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Synaptophysin expression on circulating tumor cells (CTCs) in patients with castration resistant prostate cancer (CRPC) undergoing treatment with abiraterone acetate or enzalutamide

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Abstract

Background—With the advent of secondary androgen receptor (AR)-targeted therapies in metastatic castration resistant prostate cancer (CRPC), non-adenocarcinoma prostate cancers are becoming more prevalent. Many of these cancers express neuroendocrine markers, which may provide biomarkers for emergence of this disease state. We aimed to quantify the expression of synaptophysin (Syp) on circulating tumor cells (CTCs) from serial samples of patients being treated with abiraterone acetate or enzalutamide.

Methods—CTCs were isolated from 44 CRPC patients prior to starting abiraterone or enzalutamide, at 4, 8, and 12 weeks on therapy, and at progression. Patients were stratified into three groups: *de novo* resistance, short response, and long response. CTCs were enumerated on the CellSearch platform and Syp expression was quantified using the open fluorescent channel on the platform. Correlative analyses were performed.

Results—A baseline CTC count of five or greater was associated with a more rapid time to progression (TTP) and increasing CTC counts correlated with emergence of drug resistance. Syp

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8. Author contributions

SKP helped with the design of the study, acquisition of materials, and writing of the paper. MH carried out experiments. LY and LC carried out CTC staining development and processing under the supervision of RP. PT consented patients and procured patient samples. JH coordinated sample acquisition. MK helped with the design of the study. JOJ managed the project, assisted with experimental design and execution, and writing of the paper. All authors assisted in editing of the paper.

9. Competing interests

No authors have conflicts to report.

was readily detectable on the surface of CTCs, and baseline percent CTC Syp expression was significantly associated with TTP. Furthermore, in evaluable patients, percent CTC Syp expression increased with the emergence of drug resistance. We also found that prior exposure to AR-targeted therapies was inversely associated with progression free survival.

Conclusions—We have demonstrated that Syp can be quantified on CTCs and that Syp expression correlates with resistance to abiraterone and enzalutamide. Larger studies testing Syp as a biomarker of emergence of non-adenocarcinoma disease and as a marker of response to AR-targeted therapies are warranted.

Keywords

Prostate cancer; circulating tumor cell; androgen receptor; synaptophysin; abiraterone; enzalutamide

1. Introduction

Two new androgen receptor (AR)-targeted therapies, enzalutamide and abiraterone acetate, have been approved for treatment of metastatic prostate cancer (PC) and more are in clinical trials [1]. Many patients respond to these agents, but both *de novo* and acquired resistance are common. While resistance continues to be mediated by AR signaling in some patients, emerging evidence suggests that the strong AR inhibition mediated by these drugs is giving rise to an increasing number of patients with truly AR-independent disease, often typified by a small cell appearance and expression of neuroendocrine (NE) markers [2, 3]. Synaptophysin (Syp) is one of the most often used and pertinent markers of NEPC [3–5]. While recent studies have suggested that there are multiple intermediate phenotypes of cells leading to NE disease, including variable AR expression, Syp expression is critical in defining the final transdifferentiated NE state [6, 7]. These data suggest that expression of Syp on tumors is the ideal marker for the emergence of NE disease. Therefore, we have created an assay for identifying emergence of NE disease using Syp expression on circulating tumor cells (CTCs).

CTCs provide a powerful alternative to biopsies for determining the molecular characteristics of cancers that emerge following development of resistance. The presence of ≥ 5 CTCs measured with the CellSearch platform [8] is associated with poor survival in patients with mCRPC. Furthermore, increases in the number of CTCs following treatment with AR targeted agents, abiraterone or enzalutamide, appears to be indicative of a lack of clinical response [9]. Such association with clinical outcome suggests that CTCs play an important role in metastatic process. In this study, we used the open channel on the CellSearch platform to quantify expression of Syp on CTCs from patients undergoing treatment with enzalutamide or abiraterone and found a correlation between increasing Syp-positive CTCs and the emergence of drug resistance.

