RESEARCH ARTICLE

Roles of MicroRNA-21 and MicroRNA-29a in Regulating Cell Adhesion Related Genes in Bone Metastasis Secondary to Prostate Cancer

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Abstract

Background: There is an increasing concern in the role of microRNA (miRNA) in the pathogenesis of bone metastasis (BM) secondary to prostate cancer (CaP). In this exploratory study, we hypothesized that the expression of vinculin (*VCL*) and chemokine X3C ligand 1 (*CX3CL1*) might be down-regulated in clinical samples, most likely due to the post-transcriptional modification by microRNAs. Targeted genes would be up-regulated upon transfection of the bone metastatic prostate cancer cell line, PC3, with specific microRNA inhibitors. Materials and Methods: MicroRNA software predicted that miR-21 targets *VCL* while miR-29a targets *CX3CL1*. Twenty benign prostatic hyperplasia (BPH) and 16 high grade CaP formalin-fixed paraffin embedded (FFPE) specimens were analysed. From the bone scan results, high grade CaP samples were further classified into CaP with no BM and CaP with BM. Transient transfection with respective microRNA inhibitors was done in both RWPE-1 (normal) and PC3 cell lines. QPCR was performed in all FFPE samples and transfected cell lines to measure *VCL* and *CX3CL1* levels. Results: QPCR confirmed that *VCL* messenger RNA (mRNA) was significantly down-regulated while *CX3CL1* was up-regulated in all FFPE specimens. Transient transfection with microRNA inhibitors in PC3 cells followed by qPCR of the targeted genes showed that *VCL* mRNA was significantly up-regulated while *CX3CL1* mRNA was significantly down-regulated compared to the RWPE-1 case. Conclusions: The down-regulation of *VCL* in FFPE specimens is most likely regulated by miR-21 based on the *in vitro* evidence but the exact mechanism of how miR-21 can regulate *VCL* is unclear. Up-regulated in CaP, *CX3CL1* was found not regulated by miR-29a. More microRNA screening is required to understand the regulation of this chemokine in CaP with bone metastasis. Understanding miRNA-mRNA interactions may provide additional knowledge for individualized study of cancers.

Keywords: Prostate cancer - bone metastasis - miR-21 - miR-29a - *VCL* - *CX3CL1*

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Introduction

Prostate cancer (CaP) is the second most common diagnosed cancer and the sixth leading cause of cancer deaths in men worldwide (Torre et al., 2015). It is a complex disease with an unpredictable nature and when metastasis occurs, the survival is only 32% (Jemal et al., 2010). Due to its indolent nature, patients are usually diagnosed late and when metastasis has occurred (Hong et al., 2010; Ooi et al., 2012). The aberrant expression of microRNA (miRNA) has implicated in CaP development and progression leading to a review of its role in CaP personalized medicine (Coppola et al., 2010; Gandellini et al., 2010; Liu et al., 2012). MicroRNA as an endogenous small RNA (~22 nts) has both the oncogenic (oncomiR) as well as tumor suppressor (onco-suppressor) properties. The single strand part (especially the "seed" region) of miRNA along with a protein complex, namely RNA induced silencing complex (RISC) will determine the target site of the complementary base pairing (Lewis et al., 2005; Pratt et al., 2009). Human miRNA can repress gene expression by targeting the 3' untranslated region of its targeted mRNA at the post-transcriptional level and translational repression (Fang et al., 2011; Thomson et al., 2011). Their roles as metastamiR in CaP metastasis also had been reported (Watahiki et al., 2011; Coarfa et al., 2015).

One of the potential microRNA to be studied is miR-

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21. Being an oncomiR in various cancers as well as in CaP, publishing studies had shown that miR-21 has the capability to regulate numerous tumor suppressor genes including those involved in CaP metastasis (Li et al., 2009; Volinia et al., 2010; Buscaglia et al., 2011; Schramedei et al., 2011; Mishra et al., 2014). One of the predicted targets of miR-21 is vinculin (*VCL*). Vinculin is a tumor suppressor in CaP where its presence was found could inhibit the cell motility (Rodriguez et al., 1992; Zhu et al., 2010). Through its link to actin cytoskeleton, it is one of the important genes in regulating the migration, invasion and metastatic process (Ezzell et al., 1997; Alenghat et al., 2000; DeMali et al., 2003; Li et al., 2014). It also acted as the cell mechanosensor and mechanotransducer enhancer (LeDuc et al., 2010). However, the roles of *VCL* were postulated to be dependent on the cellulo situation and its environment (Mierke et al., 2009).

