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RTN3 (Reticulon 3) as a Novel Prostate Cancer-Specific Biomarker derived from Exosomes

Ye-Eun Kim[1], Anh-Thu Nguyen[2], Jin woo Min[3], Mi Jung Kim[3], Ki-Young Kim[2*], Hee cheol Kang[3]

[1]Department of Oriental Medicine Biotechnology, Kyung Hee University, Republic of Korea
[2]Department of Biotechnology, Kyung Hee University, Republic of Korea
[3]GFC Life Science Co. LTD, Republic of Korea

ABSTRACT

Objective: Prostate cancer accounts for a large proportion of male cancer deaths worldwide.

Commonly used prostate-specific antigens (PSA) lead to over diagnosis, so novel prostate cancer diagnostic markers are needed. Several substances derived from exosomes affect the progression of cancer. Therefore, it is possible to predict the cancer progression through exosome derived markers.

Design: In order to find a candidate protein that can be used as a biomarker among the exosomal protein coding 7145 genes, screening was performed using qRT-PCR. The genes selected through screening were subjected to western blot to confirm protein expression in cells and protein expression in cancer cell derived exosomes. Afterwards, the function of the corresponding exosomal protein was confirmed through Invasion Assay.

Result: As a result of screening of the expression level of RNAs in various cancer cell lines, RTN3 showed a high expression level in the prostate cancer cell lines but not in the normal prostate cell line, RWPE-1. RTN3 was also highly expressed in prostate cancer exosomes except for the normal cell line.

To verify the function of exosomal RTN3, a cell line (Malme-3M) with low RTN3 expression was treated with exosomes extracted with high RTN3 expression and showed increased metastasis.

Conclusion: RTN3 can be used as a diagnostic marker for prostate cancer because of the high expression level of RTN3 in prostate cancer derived exosomes compared with normal prostate cell derived exosome. Exosomal RTN3 is also important for cancer metastasis.

Keywords: Prostate cancer; Diagnosis; Prognosis; Biomarker; Exosome

ABBREVIATIONS

Prostate cancer (PCa)

Prostate-specific antigen (PSA)

Reticulon-3 (RTN3)

INTRODUCTION

Prostate cancer (PCa) is one of the most common cancers and the leading cause of cancer death in men, occurring mainly in men over the age of 50 [1]. PCa is one of the solid tumours [2]. Most prostate cancers could be diagnosed with Prostate specific antigen (PSA). However, PSA has low specificity. It can only diagnose the LNCaP clone FGC among prostate cancer cell lines [3]. Overgrowth conditions can also be associated with an increasing PSA level [2]. Elevated serum PSA levels are often detected in patients with non-malignant conditions such as benign prostatic hyperplasia, resulting in an over diagnosis of

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Corresponding author Ki-Young Kim, Department of Biotechnology, Kyung Hee University, Republic of Korea, E-mail: kiyoung@khu.ac.kr

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prostate cancer [4]. However, the use of PSA has been shown to reduce the prevalence of advanced PCa and reduce PCa-related mortality by 20% [5,6]. Based on that, a new PCa biomarker is needed to diagnose prostate cancers including DU145 and PC3 to increase the specificity of prostate cancer and to reduce the over diagnosis. Recently, a lot of research on extracellular vesicles has been conducted. Extracellular vesicles are secreted by prokaryotic and eukaryotic cells, and play an important role in transferring proteins, nucleic acids, and enzymes between cells. Exosome is one type of extracellular vesicles originating from endosomes and is released in various forms with 30-150 nm in size. Various amounts of exosome can be generated depending on the advanced stage of cancer and also transferred from primary tumors to others. Primary tumor delivered exosomes affect cancer metastasis and invasion [7]. Cancer cell released exosomes can also affect tumor initiation, growth, progression and drug resistance [8]. Duijvesz et al. [9] showed that exosomal biomarkers such as CD63 and CD9 were more prevalent in PCa patients after digital rectal examination. In intercellular signaling, exosomes play a role in carcinogenesis. Tumor-derived exosomes exchange information of other cell types, and create favorable conditions for oncogenetic growth and invasion. Exosomes stimulate target cells in a variety of ways, including interactions with specific membrane receptors, horizontal transfer of protein and RNA species, and endocytosis [10]. However, there are very few studies on exosome derived markers to diagnose PCa and the function of exosomal proteins, so it is necessary to find exosome derived proteins. RTN3 is a member of the reticulon RTN family which is mainly located in the endoplasmic reticulum membrane. It is involved in membrane trafficking at the early secretory pathway, and inhibits BACE1 activity and amyloid precursor protein processing. RTN3 induces caspase-8 cascade and apoptosis [11], and the endoplasmic reticulum tubules' formation [12,13]. And RTN3 regulate the expression level of CXCR4 and that effect to regulate cancer metastasis [14].

