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Original Research Article

Silencing of *HN1L* suppresses the proliferation and migration of cancer cells

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Nonstandard Abbreviations

BRCA – Breast Cancer
CESC – Cervical Squamous Cancer
CHOL – Cholangiocarcinoma
COAD – Colon Adenocarcinoma
ESCA – Esophageal Carcinoma
HN1 – Hematological and Neurological Expressed Sequence 1
HN1L – Hematological and Neurological Expressed Sequence 1 Like
LIHC – Liver Hepatocellular Carcinoma
LUAD – Lung Adenocarcinoma
LUSC – Lung Squamous Cell Carcinoma
NS siRNA – Non-specific siRNA
PRAD – Prostate Adenocarcinoma
STAD – Stomach Adenocarcinoma
TCGA – The Cancer Genome Atlas
GO – Gene Ontology

Keywords: *HN1L*; proto-oncogene; cancer; malignant behavior

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Abstract

Background and purpose: *HN1L is a member of the HN1 gene family and shares about 30% similarity with HN1 which is another member of the family on the primary protein sequence. Since HN1 is an important gene that is involved in various cellular mechanisms and also differentially expressed in carcinogenesis, we investigated the effect of HN1L on some malignant behaviors of various cancer cells.*

Material and methods: *Co-expression analysis, Gene Ontology enrichment, and database searches were performed to predict the cellular roles of HN1, and to investigate its expression in cancers and their corresponding normal tissues. Western blotting and Real-Time PCR were used to compare the expression of HN1L in the normal prostate cells and prostate cancer cells. Cell proliferation and migration assays were used to investigate the effects of HN1L depletion on cell proliferation and migration.*

Results: *The results of co-expression and Gene Ontology enrichment analyses showed that HN1L is co-expressed with DNA replication and DNA damage response/repair associated genes. The database search results revealed that HN1L expression increases in at least 10 diverse cancer types compared to their normal corresponding tissues. This result was confirmed in the prostate cancer cell model, experimentally. Silencing of HN1L inhibited proliferative and migrative behaviors of prostate, breast, colon, and cervix cancer cells.*

Conclusions: *HN1L probably is a novel proto-oncogene that is involved in the DNA metabolism-related mechanisms, and high HN1L level promotes further proliferation and migration in the cancer cells.*

INTRODUCTION

Hematological and neurological expressed 1-like (HN1L) is an evolutionarily conserved gene that encodes a small protein of 190 aa in humans *(1)*. Although the molecular functions of HN1L in the cells are poorly understood, recent studies partially uncovered its cellular functions. HN1L has been shown to be upregulated in many human cancers, such as prostate, breast, hepatocellular, non-small cell lung, and esophageal cancers, which is significantly associated with further malignant behaviors of these cancers *(2-6)*. HN1L contributes to the regulation of cell cycle progression through controlling the cellular levels of some key genes such as Cyclin D1, Cyclin E1, CDK2, CDK4, CDK6, p53, p21, and p27 *(2,5-7)*. Moreover, HN1L has been shown to be involved in multiple signaling pathways to support cell survival and thereby tumor development and progression, in addition to the cell cycle regulation. Increased HN1L level causes sustained activation of the

LEPR-STAT3 pathway in breast cancer stem cells and thereby promotes tumor development *(8)*. Additionally, increased expression of HN1L also causes an increase in the TGF-β activity *(4)*. It was shown that HN1L increases the expression of TCF-3 and ZEB1 in an AP-2γ/METTL13 dependent manner and thereby induces further malignant behaviors in the liver cancer cells *(5,6)*. HN1L also directly interacts with intracellular Ca^{2+} channels and mediates enables NAADP to activate Ca^{2+} release from the endoplasmic reticulum *(9,10)*.

In the present study, we sought to gain evidence of the effects of HN1L expression level on cell proliferation and migration in various cancers. The results of co-expression and gene ontology (GO) term enrichment analyses have shown that HN1L is co-expressed with DNA replication and DNA damage/repair associated genes. Database searches show increased HN1L expression in 10 different cancer types compared to their normal counterparts. Furthermore, experimental results showed that silencing of HN1L represses proliferation and migration in Prostate Adenocarcinoma (PRAD), Breast Cancer (BRCA), Colon Adenocarcinoma (COAD), and Cervical Squamous Cancer (CESC) cells.