Another potential microRNA is miR-29a which was found to play dual roles either as oncomiR (indolent stage) or tumor suppressor (aggressive stage) in chronic lymphocytic leukaemia (Pekarsky et al., 2006; Pekarsky et al., 2009). Its role in bone microenvironment through osteoblast and osteonectin is mediated through Wnt signaling pathway (Kapinas et al., 2009; Kapinas et al., 2010). *CX3CL1* as its targeted gene was shown to have a potential role in CaP bone tropism in human prostate cancer and bone cell lines by activating PI3K/Akt survival pathway (Jamieson et al., 2008; Koizumi et al., 2009). In the bone, *CX3CL1* is expressed by osteoblasts and plays a role in osteoclast differentiation and bone resorption (Shulby et al., 2004).

In silico analysis reported that *CX3CL1* and *VCL* were found to be among the top most down-regulated genes in CaP development and related with bone metastasis. They were categorized as the cell adhesion associated genes under the functional annotation group (Gorlov et al., 2010). Since the PI3K/Akt pathway was implicated in CaP development and metastasis (Shukla et al., 2007; Sarker et al., 2009), *CX3CL1* and *VCL* were found affected through this pathway (Jiang et al., 2004; Shulby et al., 2004). The aim of this study was to determine the regulation of these two selected genes by the predicted miRNA using both clinical samples as well as the in vitro model.

Materials and Methods

Gene and microRNA selection

In order to identify the most probable targeted genes that are regulated by selected miRNA, *in silico* prediction of the microRNA targeted genes was performed using the PicTar, TargetScan and miRanda. Human microRNAs that are displayed in the interspecies conservation were identified and were analyzed by performing Venn diagram. *CX3CL1* was predicted to be regulated by miR-29a while *VCL* was predicted to be regulated by miR-21 (Figure 1a,b).

Clinical Samples

This study was approved by the Ethics Committee of the Universiti Kebangsaan Malaysia and registered at the National Medical Research Registry (NMRR). Thirty-six FFPE samples, which were anonymized, were taken from the Histopathology Unit, Pathology Department, Kuala Lumpur Hospital (HKL), Malaysia. These 36 samples were from the transurethral resection of prostatectomy (TURP) in patients who had lower urinary tract symptoms or acute urinary tract obstruction due to enlarged prostate glands. There were 20 samples from benign prostatic hyperplasia (BPH), which were regarded as non-cancerous tissues and 16 high grade CaP samples with Gleason scores between 8 to 10 and Gleason pattern 3 to 5 (Figure 2a-c). From the bone scan results, the high grade CaP samples were further classified into CaP with no BM (10 samples) and CaP with BM (6 samples). BPH samples were taken from January 2011 till March 2012 while the high grade CaP samples were from January 2006 till March 2012. BPH patients with no other medical or surgical history except hypertension, diabetes mellitus and dyslipidemia were chosen. For the CaP samples, the selection criteria were: 1) Gleason score of 8-10 and confirmed by the histopathological report, 2) undergone TURP procedure for the enlarged prostate only, 3) no medical/surgical illness other than hypertension, diabetes mellitus and hyperlipidaemia, 4) no other types of malignancies present, 5) no distant metastasis present other than to the bone, 6) presence of bone metastasis confirmed by the radiologist via bone scan result. Only FFPE samples with >80% cancerous tissues as confirmed by the histopathologist were taken for the study (Figure 3).

Cell cultures and transfection

The human PC3 (bone metastatic androgen independent CaP) and RWPE-1 (normal prostate epithelial) cell lines were obtained from the American Type Culture Collection (ATCC, Manassos, USA). PC3 cells were cultured in F-12K medium (Invitrogen, Carlsbad, CA) and 10% fetal bovine serum (FBS). RWPE-1 cells were grown in Keratinocyte Serum Free Media (K-SFM) supplemented with 0.05 mg/ml bovine pituitary extract (BPE) and 5ng/ml epidermal growth factor (EGF) (Invitrogen, Carlsbad, CA). Both cell lines were grown in the 37ºC incubator under humidified 5% CO_2 atmosphere. Transient transfection was done using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) along with anti-miR21 locked nucleic acid (LNA), anti-miR29a LNA and control A LNA as a negative control (Exiqon, Vedbaek, Denmark). Cells were seeded on 24-well plates for 24 hours prior to transfection. All treatments were tested in biological duplicates. Transfection was first optimized with anti-miRs and negative control. The final concentration of 300nM and 200nM for anti-miR21 LNA and anti-miR29a LNA respectively were used in PC3. While for anti-miR21 LNA and anti-miR29a LNA, the final concentrations used were 200nM and 100nM respectively in RWPE-1. The negative control was co-transfected with the anti-miRs. All treatments were incubated for 48 hours. QPCR data from PC3 were normalized to the data from the RWPE-1 using the comparative method as described in the previous studies (Livak et al., 2001; Chum et al., 2012).

RNA extraction

Total RNA was extracted from the FFPE specimens

using the deparaffinization solution and FFPE miRNeasy kit (Qiagen, Hilden, Germany). For the cell lines, the total RNA was extracted using QIAzol lysis reagent and miRNeasy Mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA purity and concentration were determined by using Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). RNA integrity was determined using Agilent 2100 Bioanalyzer (Santa Clara, CA, USA) with RNA 6000 Nano Labchip kit. Only RNA purity and integrity within 1.80 to 2.10 and 1.80 to 2.60 respectively were used. The extracted RNA was kept at -80°C until further use.

