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Original Article

High frequency of the *SDK1:AMACR* fusion transcript in Chinese prostate cancer

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Abstract: Chromosomal rearrangements and fusion genes play important roles in tumor development and progression. Four high-frequency prostate cancer (CaP) specific fusion genes, *SDK1:AMACR*, *RAD50:PDLIM4*, *CTAGE5:KHDRBS3* and *USP9Y:TTTY15* have been reported in Chinese CaP samples through a transcriptome sequencing study. We previously reported that *USP9Y:TTTY15* is a transcription-mediated chimeric RNA, which is expressed in both tumor and non-malignant samples, and here we attempted to confirm the existence of the other three fusion genes *SDK1:AMACR*, *RAD50:PDLIM* and *CTAGE5:KHDRBS3*. We detected *SDK1:AMACR* fusion transcript in 23 of 100 Chinese CaP samples, but did not detect *RAD50:PDLIM4* and *CTAGE5:KHDRBS3* transcripts in any of those samples. *SDK1:AMACR* fusion transcript is Chinese CaP specific, which was neither detected in non-malignant prostate tissues adjacent to cancer from Chinese patient nor in CaP samples from UK patients. However, we did not detect genomic rearrangement of *SDK1* gene by fluorescence *in situ* hybridization analysis, indicating that *SDK1:AMACR* is also a transcription-mediated chimeric RNA. Quantitative analysis demonstrated that high level *AMACR* expression was associated with *SDK1:AMACR* fusion status (P=0.004), suggesting that *SDK1:AMACR* fusion transcript may promote prostate carcinogenesis through increasing *AMACR* expression. However, the fusion status was not significantly correlated with any poor disease progression clinical features. The identification of the *SDK1:AMACR* fusion transcript in CaP cases from China but not from UK further supports our previous observation that different genetic alterations contribute to CaP in China and Western countries, although many genetic changes are also shared. Further studies are required to establish if CaPs with *SDK1:AMACR* represent a distinct subtype.

Keywords: Chinese CaP, chromosomal rearrangements, *SDK1:AMACR*, chimeric RNA

Introduction

Prostate cancer (CaP) is the most commonly diagnosed tumor and the second leading cause of deaths due to cancer in the Western male population [1]. The serum prostate-specific antigen (PSA) is helpful for early detection and monitoring disease progression of CaP, therefore improves the survival in some circumstances [2, 3]. However, PSA level is specific for prostate tissue, but it is not specific for CaP. Benign prostatic hyperplasia (BPH), prostatitis and the effect of some medications can also lead to an elevated PSA, which weaken its specificity as a diagnostic marker for CaP. PSA screening remains controversial, since it results in over-diagnosis and over-treatment [4-6]. Extensive searches have been carried out to develop more CaP specific molecular markers, in particular those predicting cancer aggressiveness [7, 8].

There is compelling evidence to consider genomic rearrangements as an initial event in tumorigenesis [9, 10]. Fusion genes, initially identified in hematological malignancy and soft tissue sarcomas, have been used for tumor diagnosis, sub-classification, prognosis, recurrence monitoring and as therapeutic targets, such as *BCR:ABL* fusion gene in chronic myeloid leukemia [10]. It has been demonstrated that fusion genes play a critical role in CaP development and progression [9] and *TMPRSS2:ERG*, which was frequently detected in CaP, is the

Table 1. Clinicopathological details of the 100 Chinese CaP cases

	Values
Age (year)	Mean (SD)
	66.45 (7.37)
Clinical stage	
T1	2
T2	39
T3	56
T4	3
Gleason score	
6	18
7	56
8	17
9	9
Total PSA (ng/ml) at diagnosis	Mean (SD)
	22.28 (22.15)

SD: standard deviation; PSA: prostate specific antigen.

most commonly reported fusion gene to date in human malignancies [10-13].

CaP has an obvious difference in the incidence and mortality among different populations [14]. Little is known of the genetic mechanisms contributing to this discrepancy in prostatic cancer prevalence. We previously identified certain genetic difference between Chinese and Western CaPs [15-17]. Recently, it was reported that four new high-frequency fusion genes, *USP9Y:TTTY15* (19/54), *RAD50:PDLIM4* (15/54), *CTAGE5:KHDRBS3* (20/54), *SDK1:AMACR* (13/54) were identified by transcriptome sequencing analysis of CaP cases from China [18]. Once confirmed, this finding may not only further highlight the genetic difference between Chinese and Western CaPs, but also provide new insight of prostate carcinogenesis and new population-based treatment strategy.