Taken as a whole, our results suggested that HN1L may be associated with DNA metabolism-related mechanisms such as DNA replication, DNA damage response/repair. Furthermore, HN1L expression increases in many types of cancer, and elevated HN1L induces further malignant behaviors in cancer cells.

MATERIAL AND METHODS

Bioinformatics analysis

The co-expression analysis was performed in the Oncomine database to investigate the HN1L function (http://oncomine.org) *(11,12)* as previously described *(13,14)*. Only clinical samples were used in this analysis and the threshold was adjusted to p-value < 1E-4, fold change ≥ 2, and gene rank top 1%. Seven arrays met these criteria (Table 1) and each list was created with the top 200 co-expressed genes. These filtered gene lists were then compared with each other to search genes that are repeatedly co-expressed in multiple arrays. Consequently, the genes listed commonly in at least three arrays (> 40% of 7 arrays) were considered as genes co-expressed with HN1L. The HN1L co-expressed genes were analyzed in the web-based DAVID (http://david.abcc.ncifcrf.gov) to determine GO terms *(15,16)*. UALCAN, a web-based platform that is used for accessing data deposited in The Cancer Genome Atlas (TCGA) project for various cancer types, was used to compare the expression of HN1L in tumors and their corresponding normal tissues *(17)*.

Cell lines and culture conditions

PC-3, DU145, LNCaP, MCF-7, CaCo-2, and HeLa cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI1640 or DMEM F/12 (Invitrogen, USA) supplemented with 5–10% fetal bovine serum (FBS). RWPE-1

Table 1. Arrays used in co-expression analysis, HN1L co-expressed genes, and their functional enrichments. *HN1L is co-expressed with DNA replication and DNA damage response/repair associated genes. The co-expression analysis and functional enrichments were performed using the Oncomine, and David, respectively (11,12,15,16).*

Used Arrays				
Kang Leukemia *(23)*, Bourquin Leukemia *(24)*, Zhao Renal *(25)*, Bhojwani Leukemia *(26)*, Bhojwani Leukemia 2 *(27)*, Iacobuzio-Donahue Pancreas 2 *(28)*, Ishikawa Pancreas *(29)*				
HN1L co-expressed genes				
DYNLL1, CCT5, PAICS, RPA1, RUVBL1, PYCARD, PROSC, ANXA4, DPP3, CEP63, KRCC1, SMARCAL1, TRIM14, HTATIP2, TST, HEMK1, DENND2D, ORC3L, GBAP1, SAC3D1, FEN1, TMEM106C, BTN3A2, SRR, BRCA1, UBE2T, TMCO6, BCKDHB, RAD51C, ACADM, PDSS2, RAD50, TTC15, ACYP1, HIBCH, RBM8A, RNF170, EHBP1, HERC4, BICD1, ARFIP1, ZNF280D, PTER, ATR, RRM2, HMMR, TOP2A, CENPM				
Functional enrichment of HN1L co-expressed genes				
GO Term	P-value	Fold	FDR	Genes
0006259 DNA metabolic process	<10E-4	>9	3.35E-05	RPA1, RAD51C, ORC3L, RRM2, SMARCAL, RUVBL1, ATR, TOP2A, RAD50, FEN1, BRCA1, HEMK1
0006260 DNA replication	<10E-4	>16	5.35E-04	RPA1, ORC3L, RRM2, ATR, TOP2A, RAD50, FEN1, BRCA1
0006974 Response to DNA damage stimulus	<10E-4	>8	0.046019	RPA1, RAD51C, ATR, CEP63, TOP2A, RAD50, FEN1, BRCA1
0006281 DNA repair	<10E-4	>9	0.094593	RPA1, RAD51C, ATR, TOP2A, RAD50, FEN1, BRCA1

fold – fold change; GO – gene ontology; FDR – false discovery rate

cells were kindly provided by Prof. Dr. Kemal Sami Korkmaz from Ege University, Department of Bioengineering-Turkey and were propagated using keratinocyte – serum free growth medium (K-SFM) (Invitrogen, USA) as recommended. All cells were propagated in a 37 °C incubator in a humidified atmosphere of 5% CO_2, and the experiments were performed in the logarithmic growth phase.