2. Materials and Methods

2.1 Patient Selection

44 patients were enrolled between April 2014 and November 2016. To be eligible for this study, patients must have had a cytologically or pathologically verified diagnosis of prostate cancer, and radiographic evidence of metastatic disease. Patients must be defined by the clinician as having CRPC, typically constituting failure of combined androgen blockade with leuprolide and bicalutamide followed by anti-androgen withdrawal. With appropriate counseling regarding treatment options, patients must have opted to receive either abiraterone or enzalutamide with an anticipated monitoring plan that included serial collections of PSAs simultaneous with collection of blood for correlative studies.

In line with Prostate Cancer Working Group 3 (PCWG3) guidelines [10], three cohorts of patients were identified: (1) patients who have primary or *de novo* resistance (Group A), patients who have a short response to treatment (Group B), and patients with an exceptional response (Group C). No firm criteria exist for delineating these groups; we therefore propose that Groups A, B and C can be defined by the duration of abiraterone or enzalutamide being <3 months, 3–12 months, and > 12 months, respectively. As emphasized in the guidelines, patients were maintained on abiraterone or enzalutamide until no longer clinically benefitting.

2.2 CTC Enumeration and analysis of Syp expression

Blood from eligible patients with mCRPC were collected into CellSave tubes at baseline, at weeks 4, 8 and 12 of therapy, and at the time of progression. The standard CellSearch platform was used for enumeration by a trained operator. The CellSearch platform maintains an open channel for assessment of an additional marker on the CTCs, which was used to assess the expression of a FITC-conjugated Syp antibody (Biorbyt 16373). Staining and exposure times were optimized using HEK293 cells transfected with a Syp expression vector (Harvard repository clone ID# HsCD00338768) spiked into normal donor blood samples. The optimal exposure time was found to be 0.8 seconds and the optimal antibody dilution was 1:50. Positive events were determined by the trained CellSearch operator (LC). To test the accuracy of the assay, 150 untransfected and 150 Syp-transfected HEK293 cells were spiked into 7.5mls of healthy donor blood in a CellSave tube. 264 CTCs were identified and 48.86% of them were determined to be SYP+, suggesting our optimized staining procedure was highly accurate (Figure 1). Furthermore, a sampling of three patients demonstrated that no DAPI+/CD45+/CK- cells (leukocytes) were Syp positive, suggesting that background staining of contaminating white blood cells is absent.

2.3 Statistical Analyses

A Kruskal-Wallis test was performed to test the association between baseline PSA or CTC count with group membership. A log-rank test was used to determine differences between CTC groups and time to progression (TTP) while regression analysis was used to determine an association between Syp-positivity and TTP; multivariate regression analysis was used to determine an association between Syp-positivity and TTP while controlling for age, baseline

PSA, and baseline CTC count. A chi-squared test was used to determine differences between CTC Syp-positivity as a categorical variable and group membership.

3. Results

3.1 CTC numbers correlate with response to abiraterone and enzalutamide

The baseline characteristics of patients and treatments are shown in Table 1. Three general response patterns to abiraterone and enzalutamide have emerged in our practice: *de novo* resistance, typically with no decrease in PSA levels and progressive disease, short term response, with only one or two PSA declines prior to increasing PSA and progressive disease between 3–12 months of treatment, and long term response, with continued decreased PSA levels and no sign of progressive disease for one to several years. Therefore, we grouped patients into these categories and determined if baseline variables and trends over sequential blood draws correlated to drug response category.

Analysis of the clinicopathological and CTC data corroborate previous findings regarding baseline PSA, CTC counts, and response (Table 2). There was no association between baseline PSA value and response category; however, there was a significant association between CTC count and each response category, as median CTC count was significantly higher in patients with *de novo* resistance than with short term response, and significantly higher in patients with short term response than with long term response ($p=0.0036$). Additionally, a baseline CTC count of ≥5 was associated with worse progression free survival (PFS, Figure 2, $z = 3.36$, $p < 0.001$).

We next evaluated changes in PSA and CTC number over the course of treatment (Figure 3). Both CTC and PSA values rose rapidly on average in patients with *de novo* resistance to abiraterone or enzalutamide, while both CTCs and PSA continually decreased on average in patients with a long response, only rising at progression. Both PSA and CTC values initially decreased in patients with a short response but began rising at later blood draws.

