Malays J Pathol 2022; 44(1): 29 – 38

ORIGINAL ARTICLE

Silencing of hepsin and inosine 5-monophosphate dehydrogenase 2 by siRNA reduces prostate cancer cells proliferation

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Abstract

Prostate cancer (PCa) is a multifactorial disease, which arises from the activation of genes related to cell proliferation and DNA synthesis. Two important genes namely *hepsin* (*HPN*) and *inosine 5-monophosphate dehydrogenase type 2* (*IMPDH2*) were studied to understand the pathogenesis of this disease. This study aimed to determine the transcript level of PCa-related genes, *HPN* and *IMPDH2*, in archived tissues. Their functional roles were further determined using an *in vitro* model of PCa. Total RNA extraction was done from formalin-fixed paraffin-embedded PCa tissues, and benign prostatic hyperplasia (BPH) tissues acted as the control. Quantitative real-time polymerase chain reaction (qPCR) was performed to measure *HPN* and *IMPDH2* expression. The functional assay was performed in a prostate cancer cell line (DU145) on these two genes by silencing their RNA. We discovered a significantly higher expression of *IMPDH2* in PCa samples compared to non-cancerous tissues ($P < 0.001$). While *HPN* expression level showed a downward trend in PCa but the result was not statistically significant compared to the control. SiRNA-mediated knockdown of *IMPDH2* expression in the cell line significantly decreased cell proliferation. The silencing of *IMPDH2*, however, did not affect cell migration, invasion, and apoptosis of the DU145 cell line. Our study demonstrated that *IMPDH2* plays an essential role in clinical samples as well as *in vitro* models of this cancer. Inhibition of this gene through siRNA causes retardation of cell proliferation suggesting that *IMPDH2* plays an essential role in prostate cancer.

Keywords: Prostate cancer; hepsin (HPN); inosine 5-monophosphate dehydrogenase 2 (IMPDH2); small interference RNA (siRNA); DU145 prostate cancer cell line

INTRODUCTION

Prostate cancer (PCa) consistently becoming a major focus due to the increasing incidence rate worldwide. Socioeconomic and lifestyle may play a role in the increased incidence and mortality rates of PCa.[1,2] The five-year survival rate for prostate cancer in the USA was very high, which is likely due to the early detection of cancer.[2] In the United States, the incidence of PCa has been decreasing since 2012. Recent studies have suggested that the decline has been associated with the U.S. Preventive Services Task Force against routine prostate-specific antigen (PSA) testing.[3] In the past, the incidence of clinically diagnosed PCa in Asian countries was lower than in Western countries. Lately, PCa rates have increased in many Asian countries, which might be due to environmental factors and lifestyle changes.[4]

Prostate cancer is still organ-confined in 90% of cases.[5] Despite the extended efforts from researchers from all over the world, the current treatment approaches and surgical interventions have been proven to be inadequate for the management of the disease. Understanding the underlying mechanisms of how certain genes could influence the tumour behaviour may open up opportunities for a newer approach to PCa therapy. Previous studies reported

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two genes, namely hepsin (*HPN*) and inosine 5-monophosphate dehydrogenase type II (*IMPDH2*) were found to be up-regulated in PCa suggesting their possible roles in the carcinogenesis.[6,7]

Hepsin, one of a type II transmembrane serine protease family plays a key role in epithelial differentiation and cell homeostasis. Many studies on cancer tissues reported increased expression of *HPN* gene including cancer of the breast,[8] prostate,[9] stomach,[10] and cervix.[11] Tumour progression and invasion involve the action of transmembranes proteolytic enzymes such as serine protease.[12] This finding is supported by a study that revealed the involvement of *HPN* in the progression and invasion of PCa by causing disorganisation of the basement membrane, hence allowing tumour cells to invade into the extracellular environment and promote metastasis.[13,14]

Proliferating cells need a high supply of guanine nucleotides. *Inosine 5-monophosphate dehydrogenase* (*IMPDH*) gene encodes the rate-limiting enzyme in *de novo* guanine nucleotide biosynthesis, which is needed for DNA and RNA synthesis. There are two isoforms of *IMPDH*; type I (*IMPDH1*) was expressed in normal cells, whereas type II (*IMPDH2*) was shown to be increased in proliferating malignant cells.[15,16] Inhibition of *IMPDH2* causes a reduction in guanine nucleotide pools which then results in interruption of DNA and RNA synthesis, hence the arrest of cell proliferation. A previous study on colorectal cancer patients has suggested that *IMPDH2* promoted the progression of the disease by activating PI3K/AKT pathway.[15] They also hypothesised that IMPDH2 was one of the prognostic biomarkers and therapeutic targets for colorectal cancer. Likewise, increased *IMPDH2* mRNA levels have been found in hepatocellular carcinoma tissues and were reported to be closely related to the aggressiveness of the carcinoma progression.[17] *IMPDH2* was also reported to directly cause chemo- and radioresistance in osteosarcoma cells.[18] The increased *IMPDH2* expression in methotrexate (MTX) resistance occurred as a compensation for the inhibition of dihydrofolate reductase (DHFR) by MTX, as both DHFR and IMPDH are enzymes involved in the same pathway in the *de novo* synthesis of guanylates.[19] IMPDH2 inhibition significantly increased the sensitivity of the resistant cell lines to MTX. Thus, IMPDH has been considered as a potential target for cancer therapy and also for the modulation of drug sensitivity. Besides, current studies revealed that selective inhibitors of IMPDH are beneficial as an anti-inflammation and antibacterial in the treatment of neuroinflammatory and infectious diseases.[20,21]

To date, little data on the roles of *HPN* and *IMPDH2* in the carcinogenesis of prostate cancer were reported. Therefore, the present study aimed to determine the regulation of *HPN* and *IMPDH2* expression in PCa tissues as compared to non-cancerous prostate tissues (benign prostatic hyperplasia, BPH).