METHODS AND MATERIALS

Quantitative Real-Time PCR

The experiment was performed with the same way as before we did [15]. Cells were seeded in 6 wells and incubated for 24 hours. After removing the medium, 500 ul of Trizol (Life Technology, Thermo Fisher Scientific, USA) was immediately treated and cDNA was synthesized using 1 ug of total RNA. Real-time PCR was performed using cDNA as a template, and QGreen 2x SybrGreen qPCR Master Mix (CellSafe, Korea). Primer sequence is listed in **Table 1 and 2**. GAPDH was used as a quantitative control. The amount of gene expression was calculated as the fold change value and expressed as delta-Cq.

Table 1: The primer list used for quantitative real-time PCR.

Gene	5'-3'	Primer sequence	Annealing temp. (C°)	Reference
RTN3	Forward	TAC CTG TAG CTT GAA AGG GG	54.9	In this study
	Reverse	GGA AGA TAG GAT TTG AGG GC	53.6	
RTN3L	Forward	TAC CCG ATA CGA ATG TCT CC	55	
	Reverse	TAA AGG CTG CTA TCA GGT CC	54.4	
CXCR4	Forward	AAA TCT TCC TGC CCA CCATC	56.1	
	Reverse	AAA GTA CCA GTT TGC CAC GG	56.6	
CXCR4-1	Forward	GGA GTT AGC CAA GAT GTG AC	53.9	
	Reverse	AAT CCT ACA ACT CTC CTC CC	54.2	
CXCR4-234	Forward	AGT TTG TTG GCT GCG GCA G	60.8	
	Reverse	ACT GAT CCC CTC CAT GGT AA	56.1	
GAPDH	Forward	GTG AAG GTC GGA GTC AAC G	57.1	[15]
	Reverse	TGA GGT CAA TGA AGG GGT C	55.3	

Table 2: The primer list used for PCR.

Gene	5'-3'	Primer sequence	Annealing temp. (C°)	Reference
CXCR4	Forward	ATG GAG GGG ATC AGT ATA TA	49	[14]
	Reverse	TTA GCT GGA GTG AAA ACT TG	50.4	

Exosome Purification and Western Blot Analysis

Exosome was isolated using Total Exosome Isolate (*Invitrogen*™,#4478359) by following manufacturer protocol [16]. For quantitative comparison of the amount of exosome production by cancer cell type, the amount used for western blot analysis was adjusted to the amount of media used for exosome isolation. Add 10 ml of serum free medium and incubate for 24 hours. After centrifugation at 2000 g for 30 minutes, 8 ml was transferred and followed the protocol of Total Exosome Isolate. The purified exosomes were kept in -78 degree before use. Equal amounts (20 µg) of whole-cell lysate proteins were separated using an 8, 10, and 12% acrylamide SDS-PAGE gel. For the cell pellet, cells at the time of exosome samples were used. Primary antibody (RTN-3 (#sc-374599, Santa cruz)), and secondary antibody (2nd anti-mouse (#ab6728, Abcam)) were used in this study.

Invasion Assay

This experiment was performed by slightly modifying the transmembrane assay [17]. 1.5 ml of cells added to the upper chamber with serum free medium. After 24 hours, the exosome added in the upper chamber, and the medium containing FBS is placed on the plate. 24 hours later, wash twice with PBS and scrapping to remove the remaining cells. Stain with 0.5% crystal violet and observe under a microscope.

