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Original Article

The C-terminal common to group 3 POTES (CtG3P): a newly discovered nucleolar marker associated with malignant progression and metastasis

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Abstract: A gene family expressed in prostate, ovary, testis and placenta (POTEs) is newly defined and primate-specific. POTE genes have 13 paralogs, which are dispersed in 8 chromosomes and divided into three groups. The proteins encoded by these genes contain three domains: An N-terminal, ankyrin repeats and a C-terminus. Previous studies suggest that POTE proteins are localized in the inner aspect of cellular membrane and are considered as cancer-testis antigens, because they expressed widely in cancers, but in limited benign tissues. In this study, we will study the subcellular distribution of all POTE proteins and their associations with the progress and metastasis of malignancies. By performing Immunohistochemistry, Immunocytochemistry and immunofluorescence assay on tissue microarray slides containing tissues with different pathology and origins or on cell lines, we found that the epitopes of N- and C-terminals of all detected POTEs were widely expressed in benign and malignant tissues. Among these epitopes, C-terminal common to group 3 POTEs (CtG3P) was the only portion localized in nucleoli. The nucleolar IHC scores for CtG3P was lowest in benign tissues (4.47 ± 3.43), significantly higher in localized malignancies (5.32 ± 3.36, p = 3.63E-02), and highest in metastatic malignancies (7.90 ± 2.29, p = 8.13E-12). The CtG3P was better in differentiation of benign from malignant changes, and/or in differentiation of localized from metastatic cancers as compared with Ki-67 and AgNORs. In addition, transient transfection of siRNA against mRNA of group 3 POTEs influences the growth and survival of MCF-7 cells in vitro in a dose dependent manner.

Keywords: POTEs gene family, prostate, ovary, testis and placenta, cancer, ankyrin repeats, cancer-testis antigens, nucleolar marker, malignant progression, metastasis

Introduction

The nucleolus, the cellular ribosome factory, is dramatically changed in size and number during cellular proliferation, especially in cancer cells [1, 2]. The alterations in nucleolar elements could be indicators for cancer progression. However, none of single nucleolar element has been approved as a pan-malignant and nucleolus-specific marker.

A recently defined gene family, expressed in prostate, ovary, testis and placenta (POTE), is primate-specific and includes 13 paralogs dispersed among 8 chromosomes [3, 4]. According to phylogenetic analysis, these paralogs can be sorted to three groups: group one POTE having POTE8 only; group 2 POTEs consisting of POTE15, 18 and 21; and group 3 POTEs including POTE2, 14 and 22 [5]. The proteins encoded by these genes are structured as a cysteine-rich N-terminus, 3-7 ankyrin repeats, and a C-terminus with spectrin-like helices [4]. In initial studies, POTE proteins were thought to be located at the inner aspect of the plasma membrane and were considered to be cancer-testis antigens, because they were expressed in many cancers, but in restricted normal tissues in reproductive system [6, 7]. Although POTE proteins may have roles in cytoskeletal structure [4], apoptotic pathways [8], epigenetic regulation of tumor progression [9], and linage-specif-

ic differentiation of embryonic cells [10], no exact function is known.

While seeking biomarkers for prostate cancer, we found that the C-terminal common to all group 3 POTEs (CtG3P) was clearly localized in the nucleoli of prostatic epithelia, and the expression level of nucleolar CtG3P correlated strongly with the progression and metastasis of prostate cancer (unpublished data). The current study was designed to demonstrate nucleolar localization, tissue distribution, and association of CtG3P with the progression and metastasis in a variety of benign and malignant tissues from major human organs.

Materials and methods

Human specimens and tissue micro arrays (TMAs)

This study was approved by the Institutional Research Board of the University of Mississippi Medical Center (UMMC). Two hundred sixty-six (266) subjects were admitted to the UMMC during period of 2002-2011. Formalin-fixed and paraffin embedded (FFPE) tissue blocks from studied subjects were archived in the Department of Pathology. Using a Beecher MTA1 Manual Tissue Arrayer (Sun Prairie, WI), tissue microarrays (TMAs) were made from 383 FFPE blocks (some subjects had multiple blocks) from various organs. The pathological diagnosis for each transferred tissue was reconfirmed and defined as one of three pathology categories: benign change, primary malignancy, and metastatic malignancy.

Immunohistochemistry (IHC) and scoring system

IHC stain with antibodies against POTE proteins (**Table 1**) were performed on TMA slides with protocols described previously [11]. IHC for Ki-67 was performed using a Ventana BenchMark automated analyzer with pre-diluted primary antibody for Ki-67 (Ventana, AZ). A scoring system for IHC stain was applied as previously described [11] with a maximum final score of 9. To reflect reverse and dynamic changes of expression levels of CtG3P and N-terminal common to group 3 POTEs (NtG3P) in malignant progression simultaneously, a Mean IHC Index Score (MIIS) was established and calculated as: MIIS = final IHC score of CtG3P – final IHC score of NtG3P. MIIS ranged from -9 to 9. In addition, positive rate of CtG3P, defined as the number of tissues with positive CtG3P IHC stain out of all detected tissues, was calculated for each pathology category in each individual organ/system.