We previously reported that *USP9Y:TTTY15* is a transcription-mediated chimeric RNA, which is expressed in both tumor and non-tumor samples [19]. In this study, we attempted to confirm the existence of fusion genes *SDK1:AMACR, RAD50:PDLIM* and *CTAGE5:KHDRBS3* in a separate cohort of Chinese CaP samples. We detected high frequency of *SDK1:AMACR* fusion transcript in our CaP samples, which was specific for Chinese cancers and associated with high *AMACR* expression. However, *SDK1:AMACR* fusion was not associated with disease severity. We did not detect *RAD50:PDLIM4* and *CTAGE5:KHDRBS3* transcripts in any of our CaP samples.

Materials and methods

Samples

A total of 100 pairs of fresh cancer and their matched normal prostate tissue samples from Chinese CaP patients were collected from the First Affiliated Hospital, Zhejiang University Medical College, Hangzhou, China and were preserved in -80°C. The tissue morphology and Gleason grade of cancer lesions were confirmed by two pathologists. Detailed clinical pathological information for these samples is summarized in **Table 1**. Diagnostic PSA in two patients and the age information of one cases are missing. In addition, tissue microarrays (TMAs) containing 85 of the samples were made for fluorescence in situ hybridization (FISH). This study is approved by the ethical committee of First Affiliated Hospital, Zhejiang University Medical College. Twenty eight fresh frozen tissue samples of radical prostatectomy CaP from Barts Health patients were taken with informed patient content under Orchid Tissue Bank, ethically approved by East London and City Committee. CaP cell lines LNCaP, VCaP, 22RV1, DU145 and PC3 were also used for *SDK1:AMACR* fusion transcript analysis.

RT-PCR and real time quantitative RT-PCR using SYBR green technology

Total RNA was extracted using Trizol (Invitrogen). Two micrograms of total RNA was used to synthesize cDNA in a 25 μl reaction mixture using Reverse Transcriptase M-MLV (R Nase H) and random primer as previously described [20]. The primers and annealing temperatures for the RT-PCR are listed in **Table 2**. The RT-PCR amplified product was detected by running 1.2% agarose gel.

The amplification program for quantitative RT-PCR consisted of an initial denaturation step at 95°C for 30 s followed by incubations at 95°C for 5 s, 60°C for 30 s, and 72°C for 13 s for 50 cycles. All the reactions were performed in triplicate and all gene expression values were normalized using the housekeeping gene *GAPDH* and calculated using the comparative Ct method (ΔΔCt method). The transcript-spe-

Table 2. All the primers used for RT-PCR

Primers	Sequence	Annealing temperature	Length of product
RAD50:PDLIM4-F	ACTAAGTGAATGCGAGAAACACAA	59°C or 62°C	100-250 bp
RAD50:PDLIM4-R	ACAGACAGTGTGAGGTGATCGT		
SDK1:AMACR-F	ACCTGGTCATTTCCAACATCAG	54°C or 57°C	150-400 bp
SDK1:AMACR-R	CAAAGCCAAATAGTTGATATCGTG		
CTAGE5:KHDRBS5-F	TGCTGAAAATGAAGCCACTG	59°C	400 bp
CTAGE5:KHDRBS5-R	GGACTGGTGGAGATTGGCTA		
CTAGE5:KHDRBS5-F	GTGGATGCAAGAGGCCCATTCT	59°C or 62°C	256 bp
CTAGE5:KHDRBS5-R	TAGACGCCCTTTGCTGTCCTC		

cific primers used in this study were: *AMACR*-F: GATTTGGCCAGTCAGGAAGC, *AMACR*-R: GAACACCTGACAAAGCCAAATAGTT, *GAPDH*-F: AAGGTGAAGGTCGGAGTCAA and *GAPDH*-R: AATGAAGGGGTCATTGATGG.

The forward primer for *AMACR* is located in exon 2 and the reverse primer spans the junction of exon 2 and exon3 of *AMACR*.

