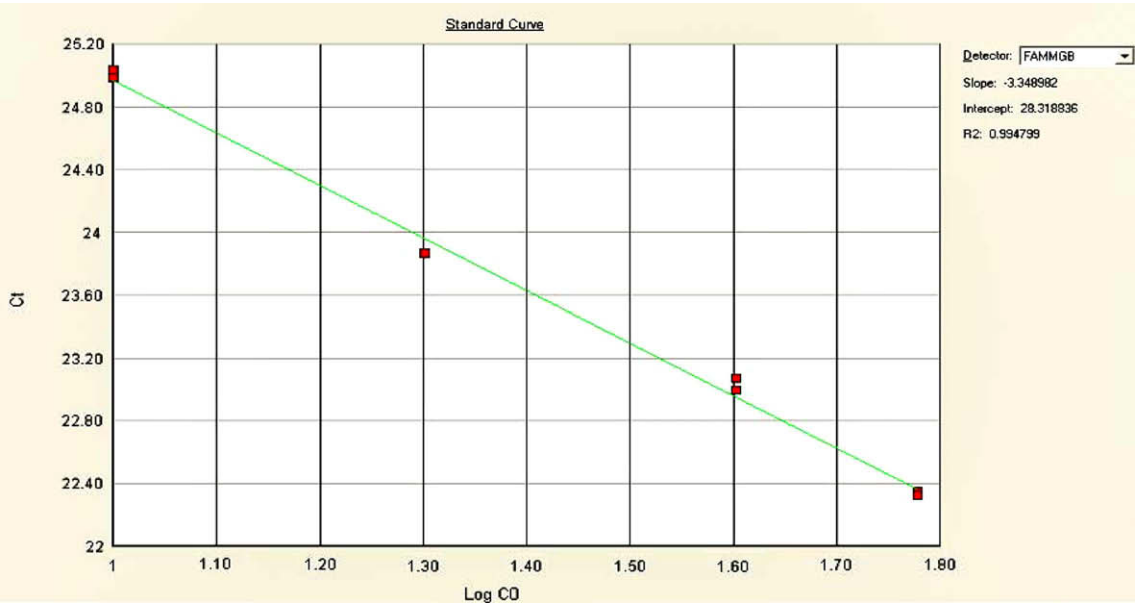

Fig. 1. Standard curve for STEAP (C_t value = threshold cycle of RT PCR at which fluorescence is detectable).