Measurement of mRNA of targeted genes using quantitative real-time polymerase chain reaction

Approximately 500ng/μL of total RNA from the FFPE specimens and 20-100ng/μL from the transfected samples were used. cDNA synthesis was performed using the Verso cDNA synthesis kit (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol. A mixture of random hexamers and oligo-dT primers in a ratio of 3 to 1 were used in a total volume of 20μL. Cycling conditions included 42°C for 30 minutes and an inactivation step at 95°C for 2 minutes. In transfected samples, cDNA was further diluted to the standardized amount before being used for qPCR. The concentration of cDNA input for the qPCR was about 600ng/μL for the transfected cells and approximately 4000 to 5000ng/μL for the FFPE samples. The amount of Thermo Scientific Solaris qPCR ROX Gene Expression Master Mix (Thermo Scientific, Waltham, MA) used was modified with an additional of 6.25μL for the FFPE samples (instead of recommended volume of 12.5μL for fresh samples) in a 25μL final reaction volume. The Human qPCR Gene Expression Assays of *CX3CL1*, *VCL* and GAPDH (the reference gene) were purchased from Thermo Scientific and the primer-probe sequences were given (Table 1). All qPCR reactions were performed using the Rotor-Gene (Corbett Life Science, AUS) in duplicates with the following parameters: One cycle at 95°C for 15 minutes, 95°C for 15 seconds followed by 60°C for 1 minute for 40 cycles. For the FFPE samples, the final step was run for 45 cycles. Non-template control (NTC) was added in each qPCR run of the samples to ensure there was no contamination. Analysis of relative gene expression was done by $2^{-\Delta\Delta}$Cq method [38-39]. All the samples were normalized to BPH.

Statistical analysis

SPSS 13.0 software for Windows (SPSS Inc, USA) was used for statistical analysis. Kruskal Wallis (KW) or Mann-Whitney test (MW) was used accordingly for comparison. Independent Student T-test was performed for statistical analysis of transfection experiments and $p < 0.05$ was considered to be statistically significant in all tests.

Results

Descriptive data in clinical samples

A total of 36 FFPE samples were obtained according to the selection criteria. For the 20 BPH patients, four patients were not on BPH medical treatment and one patient had no record on total PSA. For CaP with no BM, four out of ten patients were already on hormone ablation therapy while six of them had no records on CaP treatment. One of those ten patients had no PSA level recorded. For CaP with BM, four out of six patients presented as incidental findings, one patient was already on radiotherapy and chemotherapy and another one patient had no record of treatment. One sample had no record on total PSA upon the TURP.

Table 2 shows the descriptive data of the selected patients. The mean age for the high grade CaP patients was 70.9 ± 6.4 years while for CaP with no BM and CaP with BM were 72.3 ± 5.9 and 68.5 ± 7.1 respectively. Kruskal-Wallis test showed significant differences for the total PSA ($p < 0.0001$) between all the groups. However, there was no difference in terms of age between the groups ($p > 0.05$). The mean total PSA level was found to be significantly higher in high grade CaP compared with BPH by 309.2 ± 369.8 ng/mL ($p<0.0001$). There was also a significantly higher level of the mean total PSA between CaP with no BM ($p <0.0001$) and CaP with BM ($p < 0.05$) by 376.2 ± 404.0 ng/mL and 175.2 ± 278.6 ng/