Sequencing analysis

The expected band for each RT-PCR amplified product was gel-excised (GEL Extraction kit) and cloned into PMD 18-T vector (Takara, Dalian, China) with TA cloning technique following manufactory's instructions. Briefly, the molar ratio of vector DNA and insert DNA ranged from 1:2~10 and the ligation reaction was performed at 16°C for 50 min. Then the plasmid was transformed into competent *E. coli cells*, which were cultured in LB growth medium containing *Amp.* We picked up monoclones for sequencing analysis using M13+ primers and the ABI3730XL sequencing machine.

Fluorescence in situ hybridization (FISH) analysis

Interphase FISH analysis of TMA samples was performed as previously described [21]. Using BAC probes, we applied the FISH signal breakapart approach on CaP samples to detect the genomic rearrangements of *RAD50* (5' BAC clone RP11-410H12 in red and 3' BAC clone RP11-525D24 in green) and *SDK1* (5' BAC cloneRP11-133L20 in red and 3' BAC clone RP11-644L16 in green), which are required to generate the genomic fusion of *RAD50:PDLIM4* and *SDK1:AMACR*, respectively. If a fusion event occurs, it will either split apart the gene (red and green signals locate separately) or cause the deletion of the 3' genome region (loss of the green signal). A schematic presentation ofthe FISH signals for potential *SDK1* genomic rearrangements are shown in **Figure 1**.

Statistical analysis

Chi-square test was performed when a cut off value was used to categorize the cases into groups. Unpaired t test were applied when the expression level of *AMACR* was compared between the positive and negative *SDK1:AMACR* samples. All statistical analysis is performed using SPSS 16.0 for Windows (SPSS, Chicago, IL, USA) with two tailed tests. A *P* value of less than 0.05 was considered statistically significant.

Results

Confirmation of the recurrent SDK1:AMACR fusion transcripts in Chinese CaP samples

Using the previously reported PCR primers, we attempted to detect the *SDK1:AMACR* fusion in 100 Chinese CaP samples. Based on the TMs of the primers, we tried three annealing temperatures at 54°C, 57°C and 60°C in 16 cancer samples and found a PCR product at around 250 bp in one sample using the lowest annealing temperatures of 54°C. Sequencing analysis confirmed a 241 bp fusion product of *SDK1* (breakpoint after exon 22) with *AMACR* (breakpoint before exon 2) (**Figure 2**). This generated an in frame fusion with a predicted protein containing the most of *SDK1* and *AMACR*. We analyzed the other 84 cancer samples using the annealing temperatures of 54°C and found 22 samples with a product at around 241 bp, which was confirmed as *SDK1:AMACR* (exon 22 to exon 2) fusion products by sequencing analysis in all the cases. In many positive samples, additional bands were also obtained but sequencing analysis of these bands (cut from the gel and then cloned) showed un-specific PCR products. Using an annealing tempera-

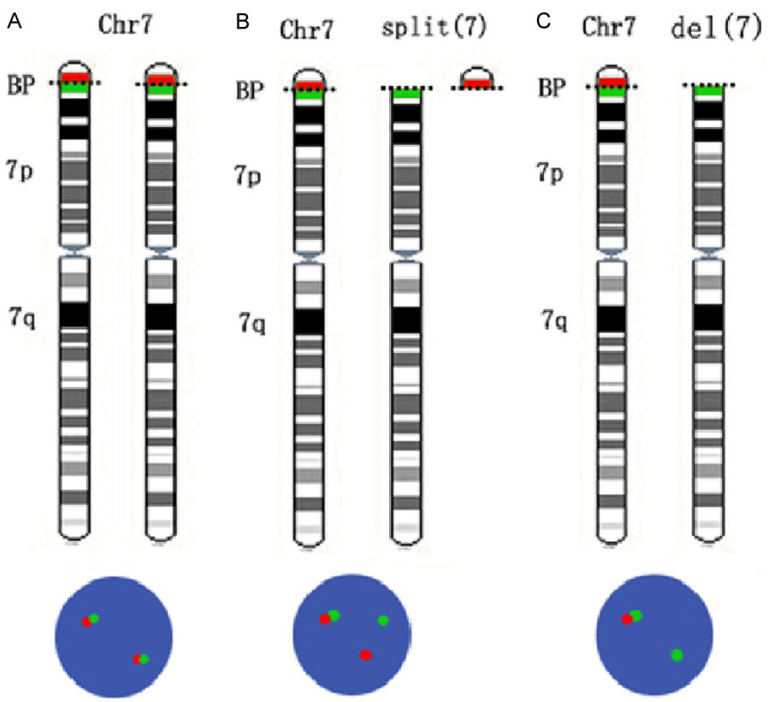

Figure 1. Schematic presentation of the detection of *SDK1* rearrangements. A. A cell with normal *SDK1* shows two pairs of green and red signals. B. Red and green signals split apart when a break at *SDK1* occurs with both chromosome fragments remaining in the cell. C. Loss of green signal when the 3' region of *SDK1* is deleted. Chr: chromosome; BP: breakpoint.