Antibodies, siRNAs, and transfections

HN1L and β-Actin antibodies were purchased from Bioss antibodies (Beijing, China) and Cell Signaling Technology (Danvers, MA, USA), respectively. Non-specific (NS) (Catalog No: SIC001) and HN1L specific (Catalog No: EHU228371) siRNAs were purchased from Sigma (St. Louis, MO, USA). siRNA transfections were performed using FuGENE HD (Promega-Madison, USA), as described in previous studies *(18-21)*. Briefly; cells were seeded into 60 mm cell culture plates one day before transfection. On the next day, the transfection mix was prepared by adding 3 µl of transfection reagent to 100 µl of pre-warmed medium in a microcentrifuge tube. After incubation of the medium-transfection medium mix for 5 min at RT, 100 pmol of NS-siRNA or HN1L siRNA was added and further incubated for 15 min at RT. Lastly, the transfection mix including siRNA, transfection reagent, and the medium was dropped on the cells.

RNA isolation, cDNA conversion, primer design, and quantitative real-time PCR

Total RNAs were isolated as described in previous studies with minor modifications *(18,19)*. In summary, the RNeasy kit (Qiagen, CA) was used to isolate total RNAs from the cells, and the amount and purity of RNA products were measured by using a nanodrop. 2 µg of total RNA was reverse transcribed using random and anchored oligo dT primers by using Omniscript cDNA synthesis kit (Qiagen, CA). To analyse gene expression changes, quantitative RT-PCR was performed using a SYBR Green RT-PCR kit and the StepOnePlus Real-Time PCR system (Applied Biosystems, USA). The qPCR conditions were set as follows: Initial denaturation at 94 °C for 4 min, followed 40 cycles of denaturation at 94 °C for 30 sec, annealing at 60 °C for 30 sec. and extension at 72 °C for 1 min, and a final extension step at 72 °C for 2 min. The abundance of each amplified cDNA was calculated using the relative quantification method and GAPDH was used as the housekeeping gene, as described previously *(18,19)*. The primers used were as follows; HN1L forward: ACACCCAAACAAACCCAAGG, HN1L reverse: GACCTTGTTGTGAGAGCGAG, GAPDH forward: CATTGCCCTCAACGACCACTTT, and GAPDH reverse: GGTGGTCCAGGGGTCTTACTCC which were designed using Primer3 primer design tool (https://bioinfo.ut.ee/primer3/).

Immunoblotting

The cells were lysed in ice-cold RIPA modified buffer [10 mM Tris-Cl (pH 8.0), 1% Triton X-100, 0.1% SDS, 0.1% Na deoxycholate, 1 mM EDTA, 1 mM EGTA, 140 mM NaCl] including complete protease and phosphatase inhibitor cocktails. Proteins were separated on 12% SDS–polyacrylamide gels and then were transferred onto PVDF membranes using a wet transfer blotter. The membranes were blocked using 5% skim milk which was prepared in TBS-T and then primary antibody incubations were performed at room temperature (RT) for 1 hour on a shaker. The membranes were washed in TBS-T and then secondary antibodies which HRP conjugated were added on the membranes and further incubated for 1 hour, at RT. After washing of the membranes in TBS-T, they were developed using ECL Prime (GE Healthcare - Inghamshire, UK) for 5 min and were photographed using X-ray films in a darkroom.

Measurement of cell proliferation and migration

The WST-1 was used to quantify cell proliferation, as described previously *(22)*. In summary, NS-siRNA or HN1L specific siRNA transfected cells were trypsinized and re-seeded in triplicates in 96-well dishes at a concentration of 4×10^3 cells/well. For designed time points (0, 24, 48, and 72 hours after plating of the cells), WST-1 solution (BioVision, USA) was added at 10 µl per well and further incubated for 4 hours at incubator at 37 °C, 5% CO_2. The absorbances were measured at 450 nm with a SpectraMax M2 microplate reader (Molecular Devices LLC, CA, USA) and obtained values were normalized to 0 hour absorbances. Then, the normalized absorbances were converted to % values, and graphs were constructed using Microsoft Excel.