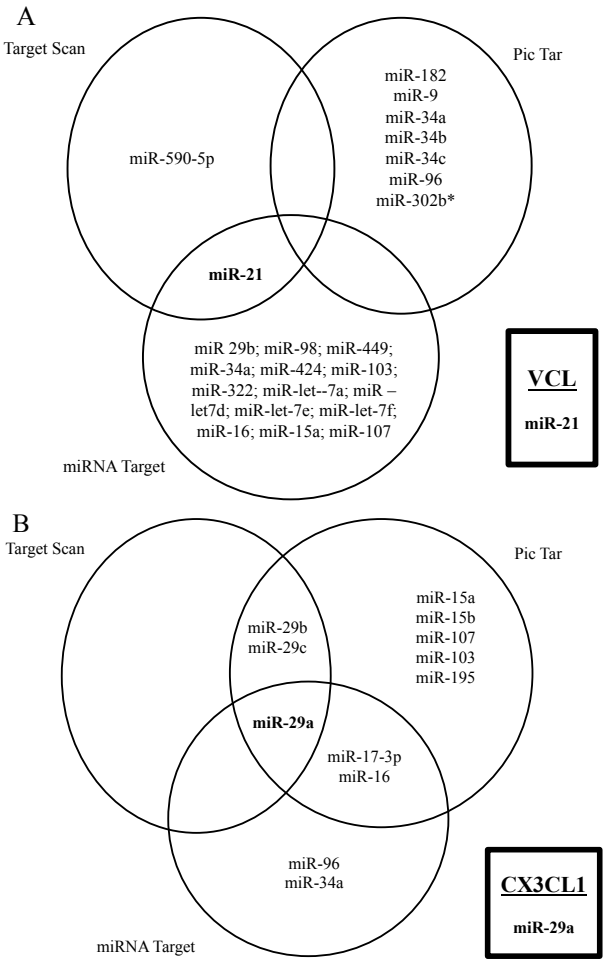

Figure 1. Venn diagram shows the intersection between three miRNA databases and how the predicted miRNAs of interest were selected. (A) MiR-21 was the predicted microRNA to target VCL as found in the intersection between Target Scan and miRNA Target. (B) miR-29a was the predicted microRNA that target CX3CL1 retrieved from all the databases

Table 1. Primers and Probes Sequence of GAPDH, VCL and CX3CL1 genes used in qPCR Reaction

Gene	Forward primer (5'3')	Reverse primer (3'5')	Probe sequence
GAPDH	GCCTCAAGATCATCAGCAATG	CTTCCACGATACCAAAGTTGTC	GCCAAGGTCATCCATGA
VCL	GCCACCAGCATTTATTAAGGTT	GTCTGACTGAAGCATCTGAG	CTTGTCCAGGCAGCTCA
CX3CL1	CGACCCGAAGGAGCAAT	CGATCTGCTTCTCGAAGG	TAACTCGAAATGGCGGCAC

Table 2. Descriptive Data of the selected patients for age, total PSA and gleason score in mean value while value in bracket is the range

Category	Age	PSA	Gleason Score
BPH (N=20)	68.4±6.5	10.9±13.2	Nil
High Grade PCa (N=16)	70.9±6.4	309.2±369.8	9.1±0.6
CaP with no BM (N=10)	72.3±5.9	376.2±404.0	8.9±0.6
CaP with BM (N=6)	68.5±7.1	175.2±278.6	9.3±0.5

PSA, prostate-specific antigen; BPH, benign prostatic hyperplasia; PCa, prostate cancer; CaP with no BM, prostate cancer with no bone metastasis; CaP with BM, prostate cancer with bone metastasis

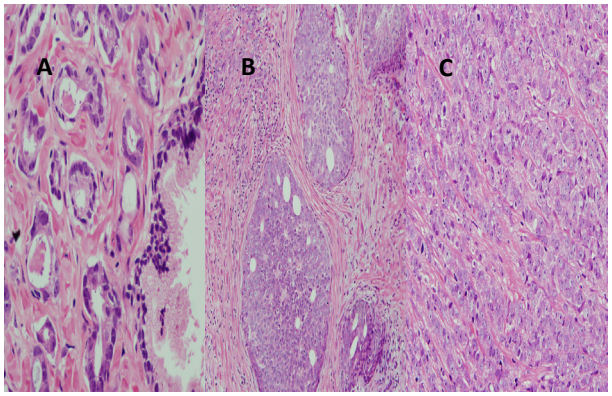

Figure 2. Gleason Patterns in Prostate Cancer Patients. (A) Gleason pattern 3 shows small singly dispersed glands lined by malignant cells with enlarge nuclei and prominent nucleoli at 600x magnification. (B) Gleason pattern 4 shows fused cribriform glands at 200x magnification. (C) Gleason pattern 5 shows the tumor does not formed gland, they are in solid sheets at 400x magnification

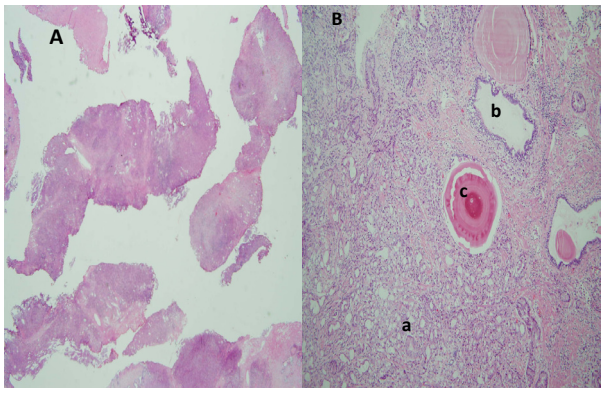

Figure 3. High Grade Prostate Cancer Samples. (A) Prostate cancer tissues from TURP containing 80% cancer tissues per sample at 20x magnification. (B) Cancerous area containing small crowded glands (a), benign prostatic gland (b) and corpora amylacea (c) at 100 x magnification