Polymerase Chain Reaction (PCR)

Cells to prep mRNA were seeded in the same way as Invasion assay. RNA was reverse transcribed into cDNA in the same manner as cDNA used in quantitative real-time PCR. PCR was performed using Excel Speed-Pfu polymerase (Inclone™, #IN5015-0250) using cDNA as a template. All methods followed the protocol of Excel Speed-Pfu polymerase [18].

Statistics

The experiments were repeated at least three times independently. Results are expressed with ± standard deviation. Using a one-way ANOVA, statistically significant differences were analysed. When only two groups were compared, the student's t-test was used (*: P<0.05, **: P<0.01).

RESULTS

General Schemes for Candidate Identification

Exosomal protein coding 7145 genes detected in exosomes of prostate, lung, kidney, breast and colorectal cancers were extracted from Extracellular vesicles database; Vesiclepedia (http://microvesicles.org/).

Among them, highly detected 168 genes in cancer cells were prepared for PCR. The expression level of 90 genes was tested by qRT-PCR and 87 genes were screened, but 3 sets of primers did not work. Among the 87 genes, 13 candidates showed specific expression in the prostate cancer cell line with 10 of them which had already been reported (TMPRSS2 etc.) (**Figure 1**).

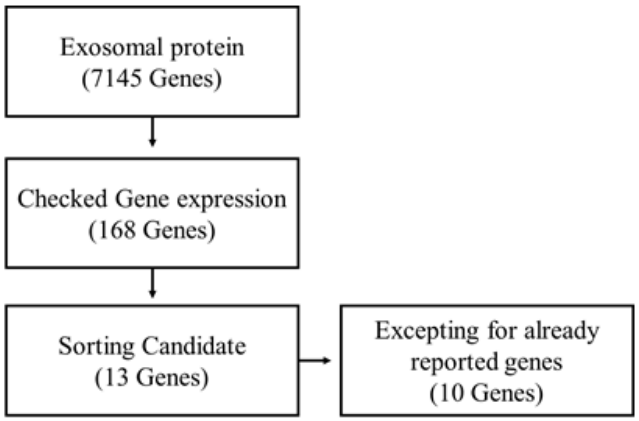

Figure 1: Exosomal protein coding 7145 genes showed specific expression in the prostate cancer cell lines.

RTN3 is Specific Cancer Marker in Prostate Cell Line

The expression level of RTN3 was checked in prostate cell lines using qRT-PCR. GAPDH was used as a reference for RTN3 and RTN3L. The expression of RTN3 showed approximately 6 to 8 times higher than that of the normal cell line, RWPE-1. RWPE-1, a normal prostate cell line, exhibited a ΔCq value of 0.037. In contrast, the prostate cancer cell lines DU145, PC3, and LNCaP clone FGC expressed ΔCq values of 0.168, 0.247, and 0.226, respectively. HaCaT, a normal keratinocyte cell line, also showed a relatively low value of ΔCq with 0.096 (**Figure 2**).

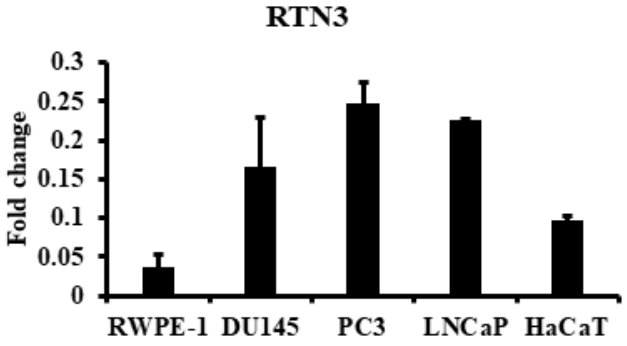

Figure 2: RTN3 highly expression in prostate cancer cell line in mRNA levels, Expression of RTN3 mRNA is higher in prostate cancer cells. RNA was extracted from the prostate-related cell line and the keratinocyte cell line, HaCaT cell, to determine the expression level of RTN3. GAPDH was used as a control to indicate the amount of fold change.