Cell migration was assessed by Cytoselect 96-well cell migration assay (Cell Biolabs Inc., San Diego, CA, USA) according to manufacturer recommendations. In summary, the cells were seeded in 60 mm plates and next day were transfected with NS-siRNA or HN1L specific siRNAs as described above. 48 hours after transfection, cells were trypsinized, counted, and seeded into upper chambers containing a serum reduced medium (containing 1% FBS) at a concentration of 10×10^3 cells/well, and 100 µl medium containing 10% FBS was added to the bottom chambers. After 24 hours incubation, migrating cells were detached from the underside of the membrane, lysed, and stained according to the manufacturer's recommendations. Then, fluorescence intensities were measured at 480 nm (Excitation)/520 nm (Emission) using SpectraMax M2 microplate reader (Molecular Devices LLC, USA). The relative values were obtained by comparing the fluorescence values of HN1L siRNA transfected cells to NS-siRNA transfected cells.

The Student's t-test was used to assess the statistical significance of any differences between NS and HN1L siRNA transfected cells, and consequently a p-value < 0.05 was considered as significant in both proliferation and migration assays.

RESULTS

GO and pathway enrichment for HN1L

To investigate the probable roles of HN1L in the cells, GO Term enrichment analysis was performed. initially, a

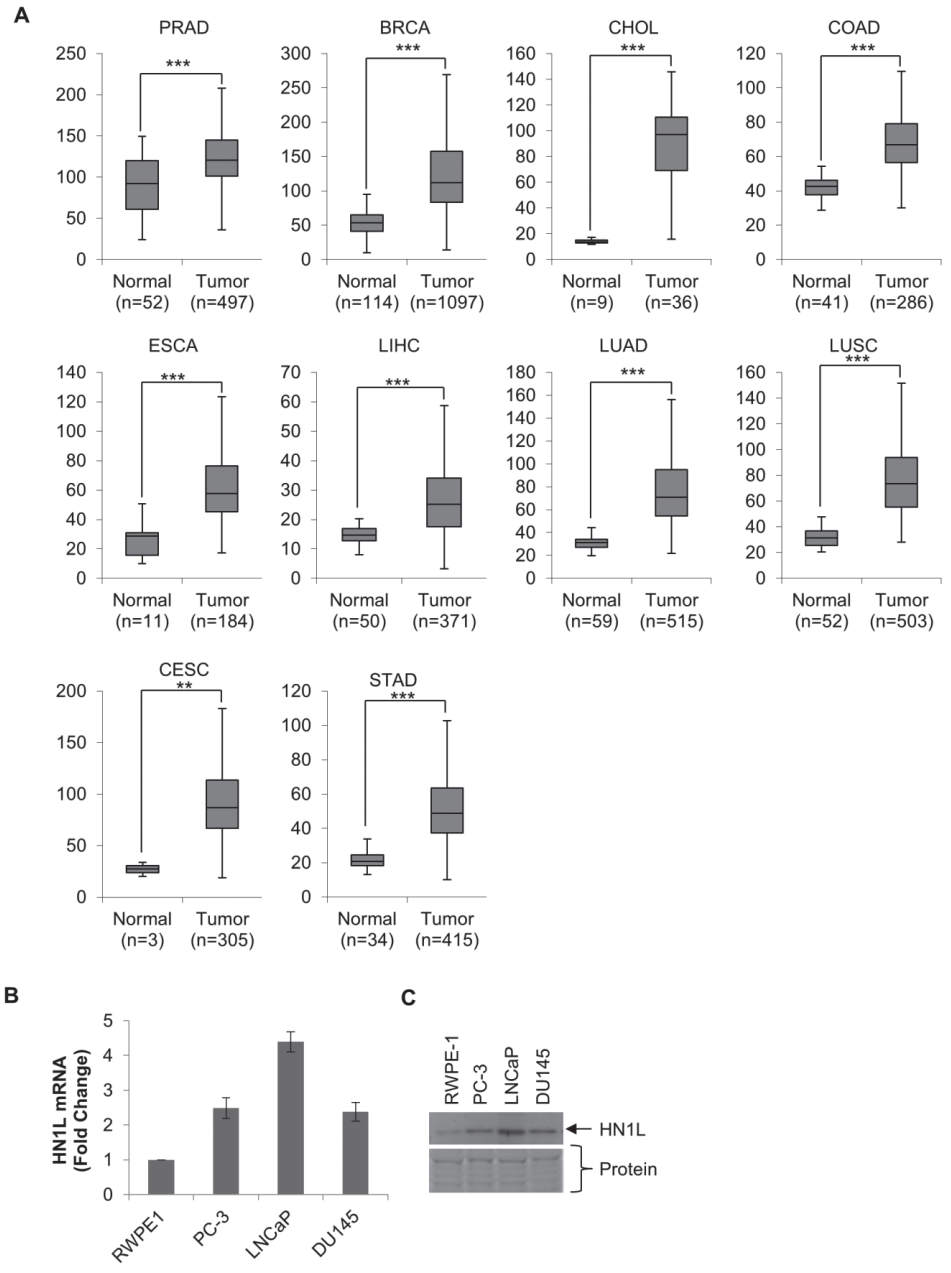

Figure 1. HN1L expression increases in cancer. *(A) HN1L expression increases in PRAD, BRCA, CHOL, COAD, ESCA, LIHC, LUAD, LUSC, CESC, and STAD, compared to their normal corresponding tissues. Statistical values were automatically generated by the database by comparing normal and cancer data (17). *** p<0.0001, and ** p<0.001. The HN1L mRNA (B) and protein (C) levels are lower in RWPE-1 normal prostate cells compared to LNCaP, PC-3, and DU145 PRAD cells. RWPE-1 is derived from the normal prostate, whereas LNCaP, PC-3, and DU145 cells are derived from lymph node, bone, and brain metastasis of PRAD, respectively. LNCaP is androgen-dependent whereas PC-3 and DU145 are androgen-independent cells. PRAD, BRCA, CHOL, COAD, ESCA, LIHC, LUAD, LUSC, CESC, and STAD represent Prostate Adenocarcinoma, Breast Cancer, Cholangiocarcinoma, Colon Adenocarcinoma, Esophageal Carcinoma, Liver Hepatocellular Carcinoma, Lung Adenocarcinoma, Lung Squamous Cell Carcinoma, Cervical Squamous Cancer, and Stomach Adenocarcinoma, respectively.*