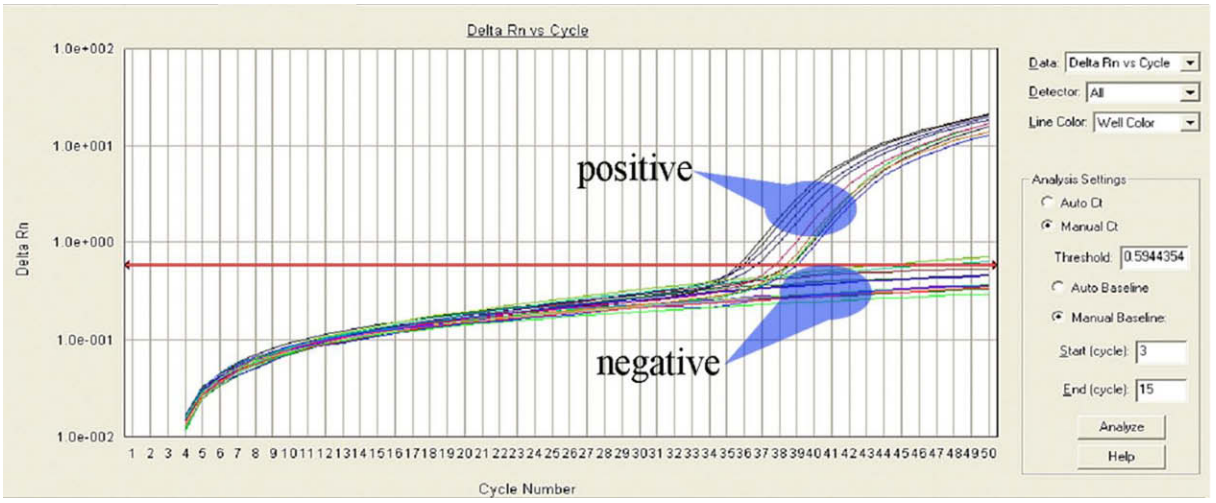

Fig. 2. STEAP mRNA amplification in serum from cancer patients and non-malignant individuals by RT Real-Time PCR.

Table 2
STEAP expression in cancer patient and non-malignant individual serum

No. tumour patients	Test results	RNA STEAP (pg/ml)	
46	Positive	Median	5
		Mean	7.39
		Ranks range	2.4–25
4	Negative	Not detectable	
No. normal donors	Test results	RNA STEAP (pg/ml)	
2	Positive	Median	–
		Mean	1.65
		Ranks range	1.5–1.8
27	Negative	Not detectable	

growth; in fact, STEAP contains a heme-binding domain called the apoptosis, cancer and redox associated transmembrane domain, which is present in a structurally related family identified recently to include members of the STEAP family, as the bacterial Nox family and the YedZ family of oxidoreductase [13].

Table 3
Performance of STEAP RT Real-Time PCR in serum of tumour patients

Parameter	Value (%)	95% Confidence interval
Sensitivity	92	84.4–99.5
Specificity	93.1	83.8–100
PPV	95.8	90.1–100
NPV	87	75.2–98.8

Table 4
STEAP levels in metastatic and non-metastatic and treated and untreated patients

Patients	RNA STEAP (pg/ml)	*p*
Treatment		
Treated	4.9 ± 4.5	ns
Untreated	7.6 ± 5.2	
Metastases		
Metastatic	6.7 ± 4.9	ns
Non-metastatic	7.4 ± 7.0	

In addition, STEAP protein is overexpressed not only in prostate cancer but also in numerous human cancer cell lines from pancreas, colon, breast, testicular, cervical, bladder and ovarian carcinoma, acute lymphocytic leukaemia and Ewing sarcoma [2]. Moreover, the characteristics of STEAP above described have validated it as a novel target with the desirable characteristics for antibody therapy. The clinical use of monoclonal antibody as cancer therapeutics has been realized in the last few years with successful commercialization of antibody products; the successful generation of two monoclonal antibodies (mAb) that bind to cell surface STEAP-1 epitopes provided the tools to study STEAP-1 susceptibility to naked antibody therapy [12].

This is the first time that STEAP-related RNA has been reported in the serum of tumour patients. The presence of STEAP RNA in the serum of tumour patients is probably the reflexion of this RNA in the tumour cells; in addition, a high percentage of sera from non-malignant donors resulted negative and an important percentage of tumour patient sera were positive. The approach by RT Real-Time PCR allowed also a quantification of this marker in the serum of patients.

We performed the analysis of possible influencing factors, including age, histological type, clinical stage of cancer and presence of the treatment. None of these factors influenced significantly the STEAP mRNA levels, even if we observed lower values of STEAP mRNA in treated with respect to untreated patients. However, in our study, the high specificity and sensibility of the method indicates that the STEAP mRNA in serum may be useful to discriminate cancer patients from normal individuals.

On the basis of this finding, STEAP RNA should be utilized as a useful cancer marker and, as previous studies have shown that this protein is frequently expressed in different neoplastic diseases, the relative serum analysis may be applied to the detection of a broad range of cancers.

Even if these findings are intriguing, further and larger studies are needed to confirm the role of this marker in detecting tumours as well as to verify the possibility to use RNA STEAP as a non invasive follow-up marker for cancer patients.

Conflict of interest statement

All the authors disclose any financial and personal conflict of interest.

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