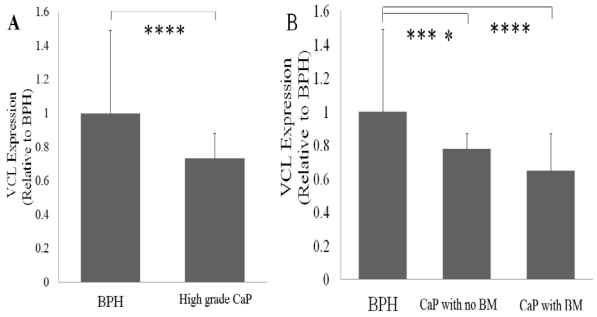

Figure 4. Relative VCL mRNA Expression in FFPE Samples. (A) VCL was found to be significantly down-regulated by 0.27-fold in high grade CaP. (B) VCL was found to be significantly down-regulated in CaP with no BM by 0.22-fold and 0.35-fold in CaP with BM. Data was normalized to BPH. Bar charts represent the mean ± S.D of duplicated samples. Asterisk denotes significant results at $p < 0.0001$ (****)

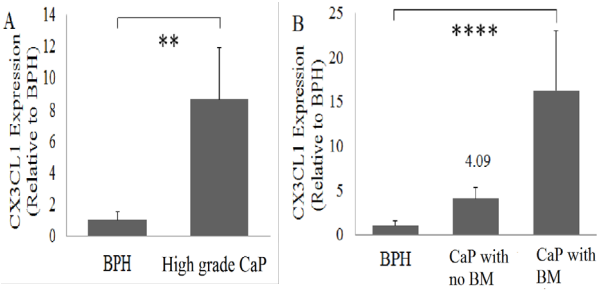

Figure 5. Relative CX3CL1 mRNA Expression in FFPE Samples. (a) CX3CL1 was found to be significantly up-regulated by 8.6 -fold in high grade CaP. (b) CX3CL1 was significantly up-regulated by 16.2 -fold in CaP with BM but was insignificant in CaP with no BM. Data was normalized to BPH. Bar charts represent the mean ± S.D of duplicated samples. Asterisk denotes significant results at $p<0.0001$ (***) and $p<0.05$ (**)

mL respectively compared to BPH.

Transcript level of targeted gene in clinical samples

VCL mRNA was found to be significantly down-regulated in high grade CaP by 0.73-fold, 0.78-fold in CaP with no BM and 0.65-fold in CaP with BM as compared to BPH ($p < 0.0001$) (Figure 4a,b). *CX3CL1* mRNA was significantly up-regulated in high-grade CaP ($p< 0.05$) and CaP with BM ($p< 0.01$) by 8.65-fold and 16.25-fold as compared to BPH respectively. However, there was no significant difference in CaP with no BM as compared to BPH for *CX3CL1* (Figure 5a, b).

Transcript level of targeted genes in untransfected and transfected cell lines

In untransfected cell lines, endogenous *VCL* mRNA was found to be significantly up-regulated by 2.94-

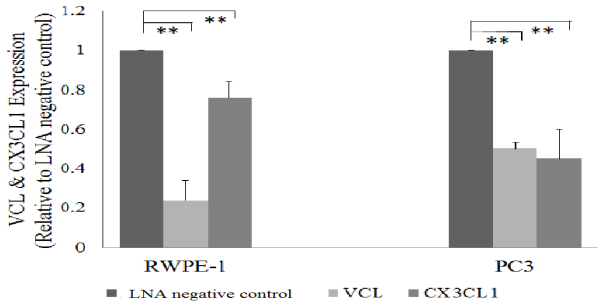

Figure 6. Relative VCL Expression between the Transfected Negative Control LNA and Transfected Anti-miR21 LNA in PC3 Cell Line. All data was normalized to RWPE-1. All PC3 results had significant finding ($p<0.05$) relative to RWPE-1 with 5.76-fold in anti-miR21 LNA and 2.58-fold in negative control LNA. There was also significant increase between transfected anti-miR21 LNA PC3 and negative control LNA PC3 ($p<0.05$). Data reported as mean ± S.D of the duplicated samples. Asterisk indicates results are significant at $p<0.05$ (**)

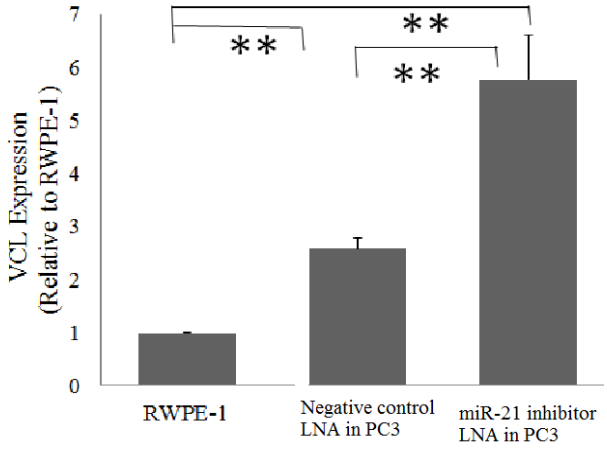

Figure 7. Relative CX3CL1 Expression between the Transfected Negative Control LNA and Transfected Anti-miR29a LNA in PC3 cell line. All data was normalized to RWPE-1. All PC3 results had significant finding ($p<0.05$) relative to RWPE-1 with 0.11-fold in anti-miR29a LNA and 0.18-fold in negative control LNA. There was insignificant increase between transfected anti-miR29a LNA PC3 and negative control LNA PC3 ($p>0.05$). Data reported as mean ± S.D of the duplicated samples. Asterisk denotes significant results at $p<0.05$ (**)