Histochemistry and scoring method

Histochemical staining for Argyrophilic Nucleolar Organiser Regions (AgNORs) was performed on TMA slides using the Ploton method [12]. In order to compare the expression levels of CtG3P with AgNORs, the same scoring system as used in the IHC stain was applied for scoring AgNOR, rather than the complicated counting method for AgNORs as reported [13].

Immunocytochemistry (ICC) and immunofluorecent assay (IFA)

ICC and IFA were performed *in vitro* on cell lines originated from breast (MCF-7 and MDA-MB-231) and from prostate (REWP-1, PC3 and DU-145). The cell suspension of each cell line was gently placed on Poly-L-Lysing coated (PLLC) slips (Neuvitro Corporation) in 6-well culture plates and incubated at 37°C in 5% CO_2. When cells reached 80% confluence (approximately at 72 h), the cells on the slips were washed with PBS × 3, fixed with 4% formalin for 15 min and washed with PBS again.

For ICC, washed cells were incubated with block/permealizer (2% BSA/PBS with 0.25% TritonX-100) for 30 min, primary antibodies for CtG3P and NtG3P for 2 h, and avidin-biotin-peroxidase complex for 30 min. Then NovaRed (Vector Laboratories, Burlingame, CA) was added as a chromogen substrate to produce a visible pink-red reaction product. Finally, the cells were counter-stained with hematoxylin.

For IFA, washed cells were also incubated with block/permealizer for 30 min, primary antibodies for CtG3P and NtG3P for 2 h, and AlexFlour 594 conjugated second antibody (goat anti rabbit IgG-Alexa 549, Life Technologies Corporation, NY, 1:250) for 1 h in the dark. Finally, the cells were counterstained with DAPI (VECTASHIELD® Mounting Medium, VECTOR LABORATORIES INC. CA). The cells were examined using a fluorescent microscope soon after counterstaining.

Table 1. List of primary antibodies used in immunohistochemistry stain

Antibody Name	POTE Group	Immunogen location	Manufacturer	Dilution
Sc-98083	1, POTEA	N-terminal, 25-75	Santa cruz biotech., Inc.	1:400
C-17980	1, POTEA	C-terminal, 391-430	Assay Biotechnology Company	1:200
Ab107416	2, POTEB	C-terminal, 381-430	Abcam PLC	1:200
14169-1-AP	2, POTEB	N-terminal, 1-381*	Proteintech Group Inc.	1:400
Ab76698	2, POTED	C-terminal, 431-480	Abcam PLC	1:50
C-17976	3, POTEG/H	C-terminal, 401-450	Assay Biotechnology Company	1:400
C-17979	3, POTEH	N-terminal, 181-230	Assay Biotechnology Company	1:400
Ab8811	3, POTEH	N-terminal, 181-290**	Abcam PLC	1:100

*Full length of POTEB isoform 3; **Monoclonal antibody, the rest are polyclonal antibodies.

Transient transfection of small interference RNAs (siRNAs) against POTEs

A breast cancer cell line, MCF-7, was used in transient knockdown of POTEG or POTEH with siRNAs. Briefly, siRNAs for POTEG (SR411722), POTEH (SR308469), or scrambled negative control (SR30004) were diluted 10-fold with Rnase-free siRNA duplex resuspension buffer (SR30005, all from OriGene Technologies, Inc., MD) to a concentration of 2 μM. Then each diluted siRNA duplex was further diluted with Opti-MEM 1 reduced serum (Life Technologies Corporation, NY) to produce the final concentrations of 1 nM, 5 nM, or 10 nM in correspondingly designated wells of the 6-well culture plates. Next, 4 μL of transfection reagents (Lipofectamine RNAiMAX, Life Technologies Corporation, NY) was added to each well. After the plates were gently mixed and incubated for 20 minutes at RT, 2.5 mL of the diluted MCF-7 cell suspensions (1×10^5 cells) were added to each well containing un-transfected control (adding 500 μL of Opti-MEM1 medium only), scramble siRNAs, and siRNA for POTEG or POTEH with different dilutions. A PLLC slip was placed on the bottom of each well designated for ICC study. Then these 6-well plates were incubated at 37°C in 5% CO_2 for ICC and Western blot studies.

ICC was performed on PLLC slips containing MCF-7 cells with different treatments after 72 h incubation as described above. Western Blot was also performed. Briefly, the cells in wells without slips were lysed with cold radioimmunoprecipitation lysis buffer. The lysates were then sonicated (for 5 x 5 seconds with 50% pulse on ice), incubated on ice for 30 minutes (vortex every 5 min), and centrifuged at ~14,000 × g for 15 minutes at 4°C to pellet the cell debris. Supernatants were obtained and used in Western Blot with antibodies against CtG3P and NtG3P.

Data analysis

Statistical analysis was performed using the software SPSS 19 and SAS (version 9.3, the SAS institute). One way ANOVA and Student's t-test were used in analysis of differences of mean IHC score and MIIS among different groups. Some ANOVA results were reconfirmed with Kruskal-Wallis test and Wilcoxon Rank Sum test. Pearson's chi-square test was used in comparison of positive rate of IHC staining for CtG3P between pathologic categories. Significant p value was set at < 0.05 for all tests. Alignment analysis of amino acid sequence of immunogens was performed by Blast/uniport online (http://www.uniprot.org/blast/uniprot/).