tures of 57°C to PCR amplify those samples, in addition to the 241 bp specific band found in the 22 fusion transcript positive samples, we identified in sample 47C a 514 bp product (**Figure 2**). Sequencing analysis confirmed that the 514 bp product was a variation of the *SDK1:AMACR* fusion gene, which include part of the intron 2 of *AMACR* in the middle of the fusion (**Figure 2C**).

We also investigate whether *SDK1:AMACR* fusion exists at genomic level in 18 *SDK1:AMACR* fusion transcript positive sampled by dual-color FISH analysis, but we did not found evidence of the truncation of *SDK1* in any of the 18 CaP cases analyzed (**Figure 3A**).

Correlation of SDK1:AMACR fusion transcripts with clinicopathological parameters in Chinese CaP cases

We further analyzed the correlation of the existence of *SDK1:AMACR* fusion transcripts with clinical data in those 100 Chinese CaP cases (23 fusion positive and 77 fusion negative), where the clinical data were available. There was no statistically significant correlation of *SDK1:AMACR* fusion with clinicopathological parameters (**Table 3**).

Correlation of AMACR expression levels with SDK1:AMACR status in Chinese CaP cases

We further analyzed the expression difference of the *AMACR* by quantitative RT-PCR between *SDK1:AMACR* fusion positive (n=18) and negative (n=25) samples. The fusion positive samples expressed a significantly higher *AMACR* than the negative cases (P=0.004). While in 12 *SDK1:AMACR* positive cases, *AMACR* expressed at a level closed to the median expression detected in the *SDK1:AMACR* negative cases, in six *SDK1:AMACR* positive cases, >2 fold higher expression of *AMACR* than any of the expression level of the fusion negative samples was detected (**Figure 4**). The patients with both *SDK1:AMACR* fusion and relatively higher expression of *AMACR* did not have a history of using anti-androgen or taking other therapeutic drugs.

Lack of RAD50:PDLIM4 and CTAGE5:KHDRBS3 fusions in our CaP samples

While we confirmed the *SDK1:AMACR* fusion transcripts, we did not detect any *RAD50:PDLIM4* and *CTAGE5:KHDRBS3* fusion transcripts using a considerable number of our Chinese CaP samples. For *CTAGE5:KHDRBS3*, we initially tried to detect the fusion product in fourteen cancer samples used the previously described primer pair [22] and a range of annealing temperatures at 51°C, 53°C, 55°C, 57°C and 59°C. We found many PCR product bands with the most dominant one at around 300 bp in all cases even at the highest annealing temperatures of 59°C. When 51°C and 53°C were used for annealing temperatures, there was a band at the expected fusion product size of 400 (**Figure 5A**). Sequencing analysis of this PCR product (cut from the gel) from