fold ($p<0.05$) while endogenous *CX3CL1* mRNA was significantly down-regulated by 0.23-fold ($p<0.05$) in PC3 compared with RWPE-1 (normal prostate epithelial cell line). Upon transfection with negative control LNA until the significant value of $p<0.05$ (for optimization), *VCL* mRNA was found to be significantly down-regulated by 0.24-fold in RWPE-1 and 0.50-fold in PC3 compared with negative control LNA. *CX3CL1* mRNA was found to be down-regulated by 0.76-fold in RWPE-1 and 0.45-fold in PC3 compared with negative control LNA (Figure 6).

When PC3 was transfected with negative control LNA and miR-21 inhibitor, there was significant increase of *VCL* mRNA in negative control LNA (2.58-fold with $p<0.05$) and significant increase of *VCL* mRNA in miR-21

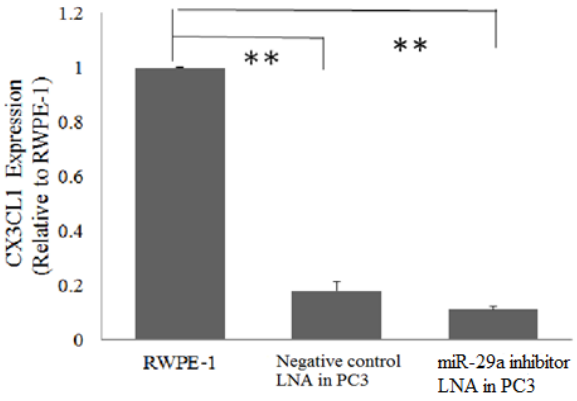

Figure 8. Relative CX3CL1 Expression between the Transfected Negative Control LNA and Anti-miR29a LNA in PC3 Cell Line. All data was normalized to RWPE-1. All PC3 results had significant finding ($p<0.05$) relative to RWPE-1 with 0.11-fold in anti-miR29a LNA and 0.18-fold in negative control LNA. There was insignificant increase between transfected anti-miR29a LNA PC3 and negative control LNA PC3 ($p>0.05$). Data reported as mean ± S.D of the duplicated samples. Asterisk denotes significant results at $p<0.05$ (**)

inhibitor LNA transfected sample (5.76-fold with $p<0.05$) compared with RWPE-1. Interestingly, there was also significant value of up-regulation in miR-21 transfected sample compared with negative control LNA transfected sample ($p<0.05$) as in Figure 7. Hence, there is possibility miR-21 regulates in vitro *VCL* mRNA in PC3 cell line.

When PC3 was transfected with negative control LNA and miR-29a inhibitor, there was significant decrease of *CX3CL1* mRNA in negative control LNA (0.18-fold with $p<0.05$) and significant decrease of *CX3CL1* mRNA in miR-29a inhibitor LNA transfected sample (0.11-fold with $p<0.05$) compared with RWPE-1. Furthermore, there was no significant value of down-regulation in miR-21 transfected sample compared with negative control LNA transfected sample (Figure 8) like *VCL* mRNA expression (Figure 7). Hence, there is no possibility of miR-29a to regulates *CX3CL1*.

Discussion

The molecular mechanisms of CaP bone metastasis are incompletely understood. The emerging roles of microRNA in this metastatic process further complicate the scenario. To obtain the best study design, we tried our best to be detailed in patient's selection criteria in the FFPE samples especially related with the patient's illness history. Since old age is implicated with the risk of various diseases which might associate with occurrence of more than one cancer at a time (Giovannucci et al., 2010; Kao et al., 2004), those with co-morbid factors were excluded in this study. This is important since *VCL* and *CX3CL1* are also involved in other cancers and diseases (Strobel et al., 2015; Sun et al., 2013). However, despite our best effort, we cannot exclude patients with diabetes mellitus, hypertension and/or hyperdyslipidaemia since those chronic diseases are rather prevalent (Alwan et al., 2010; Salwa et al., 2015). Along with that, some of our

qPCR results also had to be excluded as the results were skewed even before the statistical analysis was performed.

In silico analysis by the previous study (Gorlov et al., 2010) had shown that *VCL* and *CX3CL1* were among the top most down-regulated genes in CaP development and they had been implicated in bone metastasis. Most of the data were from gene expression profiling of various degree of CaP progression and only few samples were from bone metastatic lesions. That may be one of the reason why our findings from the FFPE samples showed only *VCL* that was down-regulated but not *CX3CL1*. Furthermore, our FFPE samples were taken from high grade CaP only (Gleason score 8-10) since these patients are at much more higher risk to get bone metastasis (Kaboteh et al., 2013).