Results

Subcellular localizations of POTE proteins and the association of their expression level with malignant progression

IHC was performed on 383 FFPE specimens using antibodies (**Table 1**) against N- and C-terminals of representative molecules in each POTE group to determine their subcellular localizations. In group 1 POTE, the IHC signals for its N-terminal were seen in the cytoplasm and nucleus, and those for the C-terminal were mainly localized in the nucleus. In group 2 POTEs, the IHC signals for both the N- and C-terminals were found in the cytoplasm. In group 3 POTEs, the IHC signals for NtG3P were solely localized in the cytoplasm; in contrast, the IHC signals for CtG3P were clearly localized

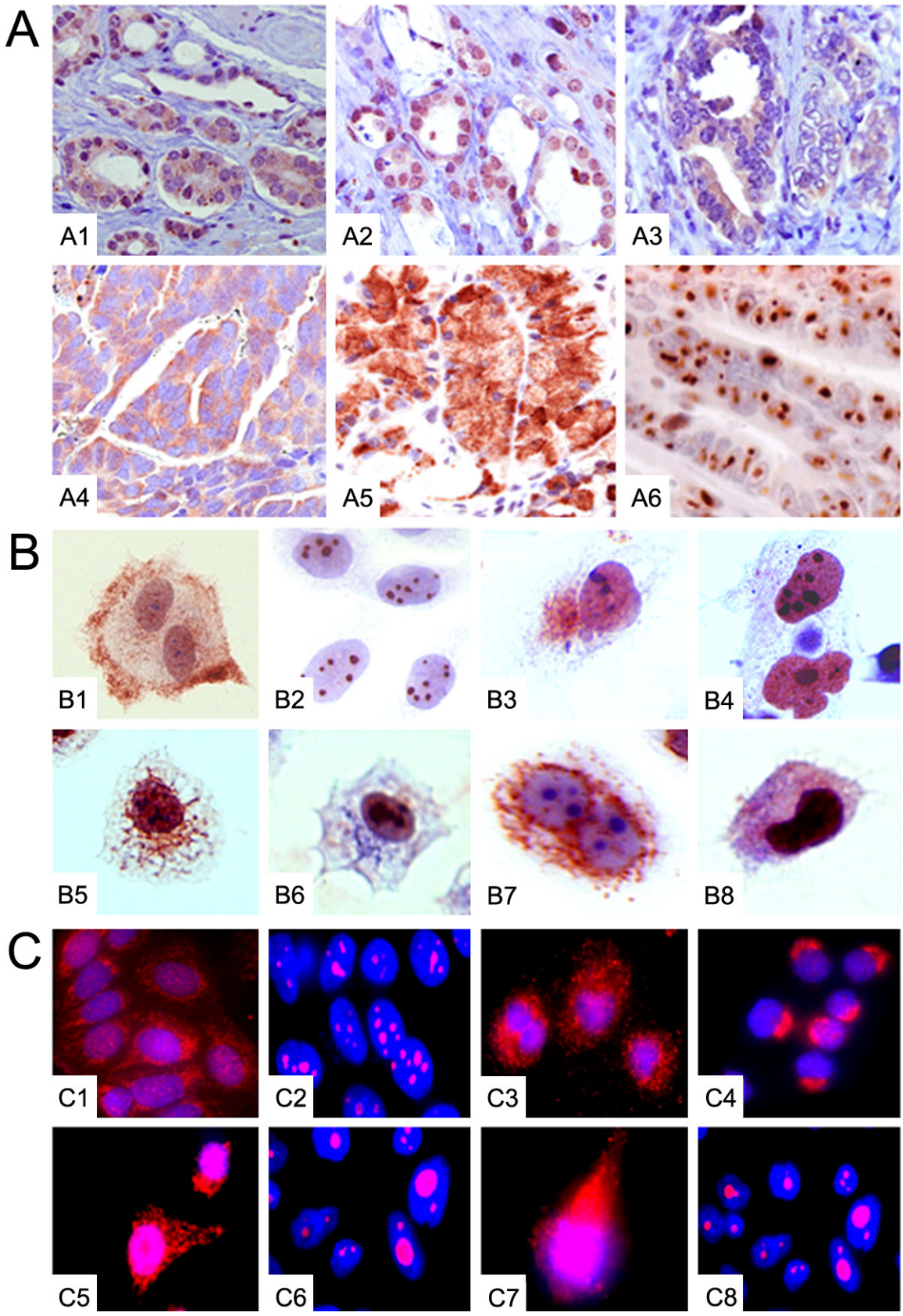
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Figure 1. Subcellular localizations of N- and C-terminals of representative POTE molecules. A: Immunohistochemistry (IHC) for the N- or C-terminal of different POTE molecules in formalin fixed paraffin embedded tissues A1: N-terminal of POTEA (group 1 POTE), cytoplasmic stain in high grade prostate cancer; A2: C-terminal of POTEA, nuclear stain in high grade prostate cancer; A3: N-terminal of POTEB (group 2 POTE), cytoplasmic stain in low grade prostate cancer; A4: C-terminal of POTEB, cytoplasmic stain in ovarian serous papillary carcinoma; A5: N-terminal common to group 3 POTEs (NtG3P), cytoplasmic stain in benign stomach; and A6: C-terminal common to group 3 POTEs (CtG3P), nucleolar stain in colon cancer. B: Immunocytochemistry (ICC) for NtG3P or CtG3P in cell lines B1: NtG3P, membranous and cytoplasmic stain in MCF-7 cells; B2: CtG3P, nucleolar satin in MCF-7 cells; B3: NtG3P, cytoplasmic/nuclear stain in MDA231 cells; B4: CtG3P, nucleolar/nuclear stain in MDA231 cells; B5: NtG3P, cytoplasmic/nuclear stain in DU-145 cells; B6: CtG3P, nucleolar/nuclear stain in DU-145 cells; B7: NtG3P, cytoplasmic stain in RWPE-1 cells; and B8: CtG3P, nucleolar/nuclear stain in PC-3 cells. C: Immunofluorescent assay (IFA) for NtG3P or CtG3P in cell lines C1: NtC3P, cytoplasmic signals in MCF-7 cells; C2: CtG3P, nucleolar signals in MCF-7 cells; C3: NtC3P, cytoplasmic signals in DU-145 cells; C4: CtG3P, nucleolar signals in DU-145 cells; C5: NtC3P, cytoplasmic/nuclear signals in PC-3 cells; C6: CtG3P, nucleolar signals in PC-3 cells; C7: NtC3P, cytoplasmic/nuclear signals in MDA-MB-231 cells; and C8: CtG3P, nucleolar signals in RWPE-1 cells.