MATERIALS AND METHODS

Clinical Samples and Cell Lines

A total of 20 formalin-fixed paraffin-embedded (FFPE) prostate samples consisting of 10 cancers and 10 benign prostatic hyperplasia (BPH) samples were selected from the Department of Pathology, Hospital Kuala Lumpur over two years (from 2009 to 2011). None of the patients received chemotherapy or hormonal therapy. All the PCa tissues were histologically confirmed by a pathologist, and samples were checked to ensure that the tumour tissue consisted of more than 80% of the specimens. Gleason score of the PCa samples determined that seven tumour samples were medium-grade, two were high-grade and only one was low grade. The BPH samples were obtained via transurethral resection of the prostate. All the samples were verified in a blinded manner by a qualified pathologist.

PCa cell line, DU145 was purchased from the American Type Culture Collection (ATCC). It is an androgen insensitive and highly metastatic cell line derived from brain metastases. The cells were cultured in Eagle's Minimum Essential Medium (GIBCO, Langley, USA) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO), at 37°C in a humidified atmosphere containing 5% CO_2.

RNA Extraction

Total RNA was extracted from tissue and DU145 cell line using RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quantity and quality of the extracted RNA were measured using NanoDrop-1000 Spectrophotometer (Thermo Scientific, Massachusetts, USA) and 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). The electropherograms of good quality RNA showed two distinct peaks of 28S/18S ribosomal peak ratio of 1.8 to 2.1. RNA integrity number of more than two and eight for

FFPE samples and cell lines respectively were accepted for cDNA synthesis.

Measurement of mRNA of Targeted Genes Using a Quantitative Real-time Polymerase Chain Reaction (qPCR)

Firstly, the RNA was reverse transcribed to cDNA using the Verso cDNA kit (Thermo Scientific, UK). The reverse transcription process followed the previous protocol.[22] The reaction mixture consisted of 12.5 µl of Solaris qPCR Master Mix (2X), 1.25 µl of Solaris primer/probe Set (20X), 1 µl of cDNA template and water to make up the final volume of 25 µl.

The program was set with enzyme activation at 95°C for 15 minutes X1 cycle, 45 cycles of denaturation at 95°C for 15 seconds and annealing/extension process at 60°C for 1 minute. *β-actin* was used as the reference gene. These experiments were performed in triplicate. Fold changes for the genes of interest, which were calculated after normalisation with the endogenous control, *β-actin*. The comparative method was used to calculate the gene expression between tumour and BPH samples. Relative quantitation of *HPN* and *IMPDH2* was performed by $2^{-\Delta\Delta}$Ct method, normalised to 18S rRNA.

Primers 5'-CGGCTCATGGTCTTTGACAA-3' and 3'-CAGCTCGGAGTGGGTCAGT-5'; 5'- AGGTGGACCTGACTTCTG-3' and 3'-AAGCCAATACCGCCTGT-5' were used to amplify *HPN* and *IMPDH2* respectively. Primers 5'- TGGAGAAAATCTGGCACCAC-3' and 3'-GGTCTCAAACATGATCTGG-5' were used to amplify the *β-actin* transcript. The sequence for the probes is shown in Table 1.

Small Interfering RNA (siRNA) Assay and Transient Transfection

Twenty-four hours before transfection, 1×10^4 DU145 cells were seeded into a 96-well microplate. Transient silencing of *IMPDH2* gene was achieved using a pool of four siRNA. The sequence of the siRNA was provided by the Dharmacon Company (Colorado, USA) using an online design tool for siRNA (siDESIGN Center).

The target sequences were GGUAUGGGUUCUCUCGAUG, AAGGGUC AAUCCACAAAUU,GCACGGCGCUUUGGU GUUC, and GGAAAGUUGCCCAUUGUAA. Non-targeting siRNA was used as a negative control (ONTARGETplusNontargetingvPool, Dharmacon) and GAPDH siRNA was used as a positive control (ONTARGETplusSMARTpool, Dharmacon). Both positive and negative controls were provided by the company, which were pre-optimised for the above cell line. Transfection of the DU145 cell was performed using DharmaFECT1 Transfection reagents, according to the manufacturer's instructions. An amount of 100 nmol siRNA was used for each well. The efficiency of IMPDH2 knockdown was detected by RT–PCR, siRNA targeting housekeeping gene GAPDH was used as a positive control for transfection efficiency. qPCR was carried out 48 hours after transfection.

Proliferation Assays

To determine the proliferation of the DU145 cell line, 1×10^4 DU145 cells of the non-transfected group, nonspecific negative control, and siRNA-IMPDH2 transfected group were plated in triplicates in a 96-well culture plate 48 hours post-transfection. Proliferation assays were performed using a kit containing a novel tetrazolium compound (MTS) reagent as described below. 20 µlCellTiter 96® AQ_{ueous} One Solution reagent (Promega, Fitchburg, Wisconsin, USA) was added to each well and incubated for four hours at 37°C. The optical density of the solution was determined spectrophotometrically at 490 nm.