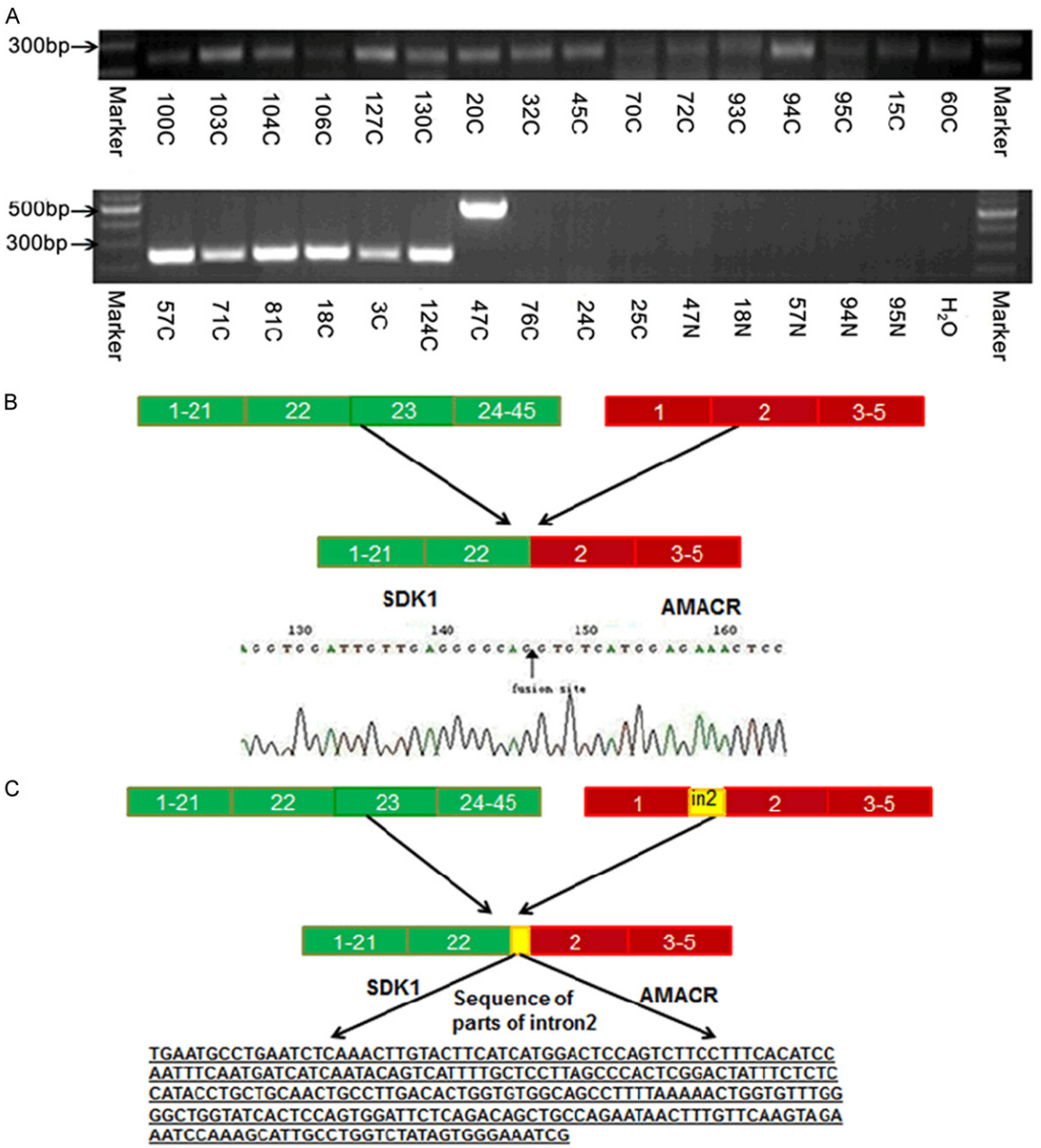

Figure 2. Detection of the *SDK1:AMACR* fusion transcript in CaP. (A) Examples of the RT-PCR products of *SDK1:AMACR* fusion transcript at 241 bp in CaP and a variation of 514 bp band (case 47C) along with case-matched adjacent normal tissue samples and a negative control without input cDNA template. (B) Schematic presentation of the alignment of SDK1 on chromosome 7 and *AMACR* on chromosome 5 and the fusion transcripts based on the sequencing data of the fusion at the end of *AMACR* exon 22 and the beginning of *AMACR* exon 2. (C) Schematic presentation of the alignment of the 514 bp fusion sequence for *SDK1* and *AMACR*. In the middle of the fusion sequence presented in (B), there is additional sequence from *AMACR* intron 2 (underline). The position of the nucleotide fusion site is indicated by the blue arrows.

all the samples showed unspecific PCR products. We then designed another pair of PCR primer with expected PCR products of 256 bp (**Table 2**), which is nested in the above primer pair. Using the annealing temperatures of 59°C and 62°C, we analyzed 32 cancer samples and found an expected size of PCR product in 14 samples (**Figure 5B**). However, none of the PCR products were fusion transcripts by sequencing analysis. Finally we applied nested PCR to