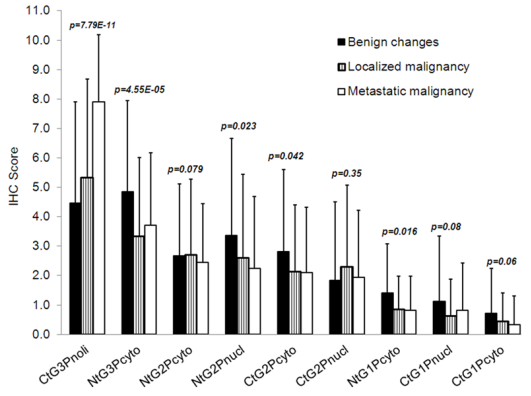


Figure 2. Expression levels (Mean ± SD of IHC score) of N- and C-terminals of representative POTE molecules among the groups with different pathology. CtG3P: C-terminal common to group 3 POTEs; NtG3P: N-terminal common to group 3 POTEs; NtG2P: N-terminal of representative group 2 POTEs; CtG2P: C-terminal of representative group 2 POTEs; NtG1P: N-terminal of group 1 POTE; CtG1P: C-terminal of group 1 POTE; noli: nucleolar; cyto: cytoplasmic; and nucl: nuclear. The *p* values in the figure were the results of ANOVA analysis of expression level of each POTE molecule among benign changes, localized and metastatic malignancies. The *p* value was 5.25E-11 for nucleolar IHC scores of CtG3P among the groups based on confirmation with Kruskal-Wallis test.

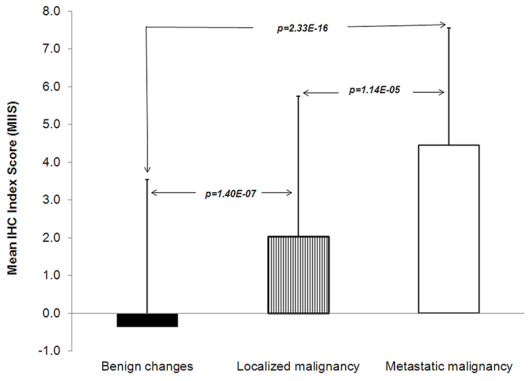


Figure 3. Comparison of MIIS (Mean ± SD of MIIS) for between the groups with different pathology. MIIS: mean IHC index score, calculated by nuclear IHC score subtracted by cytoplasmic IHC score of same POTE molecules. The *p* values in the figure were the results of ANOVA analysis of MIIS for group 3 POTEs among benign changes, localized and metastatic malignancies. Similar *p* values were confirmed with Wilcoxon Rank Sum test.

in the nucleoli with some nuclear staining (**Figure 1A**). The differential localizations of cytoplasmic NtG3P and nucleolar CtG3P were further confirmed *in vitro* by ICC and IFA on cell lines. The ICC showed that the NtG3P was localized in the cytoplasm of all tested cell lines; in contrast, the CtG3P was located in the nucleolus (with some nuclear staining in some cell lines) without any signals in the cytoplasm (**Figure 1B**). The differential subcellular localizations of cytoplasmic NtG3P and restricted nucleolar CtG3P were even clearly seen in IFA tests (**Figure 1C**).