A similar tendency was also found in RTN3L, a long isoform of RTN3 [19]. The prostate cancer cell lines DU145, PC3, and LNCaP clone FGC showed relatively high values of 0.004, 0.005, and 0.01, respectively, of ΔCq, but RWPE-1 and HaCaT showed 0.002 and 0.003, respectively, of ΔCq (Figure 3).

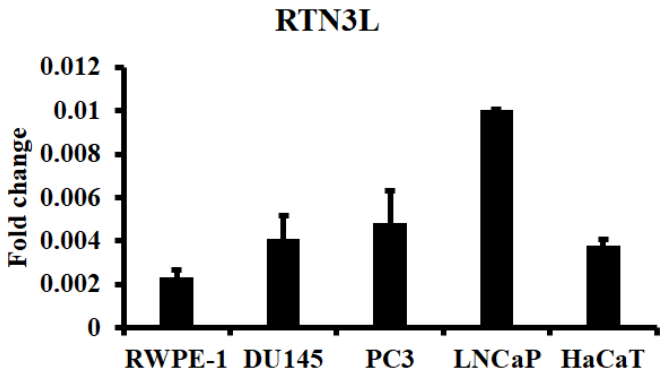

Figure 3: Expression of RTN3 variant mRNA is higher in prostate cancer cells. RNA was extracted from the prostate-related cell line and the keratinocyte cell line, HaCaT cell, to determine the expression level of RTN3L, variant of RTN3, with the same way as A.

The exosomal expression level of RTN3 showed the similar expression pattern with mRNA expression. Prostate cancer cell lines DU145, PC3, and LNCaP clone FGC showed 4 times, 7 times, and 10 times higher expression, respectively, than RWPE-1, a normal prostate cancer cell line (Figure 4).

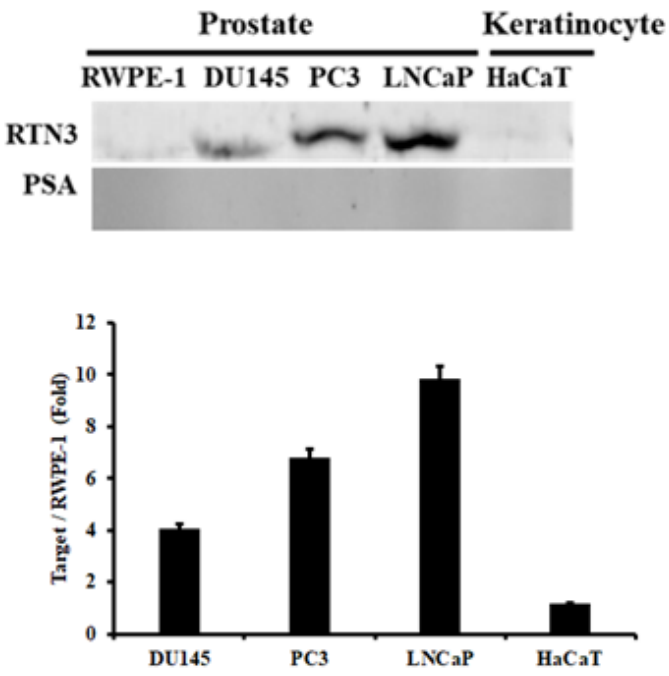

Figure 4: Exosomal RTN3 is only detected in prostate cancer cell lines Exosomal RTN3 showed high expression in cancer cells. All of the cells grew until 60% of the cell culture dish and changed with serum free media. After 24h, the media were centrifuged. Exosomes were extracted from each media and the exosome-derived RTN3 expression were checked with western blotting. Graphical view of exosomal RTN3 expression in cancer cells. The band density of western blot result was calculated with image J. RWPE-1 is prostate normal cell line, so we used that as control and calculate fold change.

Cancer Metastasis Induced by Exosomal RTN3

As a result of screening, RTN3 was hardly expressed in exosomes of Malme-3M. It was tested whether PC3 exosomes containing a large amount of RTN3 could improve the metastasis of Malme-3M with a low RTN3 expression level. The number of metastasized cells in Malme-3M treated with 200 and 400 ul of PC3 exosomes dose dependently increased by 42% and 59%, respectively (Figure 5 and 6).