Cell Migration and Invasion Assays

The migration assay was done in a migration chamber based on the Boyden chamber principle. In each well, there was an insert with an 8.0 µm pore size of polycarbonate filters. Before the assays, cells of non-transfected and transfected groups were starved by incubating for 24 hours in a serum-free medium. Then, the cells were trypsinised and suspended at a density of $1x10^6$ cells per ml in 300 µl of serum-free EMEM and placed in the upper chamber. The bottom chamber of the transwell chamber was filled

TABLE 1: Shows the probe sequences for the studied genes

Probe:	Sequence:
Human gene β-actin	ACCGCGAGAAGATGACC
Human gene *HPN*	TGGGCTTCCTCAGGGCAC
Human gene *IMPDH2*	CATAGCAATGGCGCTTAC

with 500 µl EMEM containing 10% FBS as a chemoattractant. The cells were incubated for 24 hours at 37°C in 5% CO_2. The insert was subsequently removed and stained with cell staining and incubated for 20 minutes at room temperature. The upper side of the insert was scraped with a cotton tip to eliminate cells that had not migrated through it; the stained cells extracted by extraction buffer were measured by ELISA at 560nm optical density.

Invasion assays were carried out similarly to migration assays. Cells were starved in the incubator for 18 hours with serum-free EMEM. The cells were trypsinised and suspended at a density of $5x10^4$ cells per ml in 250 µl of serum-free EMEM. The cell suspension was added to 8 µm porous inserts and placed in wells filled with 500 µl of medium supplemented with 10% FBS as a chemoattractant. Following two days of incubation, the upper side of the filter was scraped with a cotton tip to eliminate cells that had not invaded through it. The invasive ability of the cells was determined by ELISA at 560 nm after being stained and extracted using the extraction buffer.

Apoptosis Assays

Caspase-3 activity was determined by a colourimetric assay based on the ability of caspase-3 to change acetyl-Asp- Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) into a yellow formazan product, p-nitroanilide (pNA). The cells were rinsed with cold PBS and then lysed by lysis buffer for 10 minutes on ice. Cell lysates were centrifuged at 10,000 rpm for 5 minutes. The enzyme activity was measured in a 96-well microplate using a colourimetric method based on the hydrolysis of the caspase-specific substrate (Ac-DEVD-pNA at 37°C, 1 h) by caspases. A specific inhibitor of caspase-3 was used to confirm the specificity of the cleavage reaction and an increase in absorbance at 405 nm was used to quantify the activation of caspase-3 activity.

Statistical Analysis

The software of SPSS version 17.0 for Windows (SPSS Inc, Chicago, USA) was used for statistical analysis. Continuous variables were expressed as mean ± standard error mean (x ± SEM). Statistical analysis was performed with a *t*-test. Differences were considered as statistically significant when $P \leq 0.05$.

Ethics

This study was funded by the Faculty of Medicine Fundamental Research Grant, Universiti Kebangsaan Malaysia. This study received approval from the Universiti Kebangsaan Malaysia Research Ethics Committee (UKM 1.5.3.5/244/SPP/FF-233-2010) and the National Medical Research Registry (NMRR-10-1229-6840).

RESULTS

Purity, Concentration and Quality of FFPE RNA

RNA concentration of FFPE samples was more than 200 ng/µl, which was suitable for cDNA synthesis. The RNA purity ranged from 1.8 – 2.1 (A260/A280 mm), and the integrity numbers ranged from 2.1 – 2.5 (Table 2).

HPN and IMPDH2 Expression in FFPE Tissues

Quantitative PCR showed the mRNA expression level of IMPDH2 in PCa tissues was significantly higher than BPH tissues by 9.1-fold ($P < 0.0001$). There was no statistically significant for HPN mRNA expression in PCa samples compared to BPH samples (Figure 1).

Effect of SiRNA on IMPDH2 Expression in DU145 Cell Line

Forty-eight hours after IMPDH2-siRNA transfection, qPCR was performed to detect IMPDH2 mRNA expression. The results showed that mRNA expression of IMPDH2 was markedly lower in the siRNA IMPDH2 group as compared to the non-specific negative control. The percentage of knockdown was 86.7% for siRNA IMPDH2 and 89.8% for siRNA GAPDH (Figure 2). However, there was no significant difference in mRNA expression in non-targeting control before and after transfection.

Effect of IMPDH2 Down-regulation on DU145 Cell Proliferation

To better understand the role of *IMPDH2* in tumour progression, the proliferation rates of DU145 cells in different groups were determined. As shown in Figure 3, DU145 cells transfected with siRNA IMPDH2 exhibited a decreased growth rate compared to the untreated group ($P < 0.05$) and non-targeting control cells ($P < 0.05$) as determined by MTS assay. Therefore, these results suggest that siRNA IMPDH2 inhibits cell proliferation and IMPDH2 plays an important role in regulating DU145 cell growth.

TABLE 2: RNA purity, concentration and quality of 20 FFPE samples (10 BPH and 10 PCa). The mean RNA concentration for PCa samples was 394.5 ± 74.6 ng/μl and for BPH was 405.9 ± 65.3 ng/μl. The mean was expressed as concentration ± SEM

Prostate FFPE sample	Gleason score	RNA Purity (A260/A280) mm	RNA Concentration (ng/μl	Quality RNA Integrity Number (RIN)
PCa 1	3+4	1.9	168	2.3
PCa 2	9	2.0	268	2.3
PCa 3	3+4	1.9	627	2.1
PCa 4	3+4	1.9	238	2.3
PCa 5	3+3	1.9	560	2.1
PCa 6	3+4	2.0	178	2.2
PCa 7	4+3	1.9	872	2.2
PCa 8	3+4	1.9	415	2.1
PCa 9	9	1.9	171	2.4
PCa 10	3+4	2.0	448	2.1
BPH 1	-	2.0	518	2.1
BPH 2	-	2.1	206	2.3
BPH 3	-	1.9	723	2.2
BPH 4	-	2.1	571	2.3
BPH 5	-	2.0	215	2.4
BPH 6	-	1.9	190	2.5
BPH 7	-	1.9	379	2.4
BPH 8	-	2.0	426	2.4
BPH 9	-	1.9	660	2.3
BPH 10	-	1.9	171	2.5