VCL was found to be down-regulated in squamous cell carcinoma, metastatic melanoma and colorectal carcinoma samples (Guang-yuan et al., 2012; Yashimoto et al., 2014; Toma et al., 2015). Using the immunohistochemistry approach in a larger number of CaP sample size, Zhu et al., 2010 reported that *VCL* expression was generally higher in CaP than BPH. The reason for which the *VCL* mRNA was generally found to be up-regulated is that it can be influenced by stromal factors (Wong et al., 2002; Gregg et al., 2010). However, its expression was found to be progressively lower in the high grade CaP and metastatic samples (Zhu et al., 2010) study finding is in concordance with our study on the *VCL* expression. This includes our finding on the correlation between the CaP grade and PSA level which was also in agreement with their study where the variability of the PSA results was not related with the occurrence of BM.

A contradictory finding about *VCL* in CaP was reported by Ruiz et al. (2011) who performed a microarray study on human clinical samples at different levels of CaP progression and PC3. Their main emphasis was the hormone-refractory prostate cancer (HRPC) since they found that 15% of HRPC contained genomic amplification at 10q22. The array data demonstrated that *VCL* was the main gene that was significantly overexpressed. In relation with this, it is interesting to note that none of our patient's had HRPC. Moreover, four out of six patients in our study that had CaP with BM were newly diagnosed cases upon TURP and were not yet exposed to hormone ablation therapy. Hence, there was possibility that they were still in the state of hormonal dependent with metastatic progression or maybe they were not among those 15% who had the genomic amplification at 10q22.

Although a few studies (Blum et al., 2008; Gorlov et al., 2010) reported that *CX3CL1* was down-regulated in CaP, *CX3CL1* was found to be significantly up-regulated in our FFPE samples. Our finding is consistent with that of Tomlins et al., 2007 who have found that *CX3CL1* expression was high in high grade CaP tissue and even higher in metastatic tissue compared with BPH, using laser capture microdissection (LCM) approach. Trevino et al. (2011) who did the *in silico* analysis to study the influence of the normal tissue adjacent to the CaP tissue reported that *CX3CL1* was not detected in the normal tissue stroma near to the metastatic tissue but was detected in the normal tissue near the CaP tissue. The expression of *CX3CL1* at the adjacent cancer tissue was higher than in the high grade CaP tissue but lower than in the metastatic tissue. One possible explanation is that at the initial stage of CaP, the normal epithelial cells secreted the soluble *CX3CL1* in response to the presence of CX3CR1 at the CaP epithelial cell (Lucas et al., 2001; Gaudin et al., 2011). However, when CaP progresses to the metastatic stage, it has its own tumor microenvironment, in which *CX3CL1* was no more detected in the normal tissue of the surrounding tumor but is more highly expressed in its own tumor microenvironment. In this case, the main source of *CX3CL1* is probably from the reactive stroma and endothelial cells (Vindrieux et al., 2009) in the tumor itself which could mediate the cell migration towards the intratumoral neovascularization area and promotes metastasis.

Our FFPE specimen findings were found to be different with our PC3 cell line. Based on several gene expression profiling studies, *VCL* was reported to be up-regulated in the PC3 cell line (Kawakami et al., 2015; Lukk et al., 2010). Our untransfected PC3 cell line also showed the similar finding. There are a few possible reasons why *VCL* in this bone metastatic CaP cell line was not down-regulated as seen in the analysis of the FFPE tissues. Amongst the reasons are: 1) the origin of the samples (primary tumor in FFPE vs secondary tumor in PC3), 2) the different types of calibrator used (non-cancerous tissue, BPH vs normal epithelial cell, RWPE-1), and 3) the hormonal dependency state (possibility of hormonal dependent state in FFPE tissues vs hormonal independent state in PC3).

In addition, the matrix dimension, matrix stiffness and types of junction that are involved may also directly or indirectly influence the *VCL* expression level (Aniqua et al., 2016). Recently, it was found that PC3 cells migrated in groups and move in a more directional manner in a 3D matrix (Cui et al., 2013). The 3D matrix resembles the tumor microenvironment in the body more than a 2D matrix (Berrier et al., 2007; Shih et al., 2012). The migration of cells in a group approach required a constant cell-to-cell contact. Since *VCL* played more prominent roles in adherent junctions compared with the cell-matrix adhesion (Ishiyama et al., 2013), it was expected that *VCL* rate of turnover was high in 3D matrix since *VCL* was required to maintain the cell-cell contact along with the group of migratory cells. Besides that, Desai et al. (2008) showed that PC3 used invadopodia as one of their mechanisms in invasion processes. This involved the cell-matrix adhesion. It was also reported that as a tumor in the body is being encircled with stiffer matrix, more invadopodia will develop (Geiger et al., 2011; Murphy et al., 2012). Therefore, the rate of *VCL* turnover was expected to be high in cell-matrix adhesion. This synergistic action of *VCL* in adherent junctions and invadopodia (cell-matrix adhesion) could explain the down-regulation of *VCL* expression in our FFPE clinical samples most probably through PI3K activation of phosphatidylinositol (3,4,5)-triphosphate pathway (Rubashkin et al., 2014). In contrast, in the 2D matrix (as in the method used in this study), the PC3 cells were found to migrate in a single, non-contactable manner (Cui et al., 2013). Hence, less *VCL* was used and the rate of turnover