3.2 Prior secondary hormonal agent exposure decreases effectiveness of abiraterone and enzalutamide

We found that patients with prior exposure to androgen pathway antagonists (abiraterone, enzalutamide, or TAK700) had a significantly shorter TTP than those without prior exposure ($p = 0.0046$). Five, five, and one patient with prior exposure to AR-targeted agents had *de novo* resistance, short response, or long response, respectively. The secondary hormonal agent naïve patients had three *de novo* resistance, eight short responses, and eighteen long responses. Thus, it is clear that prior exposure affects response to an additional AR-targeted agent and that cross-resistance is prevalent in our study. Fourteen patients in the cohort had prior exposure to Sipuleucel-T, but there were no significant correlation between prior Sipuleucel-T use and response category or PFS.

3.3 Synaptophysin expression on CTCs increases with development of resistance

Use of advanced AR-targeted agents has coincided with an increase in the detection of cancers with non-adenocarcinoma features and expression of neuroendocrine markers [11].

As Syp is a validated marker of neuroendocrine phenotype disease, we quantified its expression on CTCs from patients in our study. Following optimization of staining (Figure 1), we were able to reliably detect Syp expression on patient CTCs. There was no correlation between number of CTCs in a sample and the percent of CTCs that were Syp-positive. When all baseline CTC events were considered together, the percentage of Syp-positive CTCs was significantly higher in the patients with *de novo* resistance compared to those with a short term or long term response ($p<.0001$, Figure 4A). Univariate regression analysis demonstrated that there was a significant association between CTC Syp-positivity and TTP ($p=0.0240$). A multivariate analysis including age, baseline PSA, and baseline CTC count also demonstrated a significant association between Syp-positivity and TTP ($p= 0.0234$); no other variable had a significant association with TTP with univariate or multivariate analyses.

Changes in Syp-positivity over time appear to correlate with drug response as well. Looking specifically at patients from the short term response group that had ≥ 5 CTCs in at least 4/5 blood draws, Syp-positivity increased 14.5% from baseline to progression on average (n=8). Individual patient trends from short term responders show that six of these eight patients either started with low Syp-positivity or had a decrease in Syp-positivity before having Syp-positivity increase (Figure 4B), suggesting that for many patients, there is an increase in the number of Syp expressing CTCs coincident with the onset of drug resistance.

4. Discussion

Here we report for the first time the measurement of Syp on prostate cancer CTCs obtained from sequential samples of patients beginning treatment with abiraterone or enzalutamide. Syp expression has been previously detected on CTCs from patients with non-prostate neuroendocrine tumors using the CellSearch platform [12], and we likewise demonstrate that Syp can be reliably detected on CTCs in CRPC patients and furthermore, that the percentage of CTCs that are Syp-positive at baseline correlates with drug response. Specifically, using linear regression analysis we found a significant association between baseline percent Syp-positivity and TTP, even when considered along with age, baseline CTC count and baseline PSA levels as co-variables. We also found an association between baseline percent Syp-positivity and group membership with Syp-positivity being higher in patients with de novo resistance to abiraterone or enzalutamide. However, this data was based on summing all CTC events and not on a per patient basis. Individual patient Syp-positive CTC percentage analysis proved difficult as it was highly influenced by patients with small numbers of CTCs, especially those with only 1 CTC, which will be, by definition, 100% positive or negative. Therefore, summing all CTC values was the more informative approach in this analysis. Our data suggests that high Syp CTC expression could be used as a predictive marker to guide therapy selection; however, a larger study with a pre-defined Syp-positive percentage cutoff must be conducted to test this hypothesis. Perhaps both baseline Syp positivity and change in Syp-positivity over consecutive blood draws will be informative biomarkers. Both could be useful for guiding therapy, especially in patients who progress without a PSA rise. Along these lines, due to the paucity of evaluable CTCs at intermediate blood draws, we were unable to investigate how the trends in CTC Syp-positivity over time differed among treatment groups and were only able to draw conclusions from the short term

response group. In this group, Syp-positivity generally was low during the period when the patient was responding to the drug and rose as progression developed, suggesting that Syp expression correlates with the development of resistant disease.