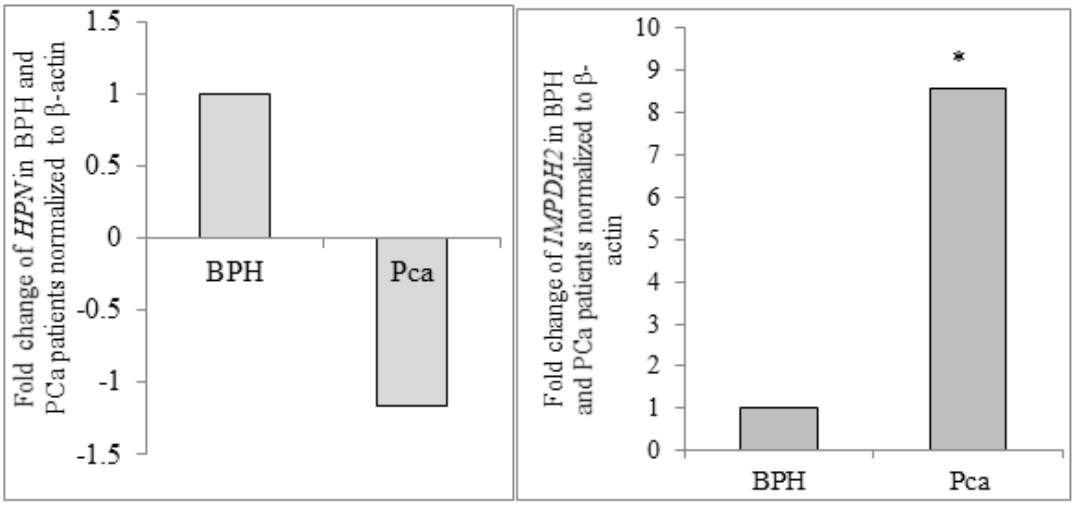

FIG. 1: Histograms of the fold change of *HPN* and *IMPDH2* gene expression in FFPE prostate tissue from the patient with prostate cancer (PCa) compared with that of BPH tissues. *HPN* gene expression in PCa tissues showed a downward trend compared to BPH tissues. *IMPDH2* was significantly up-regulated in PCa as determined by qPCR compared to BPH (*P<0.0001). Messenger RNA expression is normalised using a β-actin expression as an internal standard.

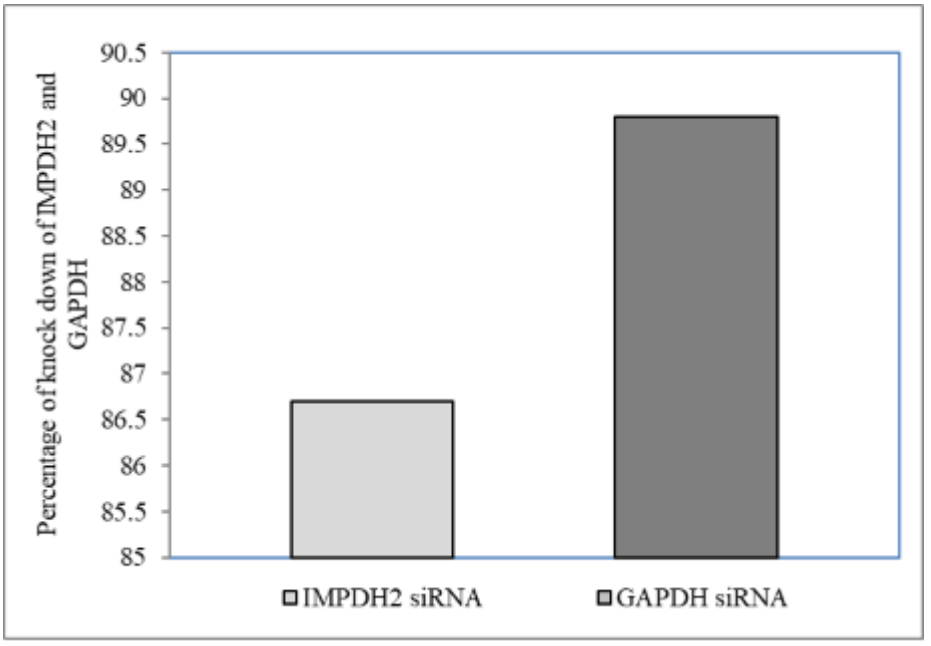

FIG. 2: The percentage of *IMPDH2* and *GAPDH* knockdown in the DU145 cell line. Total RNA was prepared from the indicated cell line, reverse-transcripted, and subjected to qPCR using oligonucleotide primers specific for *IMPDH2*. Expression was normalised using a β-actin expression as an internal standard. *IMPDH2* was successfully knockeddown by 86.7%, and *GAPDH* by 89.8%.