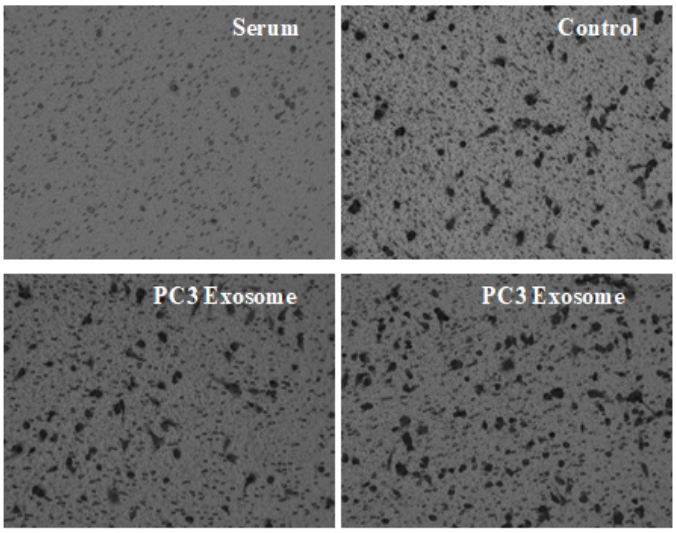

Figure 5: Exosomal RTN3 induced cancer cell invasion, Exosomal RTN3 induced the invasion of Malme-3M cells. Malme-3M is used to check that RTN3 can induce the migration of cancer cells. Malme-3M cell was seeded with serum free media in upper chamber. 24h later, the PC3 exosome was treated and media was added with serum in down chamber. Wash twice with 1X PBS, fix with methanol. After fixation.

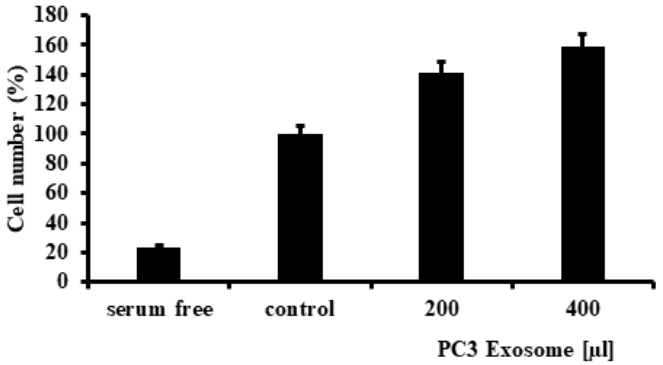

Figure 6: Graphical view of induction of cancer cell invasion by exosomal RTN3 expression. The graph showed representative number of cells that invade to lower chamber.

CXCR4 Regulated by RTN3

As a result of PCR and qRT-PCR, only the specific isoform of CXCR4 (CXCR-234) mRNA showed a tendency to increase maximally by RTN3 when 50 μl of PC3 exosome was treated. The same tendency is also seen when the entire CXCR4 is confirmed by PCR. In addition, the expression of CXCR4 mRNA was not induced when cells treated with HaCaT exosomes containing low

amount of RTN3 (Figures 7-8).