The expression levels (mean IHC scores) of different POTE proteins in different pathology categories are shown in **Figure 2**. Because there was very low expression level or no significant difference between pathology categories for group 1 and 2 POTE proteins, further analysis was only performed on group 3 POTE molecules. Both NtG3P and CtG3P had significant differences between benign changes and malignancies. The cytoplasmic IHC score for NtG3P was highest in benign tissues (4.84 ± 3.10), which was significantly higher than in localized malignancies (3.34 ± 2.67, p = 0.002) and in metastatic malignancies (3.72 ± 2.45, p = 0.015). In contrast, the nucleolar IHC scores for CtG3P was lowest in benign tissues (4.47 ± 3.43), significantly higher in localized malignancies (5.32 ± 3.36, p = 3.63E-02), and highest in metastatic malignancies (7.90 ± 2.29, p =

Table 2. The distribution and positive rate of IHC stain for CtG3P in various organs/tissues and different pathologic categories

Organs/tissues	Benign Changes			Localized Malignancies			Metastatic Malignancies		
	+	Detected	%	+	Detected	%	+	Detected	%
Appendix	2	2	100						
Bladder	2	3	100	7	7	100	5	5	100
Bone				3	4	75			
CNS									
Astrocytoma				3	3	100			
Ganglioma	2	2	100						
Meningioma	0	2	0						
Breast	7	7	100	7	7	100	6	6	100
Cervix	7	7	100	3	3	100			
Colon	6	6	100	7	7	100	7	7	100
Esophagus	2	3	67	3	3	100			
Fallopian Tube	2	2	100						
Gall Bladder	1	1	100						
Kidney	3	3	100	4	6	67	5	5	100
Wilm's Tumor				1	2	50			
Larynx	4	4	100	4	4	100	5	5	100
Liver	2	7	29	4	7	57	4	4	100
Cholangiocarcinoma				2	2	100			
Lung	4	5	80	5	5	100	3	3	100
Lymphoid Tissues	4	4	100	2	2	100			
Spleen	1	4	25						
Muscle									
Cardiac	0	4	0						
Skeletal	0	4	0						
Smooth. Artery	2	3	67						
Smooth, Umbilical vein	2	2	100						
Uterus, leiomyosarcoma				3	3	100			
Ovary	2	4	50	4	4	100	1	1	100
Pancreas	8	8	100	6	6	100	4	4	100
Parotid Gland	5	7	71	2	2	100			
Pituitary Gland, Normal	2	2	100						
Pituitary Gland, Adenoma	3	4	75						
Placenta	2	2	100						
Prostate	2	4	50	7	9	78	4	4	100
Skin	4	4	100	4	4	100	4	4	100
Soft Tissues	4	4	100	7	7	100			
Stomach	7	7	100	6	7	86	6	7	86
Testis	3	4	75	4	4	100			
Thymus	1	1	100	1	2	50			
Thyroid	11	12	92	3	4	75			
Uterus, Endometrium	5	5	100	3	4	75	3	4	75
Total	117	148	79	107	121	88	57	59	97

8.13E-12). The difference in nucleolar CtG3P IHC score was also significant between localized and metastatic malignancies ($p = 1.45E-07$).