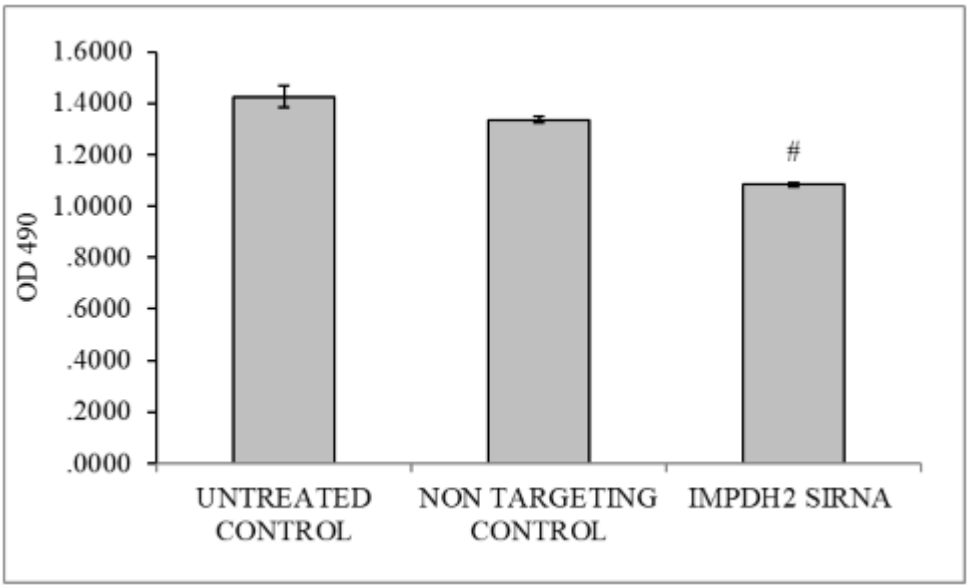

FIG. 3: *IMPDH2* enhances the proliferation of DU145 cells. Equal numbers of indicated cells were plated in triplicate and incubated under standard culture conditions. Following the MTS assay, the number of cells is significantly decreased in the cells transfected with *IMPDH2* siRNA as compared to untreated control and non-targeting control. Values are shown as the mean ± standard error of the mean (SEM).$^{\#}$ P<0.05 as compared to untreated control and non-targeting control respectively.

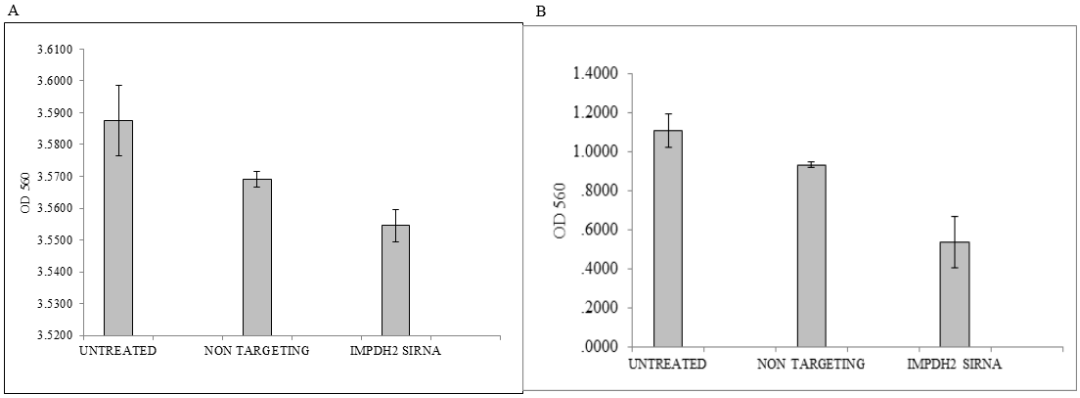

FIG. 4: *IMPDH2* is not essential for the migration and invasion of DU145 cells. (A) Inhibition of *IMPDH2* in the DU145 cell line did not affect cell migration (A) and invasion (B). Values are shown as amean ± standard error of the mean.

Effect of IMPDH2 Down-regulation on Migration and Invasion of DU145 Cells

We determined whether *IMPDH2* was critical to DU145 prostate cancer cell migration and invasion. To test whether *IMPDH2* expression affects the motility of our model cell line DU145, we assayed the capacity of cells expressing *IMPDH2* to invade and migrate through polycarbonate coated transwell inserts. The migration of DU145 cells after *IMPDH2* silencing was not significantly repressed compared to the control groups (Figure 4A). A similar result was obtained for the invasion assay of siRNA IMPDH2-mediated suppression compared with the untreated control group or non-targeting control group (Figure 4B). These results suggested that transient transfection of siRNA IMPDH2 did not have any meaningful impact on migration and invasion in our model cell line DU145.

Apoptosis Assay

Caspase-3 activity colourimetric assay was performed to determine whether the decrease in cell viability was accompanied by apoptosis. The apoptotic rate was insignificantly decreased in the cells transfected with IMPDH2 siRNA compared to those transfected with non-targeting

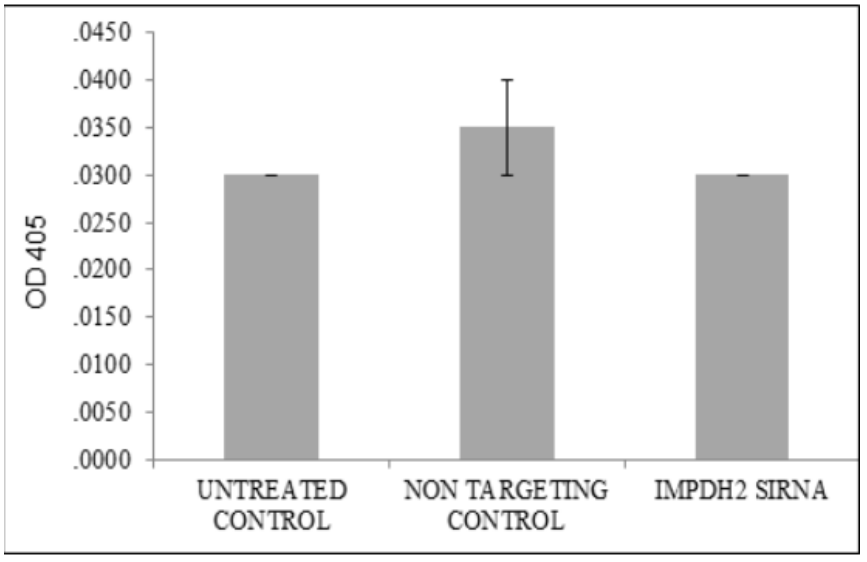

FIG. 5: Histogram of the apoptosis result based on caspase-3 activity. The values shown are mean ± SEM. Caspase-3 activity in IMPDH2 siRNA transfected DU145 cell line was not significantly decreased compared to that of the untreated group and that of non-targeting control.

control and with untreated control. There was no significant difference in apoptosis of IMPDH2 siRNA transfected cells when compared to the untreated control and non-targeting control when evaluated by apoptosis assay (Figure 5).