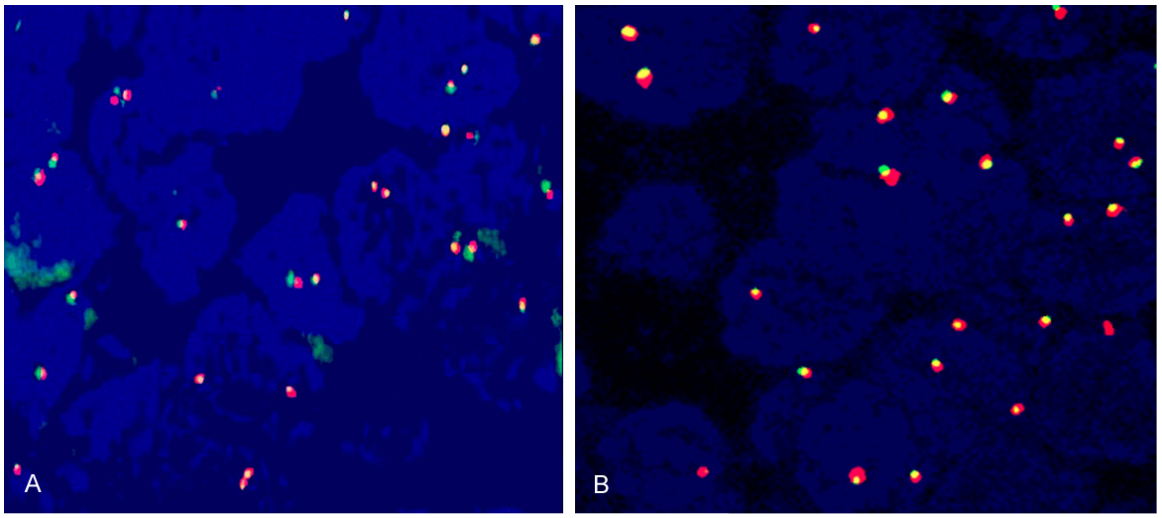

Figure 3. Interphase FISH on formalin-fixed, paraffin-embedded tissue samples to analyze (A) *SDK1* and (B) *RAD50* genomic status. The overlap of red and green indicates that no genomic rearrangement occurred to *SDK1* and *RAD50* genomic regions.

Table 3. Correlation between clinicopathological parameters and expression of *SDK1:AMACR* fusion transcript

Variable	*SDK1: AMACR* (-)	*SDK1: AMACR* (+)	*P* value
Age ≤70	52	14	P=0.554
Age >70	25	9	
PSA ≤10	24	10	P=0.292
PSA >10	52	13	
GS ≤7	54	20	P=0.106
GS >7	23	3	
Clinical stage ≤3	67	20	P=0.994
Clinical stage >3	10	3	
Meta negative	71	21	P=0.889
Meta positive	6	2	

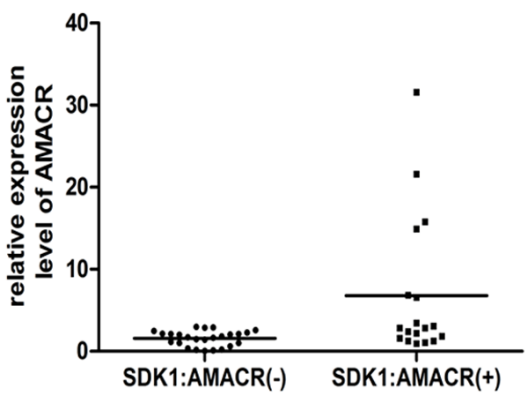

Figure 4. The expression level of *AMACR* in the fusion positive and fusion negative Chinese CaP samples. The expression level of *AMACR* in all samples was relative to a fusion transcript negative samples (101C), which was set to 1.

amplify the fusion transcript. Although we detected PCR products at around expected size of 256 bp in 16 and 13 out of 51 samples with the annealing temperature of 59°C and 62°C respectively, sequencing analysis of all those PCR products did not detected the expected fusion product.

For the *RAD50:PDLIM4* fusion, we performed the RT-PCR only with the previously reported primers [22]. The initial test using different annealing temperatures at 55°C, 57°C and 59°C in three cancer samples yielded multiple bands at all conditions, with 59°C annealing temperature better than the other two. We then tried annealing temperatures at 59°C and 62°C in eight more cancer samples and found one case with PCR product around the expected 225 bp size at both PCR conditions but with more specific PCR band at the annealing temperature at 62°C. Using the later PCR condition, we analyzed 42 more CaP samples and found the PCR product at 225 bp in four more samples (**Figure 5C**). However, sequencing analysis of the five PCR products did not identify any fusion products.