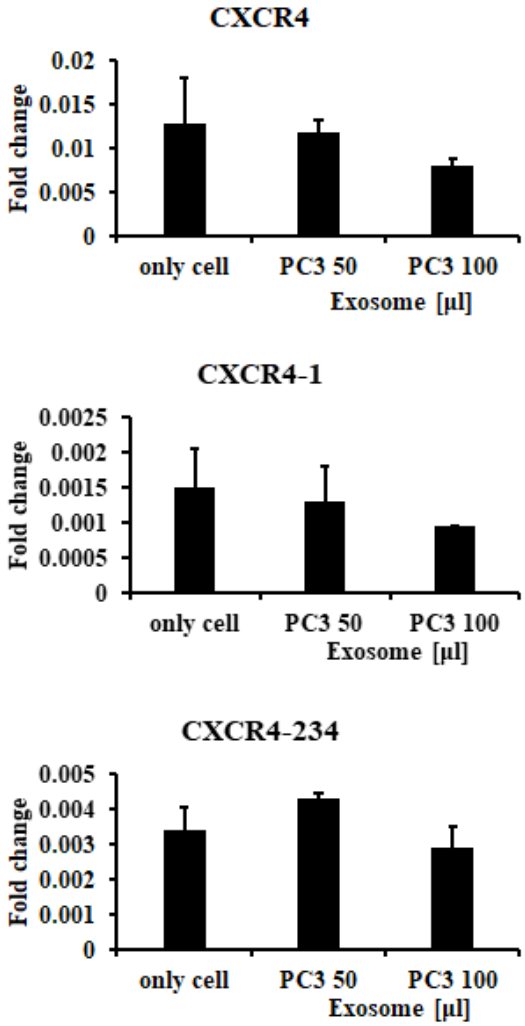

Figure 7: RTN3 changed the expression of CXCR4, Exosomal RTN3 influenced the CXCR4 mRNA expression. RNA was extracted from Malme-3M which contains PC3 exosome (50ul, 100ul) and used for cDNA synthesis. One primer set for whole mRNA and 2 primer sets for isoform were used. CXCR4 expression were checked using quantitative real-time PCR with GAPDH as a control.

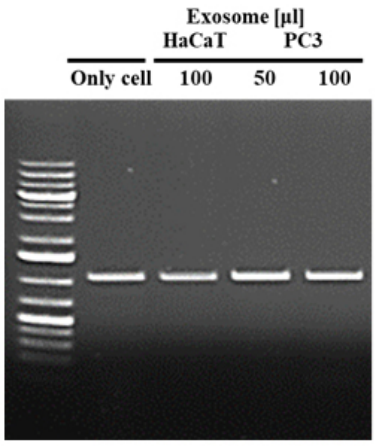

Figure 8: Exosomal RTN3 induced the CXCR4 mRNA expression. mRNA was extracted with same way and used for cDNA synthesis. CXCR4 DNA (1059bp) was amplified with Excel Speed-Pfu polymerase. PCR product was separated with 1% agarose gel with 1kB Plus ladder (Thermo Fisher Scientific, #SM1331). Images were obtained with Gel Doc™ EZ Imager.

DISCUSSION

PSA has been used as a major diagnostic marker for prostate cancers but it has a limitation as a biomarker that it can be detected only in a subset of cancer cells including LNCaP clone FGC. In addition, PSA is overexpressed depending on the patient's condition, making an accurate diagnosis difficult. [3] However, RTN3 mRNA and exosomal RTN3 were highly expressed in the DU145, PC3, and LNCaP clone FGCs used in the experiments as prostate cancer cell lines, except for HaCaT and RWPE-1. The expression level of exosomal RTN3 was high in prostate cancer cell lines but not in normal cell lines. Therefore, exosomal RTN3 should be a good diagnostic marker candidate for prostate cancer. Prostate cancer can easily metastasize to lymph nodes and bones. When metastases to the lymph nodes and bones occur, the 5 year survival rate drops to 30%-50%. Therefore, it is necessary to pay attention to the metastasis of prostate cancer [20]. Migration assay was performed using a trans-well. Malme-3M, a cell line with a low RTN3 expression level in exosomes, was used as a control, and how much it can metastasize when PC3 exosomes are treated was evaluated. Malme-3M treated with PC3 exosomes had more metastasis than untreated cells. Therefore, it could be suggested that exosome derived RTN3 affects prostate cancer metastasis.

CONCLUSION

This result suggests that inhibition of RTN3 expression or the function of RTN3 can prevent cancer metastasis. RTN3 is known to regulate cancer metastasis by regulating the expression level of CXCR4. Only certain parts of the isoform of CXCR4 mRNA appear to be affected by exosomal RTN3. Therefore, it can be expected that other factors will also be affected by RTN3 to influence metastasis of cancer. Inhibitors that modulate CXCR4 or directly inhibit RTN3 appear to be able to minimize prostate cancer metastasis. However, this requires further studies, including *in vivo* mouse assays.

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CONFLICT OF INTEREST

The authors have no conflict of interest to report.

ETHICAL APPROVAL

Not required.

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