DISCUSSION

Early identification and effective treatment of cancer are the most important challenges in cancer research. Small interference RNAs (siRNAs) are target-specific double-stranded RNA molecules designed to repress gene expression by using an endogenous cellular process of RNA interference (RNAi).[23] The present study explored whether the two selected genes, *HPN* and *IMPDH2* were upregulated in PCa and if RNAi-mediated suppression of *IMPDH2* could be used to inhibit the proliferation and motility of DU145 prostate cancer cell lines.

HPN gene has been reported to be upregulated in PCa.[24] Hepsin expression levels were reported to be correlated with Gleason score,[25] and the degree of invasiveness.[11,26] In contrast, our study showed insignificant downregulation of *HPN* in PCa samples compared to BPH samples. For the hypothesis of the present study, we expected *HPN* expression to be upregulated in PCa samples as compared to BPH samples. *HPN* has been reported to have an antitumourigenic effect on PCa cells including the DU145 cell line.[27] This finding was supported by other studies that showed the correlation between decreased HPN expression and poor PCa prognosis especially in cases with hormone-refractory metastatic PCa and elevated PSA level.[28] *HPN* is expressed both in the precursor lesion of PCa, high-grade prostate intraepithelial neoplasia, and hormone-refractory metastatic tumours.[28,29] Studies using animal model PCa showed that the upregulation of *HPN* promotes cancer progression and metastasis.[13,30] Overexpression of *HPN* leads to a disorganised basement membrane and promotes an invasive potential of cancer cells.[13] All of the cases included in our study were among localized PCa samples and a few with high PSA levels. The findings of the present study were quite comparable with other previous studies.[27,28] Those studies used fresh frozen samples that contained highly preserved RNA, while our study used FFPE tissue samples in which the RNA had degraded, thus giving poor quality of RNA. The advantage of using FFPE tissues is the ability to get all the samples at the same time.

IMPDH2 expression rate was higher in PCa clinical samples than in the noncancerous tissue (BPH). We hypothesised that *IMPDH2* might play distinct roles in cell proliferation in PCa. In this study, we demonstrated that the knockdown of *IMPDH2* by siRNA resulted in a decrease in growth in androgen-independent DU145 cells. Similarly, Floryk and Thompson reported in 2008 that AVN944 was able to inhibit the IMPDH2 and induce DU145 cell death and cell cycle arrest.[31] AVN944 is a specific, non-competitive IMPDH inhibitor and is used as an antitumour drug. The high level of IMPDH is primarily caused by the upregulation of the isoform IMPDH2 in tumour cells. They also hypothesised that AVN944 is a novel therapeutic agent, which may be combined with other therapeutic agents in the treatment of prostate cancer.[31]

Based on oncology research, increased IMPDH2 activity in neoplastic cells have made the enzyme a key target for the development of anticancer drugs. Also, IMPDH2 has become a major drug target for antiviral,[32] antibacterial,[33] parasitic infestation,[34] and immunosuppression.[35] IMPDH2 inhibitors such as azathioprine,[36] mycophenolic acid,[37] methotrexate,[19] and tiazofurin[38] have been used in clinics for many years. For example, mycophenolic acid is a reversible and uncompetitive inhibitor of IMPDH2, used as an anticancer and immunosuppressive agent.[39,40] The pharmacological IMPDH2 inhibitors directed against chemoresistant osteosarcoma cells were unable to enhance chemosensitivity in the cells by using mycophenolic acid. The assumption was that the strong anti-proliferative effects of these inhibitors on the cells would lead to cell death.[18] They concluded that there was a need for more effective pharmacological inhibitors such as siRNA to overcome chemoresistance in osteosarcomas with high *IMPDH2* expression.

Another study conducted on hepatocellular carcinoma demonstrated that overexpression of *IMPDH2* in the tissues was associated with poor survival outcomes in the patients.[17] To date, there is insufficient data regarding the effects of IMPDH2 RNAi on tumours. Our results are in accordance with previous research and we conclude that *IMPDH2* is strongly linked to cell growth and proliferation in prostate cancer. IMPDH2 was reported in most studies as an oncogene and involves in metastatic tumours. In non-small cell lung cancer, IMPDH2 promotes cell proliferation, migration and invasion through activation of Wnt/β-catenin pathway.[41]

On the other hand, overexpression of IMPDH2 in colorectal cancer induces cell proliferation and tumourigenesis through PI3K/AKT/mTOR and PI3K/AKT/FOXO1 pathways.[15] Glycogen synthase kinase-3 (GSK-3), an essential protease downstream of AKT is composed of β-catenin, Axin and adenomatous colonic polyposis. GSK-3 regulates cell-cycle proliferation and tumourigenesis by phosphorylating glycogen synthase to inhibit glycogen synthesis in cells. AKT can phosphorylate GSK-3 to inhibit its activity, thereby promoting glucose uptake and glycogen synthesis for cell proliferation.[42]

Cysteine-aspartic-acid proteases 3 (caspase-3) cleaves poly-(ADP-ribose)-polymerase in an early stage of apoptosis. Caspase-3 plays a dominant role in apoptosis pathways. *In vivo* and *in vitro* studies showed that inhibition of *IMPDH2* resulted in apoptosis of chemo- and radioresistant Saos-2 cells suggesting that *IMPDH2* plays a key role in regulating cell apoptosis that leads to malignancy and resistance to therapy.[18] Hence, we proposed that *IMPDH2* increases cell proliferation by reducing cell apoptosis.