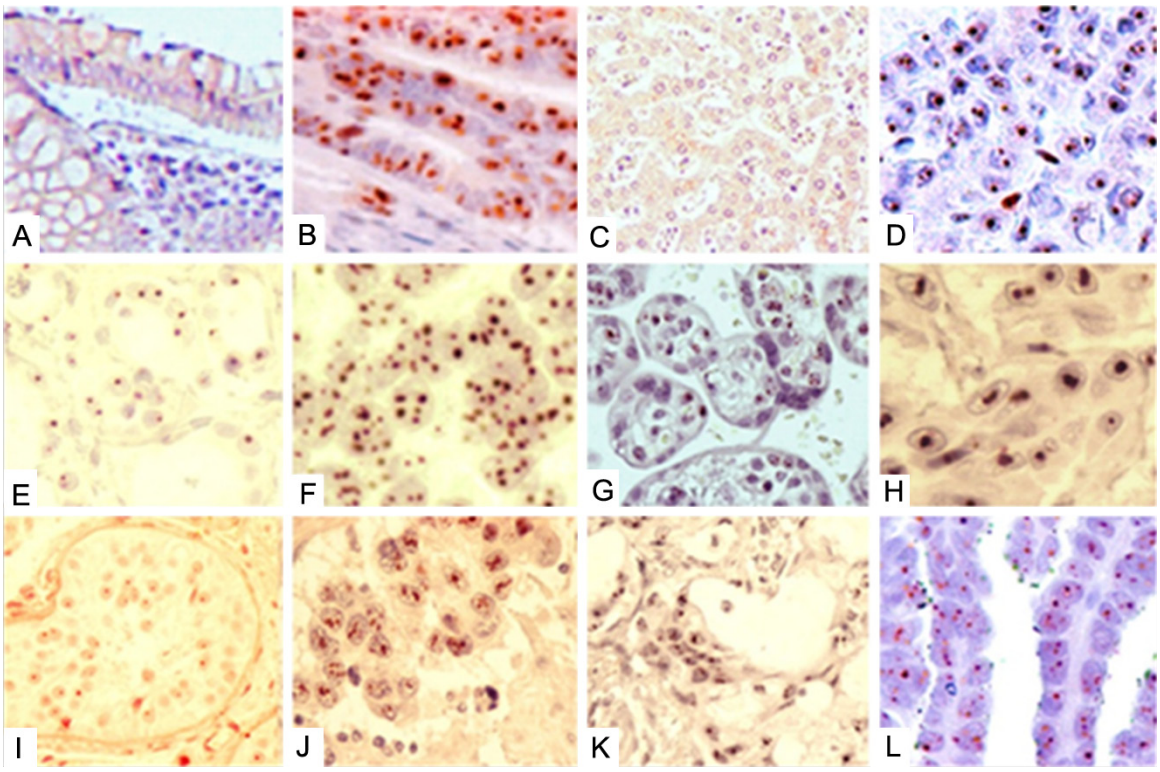


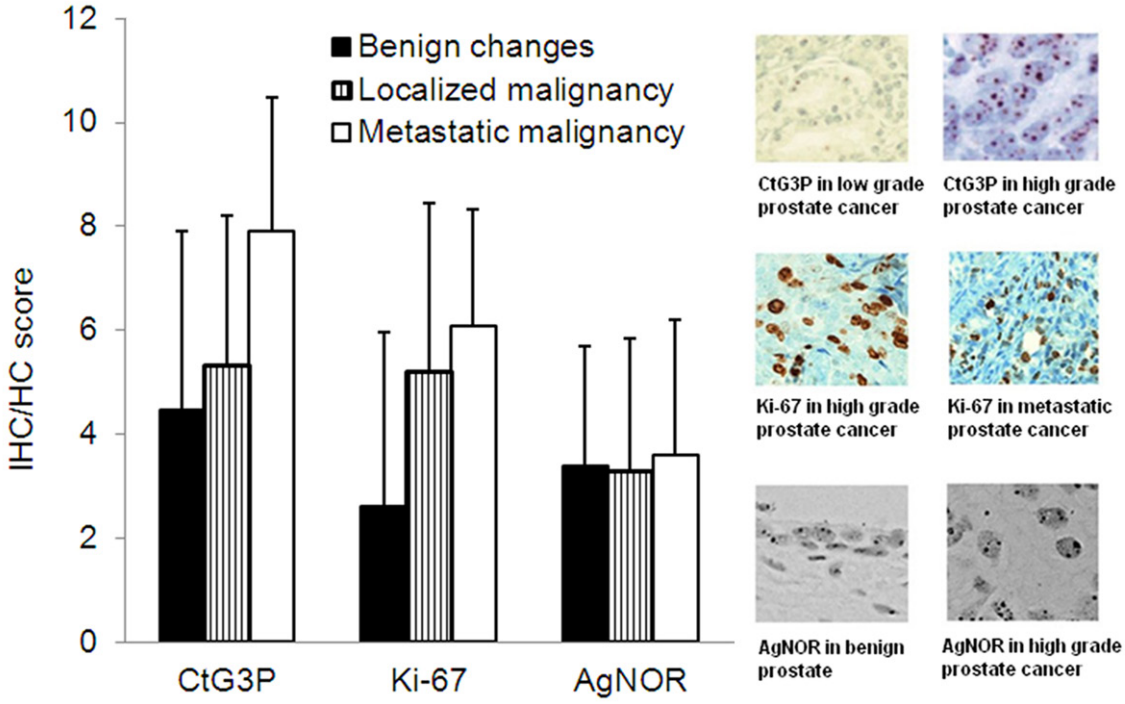
Figure 4. Immunohistochemistry (IHC) for nucleolar CtG3P in representative benign and malignant tissues. A: benign colon adjacent colon cancer, no IHC signal; B: colon cancer, strong nucleolar IHC signals; C: benign liver, no IHC signal; D: hepatocellular carcinoma, strong nucleolar IHC signals; E: prostate cancer with Gleason score of 6, moderate nucleolar IHC signals; F: metastatic prostate cancer, strong nucleolar IHC signals; G: benign placenta, weak nucleolar IHC signals; H: melanoma, strong nucleolar IHC signals; I: benign testis, scattered and weak nucleolar IHC signals; J: seminoma, strong nucleolar IHC signals; K: lung cancer, moderate to strong nucleolar IHC signals; L: ovary serous papillary carcinoma, strong nucleolar IHC signals.

To simultaneously reflect reverse changes of IHC scores for N- and C-terminals of group 3 POTEs, a mean IHC index score system is established. MIIS was calculated and analyzed for each pathology category and correlated with the malignant progression. MIIS was highest in metastatic malignancies (4.45 ± 3.08), which was significantly higher than in localized malignancies (2.04 ± 3.71, p = 1.14E-05) and in benign changes (-0.36 ± 3.89, p = 2.33E-16). The difference in MIIS between localized malignancies and benign changes was also significant (p = 1.40E-07) (**Figure 3**). When compared with single IHC score of either nucleolar CtG3P or cytoplasmic NtG3P, MIIS was a more sensitive method to separate benign changes from malignancies.

Alignment analysis for immunogens for both NtG3P and CtG3P showed that the immunogen for CtG3P has 100% homology to POTE14s and 22, 84% homology to group 2 POTEs, and no homology to group 1 POTE or any other known proteins. Similar results were obtained for the immunogen for NtG3P: 100% homology to POTE14s and 22, 90% homology to group 2 POTEs, and no homology to group 1 POTE and any other known proteins. Because N- and C-terminals of group 1 and 2 POTEs did not show nucleolar staining, it is concluded that CtG3P is the only portion localized in nucleoli among all POTE protein molecules and correlates with malignant progression.