As has been previously reported in other CRPC patient cohorts, we found that a CTC count of ≥ 5 is associated with worse progression free survival [8]. Four patients were lost to follow-up, but of the remaining 40 patients, 16 had ≥5 and 24 had <5 CTCs at baseline; 11 of these 24 had no CTCs at baseline. 14/16 patients with ≥5 CTCs have already progressed with a median of 96.5 days while 11/24 with <5 CTCs have progressed with a median response time of 205 days. Interestingly, there are three patients with ≥5 CTCs that remained on therapy for >2.5 years, two of whom are still on therapy. These exceptional responders had a few features in common; relatively low CTC counts (8, 16, and 16), and high PSA counts (113, 142, 918), which might suggest that the disease was not high abundance and still very AR-driven. We also observed strong cross-resistance among AR-targeted agents, as has been previously reported [13, 14]. The best response was 335 days (this patient only had short exposure to TAK700 in a clinical trial setting), but the majority of cross-over patients lasted on therapy less than 100 days before progression.

While the new PCWG3 guidelines advise against using PSA or any one biomarker alone to measures to dictate clinical progression [10], we did find that PSA rise nearly always coincided with clinical progression in our cohort. We only had one patient (COH12) that had clinical progression without a PSA rise. This patient's PSA dropped to ~10% of baseline value after four weeks of treatment and remained at ~5% of baseline value at the time of progression. As has been noted in other studies [2, 3], progression in absence of PSA rise is observed in the setting of abiraterone and enzalutamide treatment and may be indicative of a phenotypic change to a truly AR-independent disease state. Interestingly, this patient had the most pronounced increase in the percent of Syp-positive CTCs over the course of treatment of anyone on the study. The baseline value was 4.0% dipping to a nadir of 2.5% before surging to 58.3% positive at progression. This data would strongly suggest the development of AR-independent, neuroendocrine phenotype disease in this patient.

Our study has several shortcomings, the first being the limited sample size. Despite enrolling 44 patients, 15 patients have yet to progress, four other patients went off protocol and three others died of unrelated causes prior to disease progression. Many samples (74/165, 44.8%) had no detectable CTCs. This is to be expected for an effective therapy, but it limits the ability to correlate changes in CTC number and CTC Syp expression with outcomes. Because of the small number of evaluable samples, segregation into abiraterone or enzalutamide only comparisons was not possible. Another limitation of the study was that we only captured EpCAM positive cells. It is very likely that important CTC populations were missed, as shown using other platforms ([15]), and perhaps our results reflect only changes in cancers with strong epithelial phenotypes, presumably the cancers that remain adenocarcinomas. This might explain the slight decrease in percent Syp-positivity in two patients (COH7 and COH22) from their 12 week draw to their progression draw, despite an otherwise upward trend of Syp-positivity. Presumably, the cancers had undergone a more full neuroendocrine transition in that time period, but the CellSearch platform could only detect those CTCs that still expressed EpCAM. It will be important to assess the utility of

Syp CTC expression as a prognostic or predictive biomarker using platforms that do not rely on an epithelial protein for capture.

5. Conclusions

We demonstrated for the first time the measurement of Syp on prostate cancer CTCs obtained from sequential samples of patients beginning treatment with abiraterone or enzalutamide and that Syp expression is correlated with resistance to these drugs. Larger studies testing Syp as a biomarker of emergence of non-adenocarcinoma disease and as a marker of response to AR-targeted therapies are warranted.

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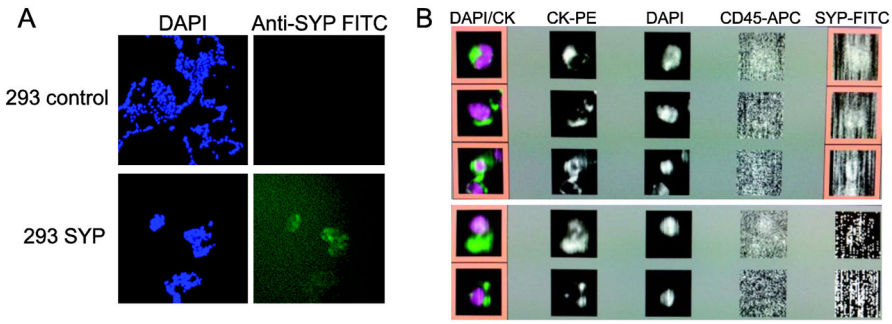
Highlights

- Synaptophysin (Syp) was quantified on prostate cancer circulating tumor cells (CTC)
- Baseline percent CTC Syp expression was associated with TTP
- Percent CTC Syp expression increased with the emergence of drug resistance
- Syp CTC expression might be a useful biomarker of response to AR-targeted therapies
- Syp CTC expression might be a useful biomarker for emergence of neuroendocrine PC



C

CTC	264	100.00
CTC SYP +	129	48.86
CTC SYP -	135	51.14

Figure 1.