was low which could be the probable reason of *VCL* up-regulation in our untransfected PC3 cells. This indicates that *VCL* roles in different environment may influence the pattern of its expression (Thievessen et al., 2015).

Many studies have shown that miR-21 was up-regulated in PC3 which indicated its role as oncomiR (Folini et al., 2010; Mattie et al., 2006). Our study had shown that *VCL* mRNA expression was significantly up-regulated following transfection of anti-miR21 LNA into PC3 compared to RWPE-1. Interestingly, there was a significant increase in *VCL* mRNA level between transfected anti-miR21 LNA PC3 and the negative control LNA PC3, which suggested further the role of miR-21. From our study, we postulate that miR-21 probably plays a role in regulating *VCL* expression at the post-transcriptional level in the bone metastatic CaP cell line. Demonstrating this effect at the protein level using Western Blot would be more beneficial to check the effect at the post-translational level. However, there was a study as opposed to our finding, reported that *VCL* was not regulated by miR-21 in the endothelial cell line (Sabatel et al., 2011).

As an oncomiR, miR-21 was shown to play a role in cell migration and invasion by regulating three tumor suppressor genes namely, tropomyosin 1 (TPM1), maspin and programmed cell death 4 (PDCD4) in the metastatic breast cancer cell line, MDA-MB-231 (Zhu et al., 2008). Another study reported that when miR-21 was inhibited, the cell invasion was found to be reduced. The reduced cell invasive activity is probably because miR-21 also has a role to revert the epithelial-mesenchymal transition (EMT) to mesenchymal-epithelial transition (MET) state through one of its targeted gene, the phosphatase and tensin homolog (PTEN) (Han et al., 2012). Despite that, Folini et al., 2010 found that it was insufficient to inhibit miR-21 alone in order to disturb or regulate the PTEN and PDCD4 expression in CaP.

Our finding on *CX3CL1* was different compared with *VCL*. Another study had denoted that *CX3CL1* was found to be progressively higher in the surrounding tissues nearby the CaP tissue from the stage of prostate intraepithelial neoplasia (PIN) to high grade CaP (Trevino et al., 2011). As in our FFPE samples, the reasons for the up-regulated expression of *CX3CL1* were probably because: 1) FFPE contained more reactive stromal tissue (Lucas et al., 2001) aggravated by inflammation caused by acute urinary retention, 2) the presence of androgen in the samples for the cleavage of *CX3CL1* membrane bound form (Jamieson et al., 2008), and 3) the possible secretion of *CX3CL1* from the remaining normal prostate epithelium in the samples (Gaudin et al., 2011; Lucas et al., 2001). However, *CX3CL1* also was reported to be absent in PC3 (Trevino et al., 2011). Been a secondary tumor in the bone, PC3 has a characteristic of being androgen independent. Hence, this could possibly explain the down-regulation of *CX3CL1* in PC3.

Since miR-29a was predicted to target *CX3CL1*, though miR-29a was found to be up-regulated in PC3 (Mattie et al., 2006), our in vitro findings, however, showed no statistical difference of *CX3CL1* mRNA between the transfected negative control LNA and transfected anti-miR29a LNA. This renders the possibility that *CX3CL1* is post-transcriptionally not regulated by miR-29a.

In conclusion, The ability of microRNA to form a multi-regulatory complex network, where single microRNA can regulate multiple genes or a single gene is targeted by multiple microRNAs, highlights the challenges in understanding the complexity of this regulation. The role of miR-21 in regulating *VCL* and miR-29a in regulating *CX3CL1* at the post-transcriptional level was the key objective in our exploration to find their relations with CaP bone metastasis. We concluded that the down-regulation of *VCL* in bone metastatic CaP is most likely regulated by miR-21 at the post-transcriptional level based on the in vitro evidence. However, the exact mechanism is still unknown. It would be interesting if the transfected gene is tested at the protein level. Based on the in vitro findings, *CX3CL1* is possibly not regulated by miR-29a at the post-transcriptional level. Therefore, more microRNA studies are required to understand the regulation of this chemokine in CaP with bone metastasis. Understanding the miRNA-mRNA interactions that may directly or indirectly affect the targeted gene in prostate cancer with bone metastasis may provide additional knowledge for personalized medicine in prostate cancer bone metastasis.

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