Systemic distribution of CtG3P

As shown in **Table 2**, the IHC signals of CtG3P were widely distributed in a variety of benign and malignant tissues in almost all body tissues/systems except in meningioma and cardiac and skeletal muscles. The positive rate of CtG3P IHC stain was not significantly different among pathologic categories in individual organ/system due to limited number of speci-



Comparison of expression levels of three markers between groups (p value)

	CtG3P	Ki-67	AgNOR
Benign vs. localized malignancy	3.61E-02	1.69E-11	0.79
Benign vs. metastatic malignancy	8.38E-15	3.66E-14	0.74
Localized vs. metastatic malignancy	3.55E-09	0.06	0.61

Figure 5. Comparison of expression levels of IHC score (mean ± SD, for CtG3P and Ki-67) and HC score (mean ± SD, for AgNOR) between benign changes, localized malignancies and metastatic malignancies. The *p* values in the figure were the results of ANOVA analysis. Similar *p* values were confirmed with Wilcoxon Rank Sum test.

mens in each organs/systems; however it was significantly different between total benign changes (79%) and total localized malignancies (88%, p = 0.04, OR = 2.03, and 95% confidential interval was 1.02 - 4.01) and between benign changes and total metastatic malignancies (97%, p = 0.002, OR = 7.55, and 95% confidential interval was 1.75 – 32.66). The nucleolar IHC signals for CtG3P were scattered with weak intensity in benign tissues, some with moderate intensity in localized malignancies, and diffuse with strong intensity in metastatic malignancies independent of metastatic sites. This ascending pattern of CtG3P IHC staining was especially seen in bladder, liver, lung, colon, ovary and prostate. The nucleolar IHC staining in representative tissues are shown in **Figure 4**. Although benign changes had a high positive rate of IHC for CtG3P, lower stain intensity in benign changes made overall expression level of nucleolar CtG3P significantly lower than that in malignancies as described before.

Comparison of CtG3P with Ki-67 and AgNORs in differentiation of benign changes from malignancies, and localized from metastatic malignancies

The ability of CtG3P to separate benign changes from malignancies and localized from metastatic malignancies was compared with Ki-67 and AgNORs. As described earlier, the mean IHC score of CtG3P was significantly different not only between benign changes and malignancies, but also between localized and metastatic malignancies. This is in contrast to Ki-67

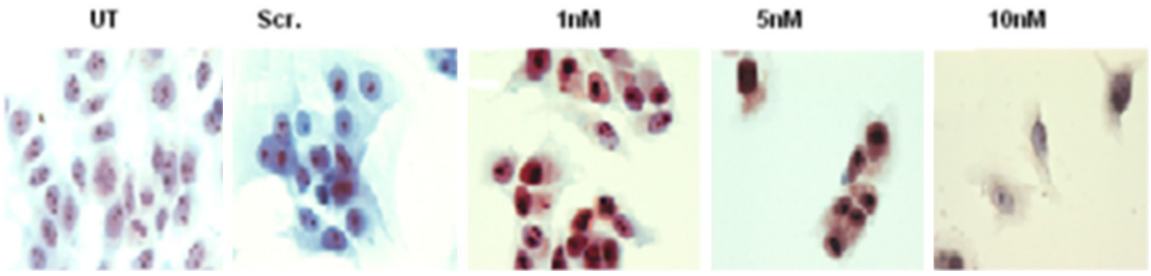


Figure 6. The effect of siRNAs against POTEG or POTEH on the morphology of MCF-7 cells UT: untreated MCF-7 cells, Scr: scramble siRNA; 1 nM, 5 nM and 10 nM: 1 nM, 5 nM and 10 nM of siRNA, respectively, for POTEG or POTEH.

where there was no significant difference in mean IHC scores of Ki-67 between localized and metastatic malignancies ($p = 0.064$). Therefore, compared with Ki-67, the CtG3P has similar ability to separate benign changes from malignancies, and a stronger ability to differentiate localized from metastatic malignancies. CtG3P is superior to AgNOR in separation of benign changes from malignancies and in differentiation of localized from metastatic malignancies under the same scoring system, because there was no significant difference in mean histochemistry score of AgNOR either between benign changes and malignancies, or between localized and metastatic malignancies (**Figure 5**).

Transient knockdown of POTEG and POTEH by siRNAs in vitro

When MCF-7 cells were transfected with siRNAs against POTEG or POTEH, detachment of cultured cells was observed in the wells with 10 nmol of siRNAs in 24 h and in the wells with 5 nmol of siRNAs in 36 h. No difference in detachment of cells was observed between wells treated with 1 nmol of siRNAs and wells with scramble siRNA.

Microscopically, cells treated with scramble siRNAs were looked the same as untreated cells, had sufficient unstained cytoplasm, and had nucleoplasm with clearly stained nucleoli. In cells treated with 1 nM siRNAs, the nucleoli were strongly stained and aggregated, the nucleoplasm was diffusely stained, and the cellular cytoplasm remained visible but unstained. In cells treated with 5 nM siRNAs, the nucleoli were fused together, stained in dark-red and smudged with diffusely stained nucleoplasm, and cellular cytoplasm was rarely seen. The cells treated with 10 nM of siRNAs showed unstained remains of dead cells without clear cellular structures (**Figure 6**).

Western blot was performed on the lysates of MCF-7 cells treated with scramble siRNA, 1 nM and 5 nM siRNAs against POTEG or POTEH. As compared with scramble siRNA, the cells treated with 1 nM siRNAs did not show changes in bands in gel. However, the cells treated with 5 nM siRNAs, a band of approximate 65 Kd was decreased with antibody for the N-terminal common to all group 3 POTEs (NtG3P), and a band of approximate 24 Kd with antibody against CtG3P was decreased as well (Figure not shown). These results suggested that group 3 POTEs link to the growth and survival of MCF-7 breast cancer cells *in vitro*.