To investigate *RAD50:PDLIM4* fusion at genomic level, we also determined the existence of any truncations of *RAD50* as a result of the fusion event by dual-color FISH analysis. We did not found evidence of the truncation of *RAD50* in any of the 85 CaP cases analyzed (**Figure 3B**).

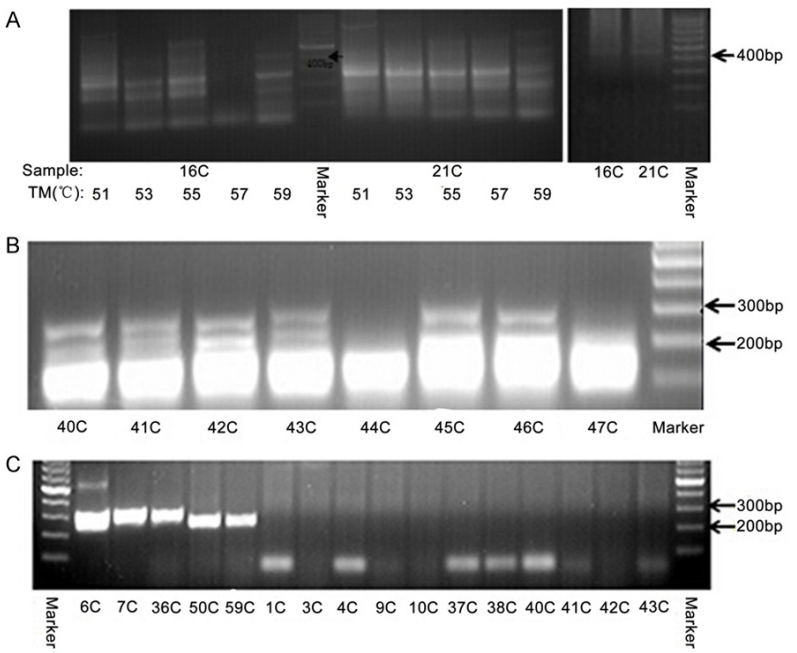

Figure 5. Examples of the RT-PCR products using primers for *CTAGE5:KHDRBS3* and *RAD50:PDLIM4* fusion transcripts. A. Examples of the RT-PCR products around the expected size (about 400 bp) for *CTAGE5:KHDRBS3* fusion transcript. B. Examples of the RT-PCR products with the expected size around 256 bp (in samples 40C, 41C, 42C, 43C, 45C and 46C) for *CTAGE5:KHDRBS3* fusion transcript. C. Examples of the RT-PCR products at the expected size between 100-250 bp in five samples, 6C, 7C, 36C, 50C and 59C for *RAD50:PDLIM4* fusion transcript.

Discussion

With the recent investigation of fusion genes in solid tumors, fusion gene has now been recognized as a common mechanism in the development and/or progression of many human tumors [10, 23]. CaP is the carcinoma where fusion gene is most commonly found. However, apart from *TMPRSS2:ERG*, all the other fusion genes have been only found at a low frequency and *TMPRSS2:ERG* was found at a much lower frequency in CaP samples in East Asian population compared to the Western countries [15, 24]. The reporting of the four high frequency fusions in Chinese CaP [22] poses the potential to reveal the specific mechanisms, genetic pathways and unique therapeutic approaches for cancer in this population. However, in our previous study we identified that one of the four fusion transcripts, *USP9Y:TTTY15*, is a transcription-mediated chimeric RNA, which was detected in both tumor and non-malignant samples as well as non-prostate tissues [19]. In this independent study, we confirmed that *SDK1:AMACR* is frequently and specifically expressed in Chinese CaP samples, but we did not detect the other two reported fusions with different PCR conditions on a considerable large number of Chinese CaP samples. Our FISH data also demonstrated that there was no genomic rearrangement of *RAD50*, which is required to generate the *RAD50:PDLIM4* genomic fusion. Therefore *RAD50:PDLIM4* and *CTAGE5:KHDRBS3* genomic fusions are unlikely to exist in Chinese CaP and even those fusion transcripts may be rare.