Optimization of synaptophysin (Syp) CTC staining. (A) HEK293 cells were transfected with human Syp or control plasmids and the next day were fixed and stained with anti-Syp-FITC and DAPI. Immunofluorescent imaging demonstrates a strong, specific signal at 1:50 dilution. (B, C) HEK293 cells were spiked into healthy volunteer donor blood, half of which had been transfected with the SYP expression plasmid and half of which had not. The sample was then processed for assessment on the CellSearch platform, using the open FITC channel to assess expression of SYP on the CTCs. (B) Examples of SYP-positive (top) and SYP-negative (bottom) CTCs are shown as is the enumeration (C).

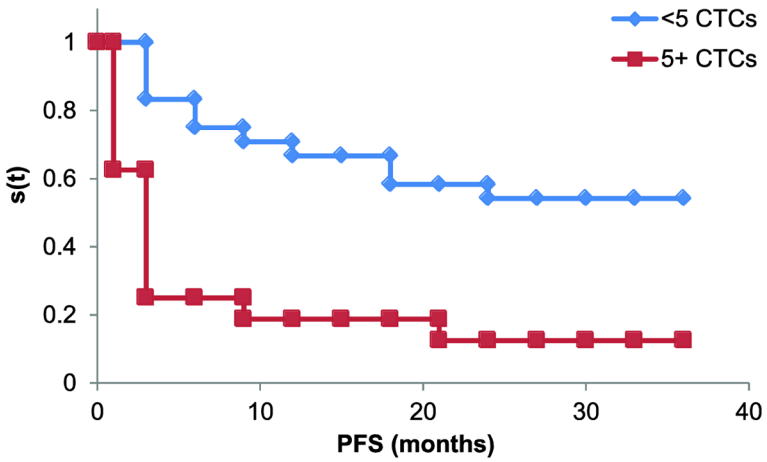


Figure 2.

Progression free survival (PFS) by baseline CTC count. The fraction of patients with <5 or ≥5 CTCs at baseline that remained progression free is plotted over time. Patients with ≥5 CTCs at baseline had a significantly worse PFS.

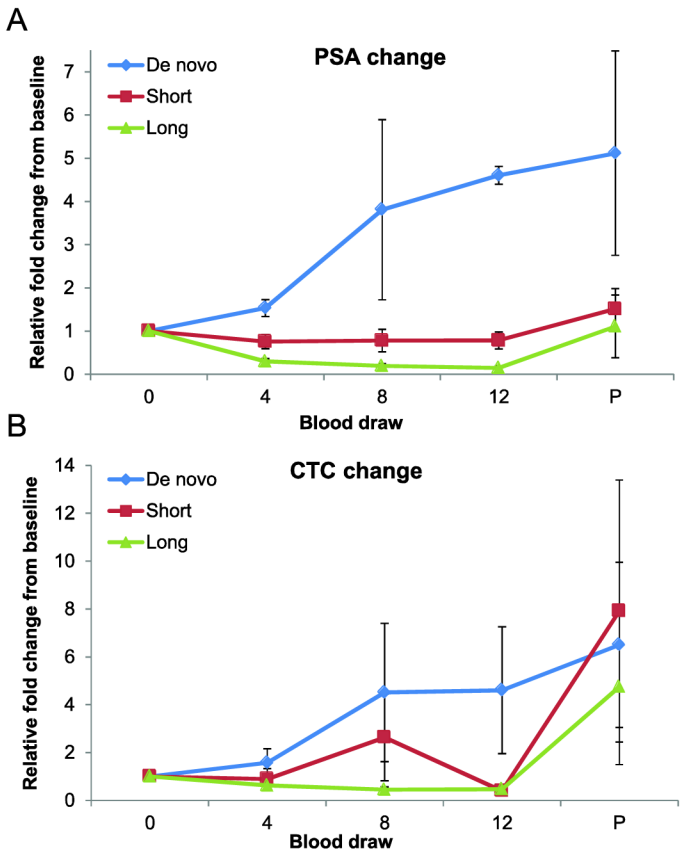


Figure 3.