Discussion

Previous studies have shown that POTE molecules are localized in the cytoplasm towards the inner aspect of cellular membrane. These results were obtained from RNA analysis (4), or from methods using antibodies only raised from immunogens within N-terminal and ankyrin repeats of POTE molecules [14]. In the current study, we first applied antibodies raised from both N- and C-terminals of all three group POTEs to determine their subcellular localizations. Our results demonstrated that different POTE protein molecules and different portions of the same POTE molecule have distinct subcellular localizations. The CtG3P is the only portion localized in the nucleolus among all POTE molecules, and importantly, it correlated well with malignant progression and metastasis in a variety of tissues/organs. Therefore, discovery of nucleolar localization of CtG3P and its association with malignant progression makes it possible to use CtG3P as a new and true nucleolar marker in diagnosis, prediction and prog-

nosis of various malignancies in future. As a cancer marker, CtG3P could be better than Ki-67, a non-nucleolar marker [15, 16], because it was better than Ki-67 in separation of localized from metastatic malignancies, and superior to AgNORs, one of most studied nucleolus-related markers since the early 1980s [12, 13], in differentiation of benign from malignant changes and localized from metastatic malignancies. It remains controversial whether AgNOR is clinically useful since some studies have indicated that AgNOR correlated with proliferation and progression of cancers [17, 18], while others did not [19, 20]. Actually, AgNORs comprise a set of argyrophilic nucleolar and nuclear acidic proteins including nucleolin and nucleophosmin [21], thus it is hard to determine which individual protein of AgNORs is associated with nucleolar function. In addition, argyrophilic histochemical staining of AgNOR is limited by many physical and chemical conditions [22, 23].

In the current study, the expression levels of NtG3P and CtG3P were almost at the same constitutive level in benign tissues, but were changed inversely in malignancies. MIIS developed in this study might simultaneously reflect dynamic changes in expression level of two ends of one group 3 POTE protein molecule. The value of MIIS in prediction and prognosis of cancer outcome needs further studies.

It is unclear why the NtG3P is decreased in malignancies; however it is possible that degradation of cytoplasmic NtG3P is accelerated after increased cleavage of CtG3P from intact group 3 POTE molecules in cancerous status. Also it is not clear how the CtG3P gets shifted to the nucleolus. Perhaps, the CtG3P, once cleaved in the cytoplasm, is rapidly translocated and accumulated in the nucleoli of tumor cells through a mechanism that other ankyrin repeat-containing molecules use. For example, after a second cleavage in cytoplasm, the C-terminal domain of Notch1, another ankyrin repeats-containing molecule, is rapidly translocated (as fast as a few minutes) to the nucleus through CBF1-dependent signal transduction cascade [24].

rRNA is usually overexpressed in the nucleoli of cancer cells [25], resulting in increased size and number of nucleoli. In parallel, the expression level of nucleolar CtG3P was increased in various tumors in this study. Interestingly, a whole set of 5 chromosomes (13, 14, 15, 21 and 22) dispersed with rRNA genes [26] are also occupied by 5 of the 8 POTE genes [4]. The genes of rRNA and POTEs are closely located at centromeric regions of all 5 chromosomes (rRNA genes at p12 and POTE genes at q11.2). This evidence implies that nucleolar translocation of CtG3P is associated with rRNA activities perhaps, through influencing rRNA biosynthesis, or other rRNA function similar to Ki-67 and nucleolin [27, 28]. This could be the mechanism through which CtG3P participates in malignant progression and metastasis. In addition to influencing rRNA, POTEs may have more pathophysiological functions because different portions of the same POTE molecule and different POTE molecules have their distinct subcellular localizations, from membrane, cytoplasm, to nucleus and nucleolus.

Transient knockdown of POTEG or POTEH by their corresponding siRNAs altered MCF-7 breast cancer cells morphologically and functionally *in vitro* in a dose-dependent manner. With smaller dose siRNAs, cancer cells showed morphological changes including diminished cytoplasm and aggregated nucleoli; however, cellular protein levels of POTEG or POTEH were not obviously decreased. With larger dose siRNAs, cancer cells showed no cytoplasm, smudged nuclei-nucleoli, and dead cell remnants, and the level of intact and truncated molecules of POTEG and POTEH were decreased. These results demonstrated that POTEG and/or POTEH are pivotal for cancer cells to grow and survive. In addition, these results suggest that group 3 POTEs could be a new therapeutic target in the treatment of cancer. This is especially true for CtG3P, because it is not a nucleolar resident; instead, it is translocated from cytoplasm into nucleoli in corresponding to malignant status. This provides multiple targets for cancer therapy, either by inhibiting expression of entire group 3 POTEs or by blocking the nucleolar translocation of CtG3P.

The limitations of this study include inadequacies of studied subjects in each type of tissue/organ and lack of enough functional studies for CtG3P. Thus, the conclusion that CtG3P correlates with malignant progression and metastasis needs to be confirmed in further studies

with larger study cohorts for individually given tumor. Aside from the limitations, CtG3P is a newly discovered nucleolar marker with potential use in diagnosis, prognosis, prediction, and therapy of pan-malignancies.

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