The *SDK1:AMACR* fusion transcripts identified in 23% samples in our study is comparable with the 24% (13/54) positive cases reported by Ren [18]. *SDK1* is regulated by androgen through the androgen-responsive Serum Response Factor (SRF) [25, 26]. AMACR was initially identified as a CaP over expressed gene through a microarray analysis of subtracted cDNA library of CaP and non-malignant tissues [27] and it is commonly over expressed in CaP [28]. Over expression of *AMACR* has been considered as a biomarker for CaP [29-33]. *AMACR* expression is not regulated by androgen, but it depends on tumor differentiation [34, 35]. Therefore, similar to *TMPRSS2:ERG* fusion, *SDK1:AMACR* fusion may also lead to *AMACR* over expression in those Chinese CaPs, driving by *SDK1* promoter under androgen control. By quantitative detection of the expression level of *AMACR* in CaP samples, we found that the *SDK1:AMACR* fusion transcripts was associated with over expression of *AMACR*. However, our FISH data rules out the genomic fusion of these two genes. Therefore, *SDK1:AMACR*, although specific to Chinese CaP, it is also a transcription-mediated chimeric RNA. The transcription of this fusion RNA may use a novel mechanism, transcription-mediated chimeric RNA, for *AMACR* over expression in CaP cells. Many mechanisms have been used to generate transcription-mediated chimeric RNA, but the biological significance and controls to use

those processes generating those fusion transcripts are not known yet [36-38].

Although the above data, both from the literature and our experiments, suggest that *AMACR* over expression and *SDK1:AMACR* contribute to prostate carcinogenesis, the fusion status was not correlated with any clinical parameters of CaP we analyzed. Further study of its clinical significant is required. In consistent with our finding of the lack of relationship between the *SDK1:AMACR* and PSA level, previous studies showed that, although *AMACR* expression was strongly associated with prostate, it was not associated with PSA level. *AMACR* over expression was detected in several human cancers and their precursor lesions, including CaP precursor, high-grade prostatic intraepithelial neoplasia [28], suggesting that *AMACR* may have a role in the early steps of cancer development. High level *AMACR* expression in normal prostate tissue is associated with increased risk of developing CaP [28]. However, the mechanism how *AMACR* promotes carcinogenesis is not clear yet. Methylacyl-CoA racemase (*AMACR*) is a mitochondrial and peroxisomal enzyme. It is essential in the metabolism of branched chain fatty acid and bile acid intermediates [39]. Red meat and dairy product, which are associated with branched chain fatty acid, are suggestive risk factor for CaP, indicating that *AMACR* may contribute to prostate carcinogenesis through branched chain fatty acid metabolism pathway.

In gastrointestinal stromal tumor, where *AMACR* is also over expressed, *AMACR* affects cell proliferation but not apoptosis [40]. *AMACR* amplification and overexpression in primary imatinib-naïve gastrointestinal stromal tumors is a driver of cell proliferation, indicating adverse prognosis [40]. In CaP, Zha S also found that *AMACR* knockdown by RNA interference inhibited the growth of cancer cells [35]. However, in gastrointestinal stromal tumor, *AMACR* over expression was associated with poor prognosis [40], but in CaP, it has been reported that decreased *AMACR* expression in localized cancer was associated with poor prognosis [41]. *AMACR* may be differentially associated with other factors involved in disease progression in different cancer cells, while have similar role in cell proliferation and cancer development for different tissue types.

As *SDK1* is controlled by androgen and *SDK1:AMACR* fusion transcript was associated with increased *AMACR* expression, this transcription-mediated fusion transcript may, similar to the genomic fusion of these genes, also put *AMACR* expression under the control of *SDK1* promoter, which is stimulated by androgen. Therefore, *AMACR* expression in those *SDK1:AMACR* fusion transcript positive cases is likely influenced by androgen stimulation or AR activity. As generation of this transcription-mediated fusion transcript needs a complicated transcript level control, it may not as efficient as the consequence of genomic fusion to stimulate the expression of the downstream gene by an androgen regulated promoter. As the product contains half of *SDK1* and most of the sequence of *AMACR*, it is also possible that the fusion protein may gain novel oncogenic functions. While the role of *SDK1:AMACR* fusion products in CaP development and progression requires further clarification, it is equally important to investigate the mechanism how promoter on a different chromosome can be used to drive a gene expression and if the generation of *SDK1:AMACR* is influenced by androgen level or AR activity, which will help us to prevent this large proportion of *SDK1:AMACR* fusion transcript positive CaP in Chinese men.

Disclosure of conflict of interest

None.

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