Changes in PSA and CTC counts over time. The fold changes of PSA (A) or CTC count (B) relative to the baseline draw (draw 0 = 1) are shown at the indicated week on therapy and at progression (P). Error bars represent standard error of the mean.

A

De novo resistance	905	658	0.579
Short response	785	2888	0.214
Long response	18	39	0.316

B

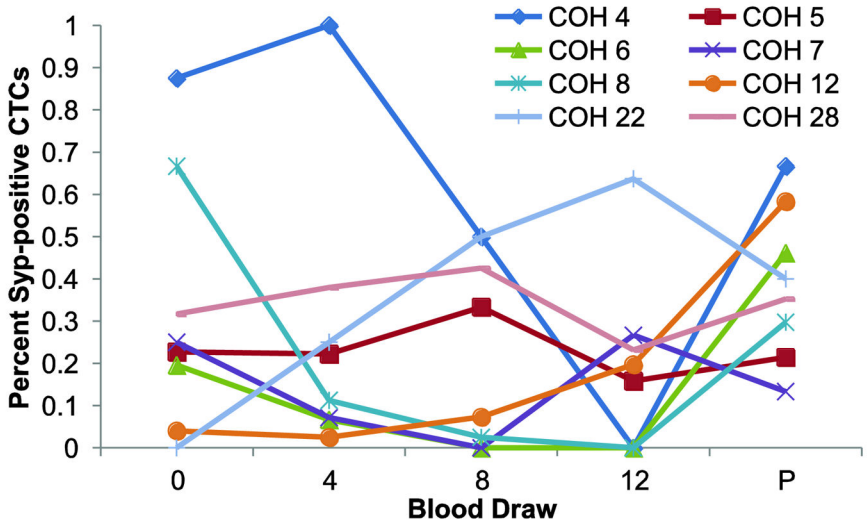


Figure 4.

Synaptophysin (Syp) staining of CTCs. (A) The number of Syp-positive and Syp-negative CTCs detected in each group are shown. (B) Percent Syp-positive CTCs are shown at the indicated week on therapy and at progression (P) for eight short term responders that had ≥5 CTCs in at least 4/5 blood draws.

Table 1

Patient characteristics

Clinical characteristics of patients (n=44)	
Age at start of treatment	71.5 (51–92)
PSA at start of treatment	18.07 (0–918.25)
Therapy	
Abi	11
Enza	21
Abi -> Enza	7
Enza -> Abi	5
Follow-up from start of treatment (median months, range)	34.4 (8.5–40.0)
Response to treatment	(4 off study)
De novo resistance	8
Short response (< 1 yr)	14
Exceptional response (≥ 1 yr)	18

Table 2

Response category characteristics

	De novo resistance			Short response			Long response		
Baseline	**n**	**PSA (median/mean/ range)**	**CTC (median/mean/ range)**	**n**	**PSA (median/mean/ range)**	**CTC (median/mean/ range)**	**n**	**PSA (median/mean/ range)**	**CTC (median/mean/ range)**
Enzalut amide	4	66.73 74.4±20.2 34.98–129.19	61 387 15–1411	6	12.05 29.5±20.8 1.09–153.85	2 7.4 0–22	15	26.01 102.6±63.9 0.26–918.25	1.5 3.7 0–16
Abirater one	4	27.99 26.7±7.7 6.54–44.18	4 3.75 2–5	4	148.44 122.8±44.5 0.67–231.69	179 722.2 0–2126	7	5.26 7.7±4.1 0–27.09	0.5 0.67 0–2
Previous AR pathway inhibitor	5	34.98 48.9±21.0 6.54–129.19	5 8.6 2–18	5	9.42 32.1±29.3 0.67–153.85	4.5 8.3 0–22	1	34.3 34.3 34.3	3 3 3
Combined	8	39.58 50.5±13.5 6.54–129.19	10 195.375 2–1411	12	15.62 68.4±25.1 0.67–231.69	11 305.3 0–2126	20	9.09 74.1±45.4 0–918.25	1 2.8 0–16