Our study did not show any significant effect of siRNA silencing of *IMPDH2* on apoptosis of the DU145 cell line measured by caspase-3 activity assay. In contrast, a study in 2008 revealed that inhibition of *IMPDH2* by AVN944 inhibitor was able to induce apoptosis in DU145 cell line.[30] Nonetheless, the cell death in DU145 cells was caspase-independent and the activation of caspase-9 and caspase-3 was not detected in DU145.[30] Therefore, further studies are advised to address the effect of *IMPDH2* silencing on the apoptosis pathway, which is caspase-independent in the DU145 cell line.

The overexpression of *IMPDH2* has been correlated with a metastatic propensity for other tumours. In our study, we found that the knockdown of *IMPDH2* did not show a significant effect on DU145 migration and invasion.

CONCLUSION

In summary, *IMPDH2* contributes to the proliferation of malignant PCa cells and is successfully inhibited by the siRNA technique. Therefore, we conclude that *IMPDH2* may be considered a potential target for PCa therapeutic strategies using a siRNA approach alone or in combination with other pharmacological strategies.

Acknowledgments: We would like to thank the supporting staff of the Faculty of Medicine, Universiti Kebangsaan Malaysia, Histopathology Unit and Record Unit, Hospital Kuala Lumpur, and their supports and technical assistance in this study.

Authors' contribution: Original draft preparation, Norhazlina A WAHAB, Huria D DARDAR; Sample collection and verification, Huria D Dardar, Rosna YUNUS, Zulkifli M ZAINUDIN; Supervision and funding, Norhazlina A WAHAB, Norfilza M MOKHTAR. All authors have agreed to the published version of the manuscript.

Conflict of interest: The authors declare no conflict of interest.

REFERENCES

1. Hassanipour S, Delam H, Arab-Zozani M, *et al*. Survival rate of prostate cancer in Asian countries: a systematic review and meta-analysis. Ann Glob Health. 2020; 86(1): 1-13.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2019. CA Cancer J Clin. 2019; 69(1): 7-34.
3. Jemal A, Fedewa SA, Ma J, *et al*. Prostate cancer incidence and PSA testing patterns in relation to USPSTF screening recommendations. JAMA. 2015; 314(19): 2054-61.
4. Baade PD, Youlden DR, Cramb SM, Dunn J, Gardiner RA. Epidemiology of prostate cancer in the Asia-Pacific region. Prostate Int. 2013; 1(2): 47-58.
5. Yap TA, Smith AD, Ferraldeschi R, Al-Lazikani B, Workman P, de Bono JS. Drug discovery in advanced prostate cancer: translating biology into therapy. Nat Rev Drug Discov. 2016; 15: 699-718.
6. Tervonen TA, Belitškin D, Pant SM, *et al*. Deregulated hepsin protease activity confers oncogenicity by concomitantly augmenting HGF/MET signaling and disrupting epithelial cohesion. Oncogene. 2016; 92: 278–83.
7. Wieczorek P, Balut-Wieczorek M, Jasinski M, Szablonski W, Antczak A. Inosine monophosphate dehydrogenase 2 as a marker of aggressive and advanced prostate cancer. Cent European J Urol. 2018; 71(4):399-403.
8. Xing P, Li JG, Jin F, *et al*. Clinical and biological significance of hepsin overexpression in breast cancer. J Investig Med. 2011; 59(5): 803-10.
9. Willbold R, Wirth K, Martini T, Sültmann H, Bolenz C, Wittig R. Excess hepsin proteolytic activity limits oncogenic signaling and induces ER stress and autophagy in prostate cancer cells. Cell Death Dis. 2019; 10(8): 601.
10. Zhang M, Zhao J, Tang W. High Hepsin expression predicts poor prognosis in gastric cancer. Scientific Reports. 2016; 6: 36902.
11. Cheng H, Wang W, Zhang Y, *et al*. Expression levels and clinical significance of hepsin and HMGB1

protein in cervical carcinoma. Oncol Lett. 2017; 14(1): 159-64.
12. Pawar NR, Buzza MS, Antalis TM. Membrane-anchored serine proteases and protease-activated receptor-2 (PAR-2)-mediated signaling: co-conspirators in cancer progression. Cancer Res. 2019; 79(2): 301-10.
13. Klezovitch O, Chevillet J, Mirosevich J, Roberts RL, Matusik RJ, Vasioukhin V. Hepsin promotes prostate cancer progression and metastasis. Cancer Cell. 2004; 6(2): 185-95.
14. Tang X, Mahajan SS, Nguyen LT, *et al*. Targeted inhibition of cell-surface serine protease hepsin blocks prostate cancer bone metastasis. Oncotarget.2014; 5(5): 1352-62.
15. Duan S, Huang W, Liu X, *et al*. IMPDH2 promotes colorectal cancer progression through activation of the PI3K/AKT/mTOR and PI3K/AKT/FOXO1 signaling pathways. J Exp Clin Cancer Res. 2018; 37: 304.
16. Xu Y, Zheng Z, Gao Y, *et al*. High expression of IMPDH2 is associated with aggressive features and poor prognosis of primary nasopharyngeal carcinoma. Sci Rep. 2017; 7: 745.
17. He Y, Zheng Z, Xu Y, *et al*. Over-expression of IMPDH2 is associated with tumor progression and poor prognosis in hepatocellular carcinoma. Am J Cancer Res. 2018; 8(8): 1604-14.
18. Li HX, Meng QP, Liu W, *et al*. IMPDH2 mediate radioresistance and chemoresistance in osteosarcoma cells.Eur Rev Med Pharmacol Sci. 2014; 18(20): 3038-44.
19. Peñuelas S, Noé V, Morales R, Ciudad CJ. Sensitization of human erythroleukemia K562 cells resistant to methotrexate by inhibiting IMPDH. Med Sci Monit. 2005; 11(1): BR6–12.
20. Liao LX, Song XM, Wang LC, *et al*. Highly selective inhibition of IMPDH2 provides the basis of antineuroinflammation therapy. Proc Natl Acad Sci USA. 2017; 114(29): E5986-E5994.
21. Juvale K, Shaik A, Kirubakaran S. Inhibitors of inosine 5'-monophosphate dehydrogenase as emerging new generation antimicrobial agents. Medchemcomm. 2019; 10(8): 1290-301.
22. Mohamad M, Wahab NA, Yunus R, *et al*. Roles of microRNA-21 and microRNA-29a in regulating cell adhesion-related genes in bone metastasis secondary to prostate cancer. Asian Pac J Cancer Prev. 2016; 17(7): 3437-45.
23. Hannon GJ. RNA interference. Nature.2002; 418(6894): 244–251.
24. Magee JA, Araki T, Patil S, *et al*. Expression profiling reveals hepsin overexpression in prostate cancer. Cancer Res. 2001; 61: 5692-96.
25. Stephan C, Yousef GM, Scorilas A, *et al.* Hepsin is highly overexpressed in and a new candidate for a prognostic indicator in prostate cancer. J Urol. 2004; 171: 187-91.
26. Matsuo T, Nakamura K, Takamoto N, *et al.* Expression og the serine protease hepsin and clinical outcome of human endometrial cancer. Anticancer Res. 2008; 28 (1A): 159-64.
27. Vasantha S, Michael V, Johng SR, Moul JW, Srivastava S. Hepsin inhibits cell growth/invasion in prostate cancer cells. Cancer Res. 2002; 62(23): 6812-6.
28. Dhanasekaran SM, Barrette TR, Ghosh D, *et al*. Delineation of prognostic biomarkers in prostate cancer. Nature. 2001; 412: 822-6.
29. Kelly KA, Setlur SR, Ross R, *et al*. Detection of early prostate cancer using a hepsin-targeted imaging agent. Cancer Res. 2008; 68(7): 2286-91.
30. Nandana S, Ellwood-Yen K, Sawyers C, *et al*. Hepsin cooperates with MYC in the progression of adenocarcinoma in a prostate cancer mouse model. Prostate. 2010; 70(6): 591-600.
31. Floryk D, Thompson TC. Antiproliferative effects of AVN944, a novel inosine 5-monophosphate dehydrogenase inhibitor, in prostate cancer cells. Int J Cancer. 2008; 123(10): 2294–302.
32. Nair V, Shu Q. Inosine monophosphate dehydrogenase as a probe in antiviral drug discovery. Antivir Chem Chemother. 2007; 18: 245-58.
33. Chacko S, Boshoff HIM, Singh V, *et al*. Expanding benzoxazole-based inosine 5'-monophosphate dehydrogenase (IMPDH) inhibitor structure-activity as potential anti-tuberculosis agents. J Med Chem. 2018; 61(11): 4739-56.
34. Shah CP, Kharkar PS. Inosine 5'-monophosphate dehydrogenase inhibitors as antimicrobial agents: recent progress and future perspectives. Future Med Chem. 2015; 7: 1415-29.
35. Duong-Ly KC, Kuo YM, Johnson MC, *et al*. T cell activation triggers reversible inosine-5'-monophosphate dehydrogenase assembly. J Cell Sci [Internet]. 2018 Sept 1 [cited 2020 June 15]; 131(17): jcs.223289. Available from: https://jcs.biologists.org/content/131/17/jcs223289
36. Peyrin-Biroulet L, Cadranel JF, Nousbaum JB, *et al*. Interaction of ribavirin with azathioprine metabolism potentially induces myelosuppression. Aliment PharmacolTher. 2008; 28(8): 984-93.
37. Cao S, Aboge GO, Terkawi MA, *et al*. Mycophenolic acid, mycophenolate mofetil, mizoribine, ribavirin, and 7-nitroindole inhibit propagation of Babesia parasites by targeting inosine 5'-monophosphate dehydrogenase. J Parasitol. 2014;100(4): 522-6.
38. Olah E, Natsumeda Y, Ikegami T, *et al*. Induction of erythroid differentiation and modulation of gene expression by tiazofurin in K-562 leukemia cells. Proc Natl Acad Sci USA. 1988; 85(17): 6533-7.
39. Chen L, Pankiewicz KW. Recent development of IMP dehydrogenase inhibitors for the treatment of cancer. CurrOpin Drug DiscovDevel. 2007; 10(4): 403–12.
40. Klotsman M, Sathyan G, Anderson WH, Garden OA, Shivanand P. Mycophenolic acid in patients with immune-mediated inflammatory diseases: from humans to dogs. J Vet PharmacolTher. 2019; 42(2): 127-38.
41. Xu H, Ma H, Zha L, *et al.* IMPDH2 promotes cell proliferation and epithelial-mesenchymal transition of non-small cell lung cancer by activating the Wnt/β-catenin signaling pathway. Oncol Lett. 2020; 20(5): 219.
42. Xu F, Na L, Li Y, Chen L. Roles of the PI3K/AKT/mTOR signaling pathways in neurodegenerative diseases and tumours. Cell Biosci. 2020; 10: 54.