co-expression analysis was performed using Oncomine and 49 genes were found to be co-expressed with HN1L in 3 or more studies (Table 1). The results of GO analysis of HN1L co-expressed genes with the DAVID functional annotation tool (GO Biological Processes) under high stringency conditions ($p<0.0001$ and fold enrichment > 2) resulted in 4 GO categories which are "DNA metabolic process", "DNA replication", "Response to DNA damage stimulus" and "DNA repair" (Table 1).

HN1L expression increases in various types of cancer

UALCAN was used to compare the expression of HN1L in tumors and their normal corresponding tissues. The results have shown that HN1L expression has meaningfully increased in PRAD, BRCA, COAD, CESC, Cholangiocarcinoma (CHOL), Esophageal Carcinoma (ESCA), Liver Hepatocellular Carcinoma (LIHC), Lung Adenocarcinoma (LUAD), Lung Squamous Cell Carcinoma (LUSC), and Stomach Adenocarcinoma (STAD) compared to their normal corresponding tissues. To verify the UALCAN results, the mRNA and protein levels of HN1L were investigated in normal prostate and PRAD cell lines. Initially, we examined the HN1L mRNA level in a normal prostate cell line (RWPE-1) and three PRAD cell lines (PC-3, DU145, and LNCaP). The results have shown that HN1L mRNA level increases in all three PRAD cell lines compared to normal prostate cells, in accordance with database results (Figure 1b). Furthermore, we also examined HN1L protein level in normal prostate and PRAD cell lines to confirm whether increased HN1L expression in PRAD cells is at the protein level or not. The results show that HN1L protein level increases in PRAD cells compared to normal prostate cells, in concordance with mRNA results (Figure 1c).

HN1L depletion represses proliferation and migration in all investigated cancer cells

To investigate the effects of HN1L on cell proliferation and migration, the cells were transfected with NS-siRNA

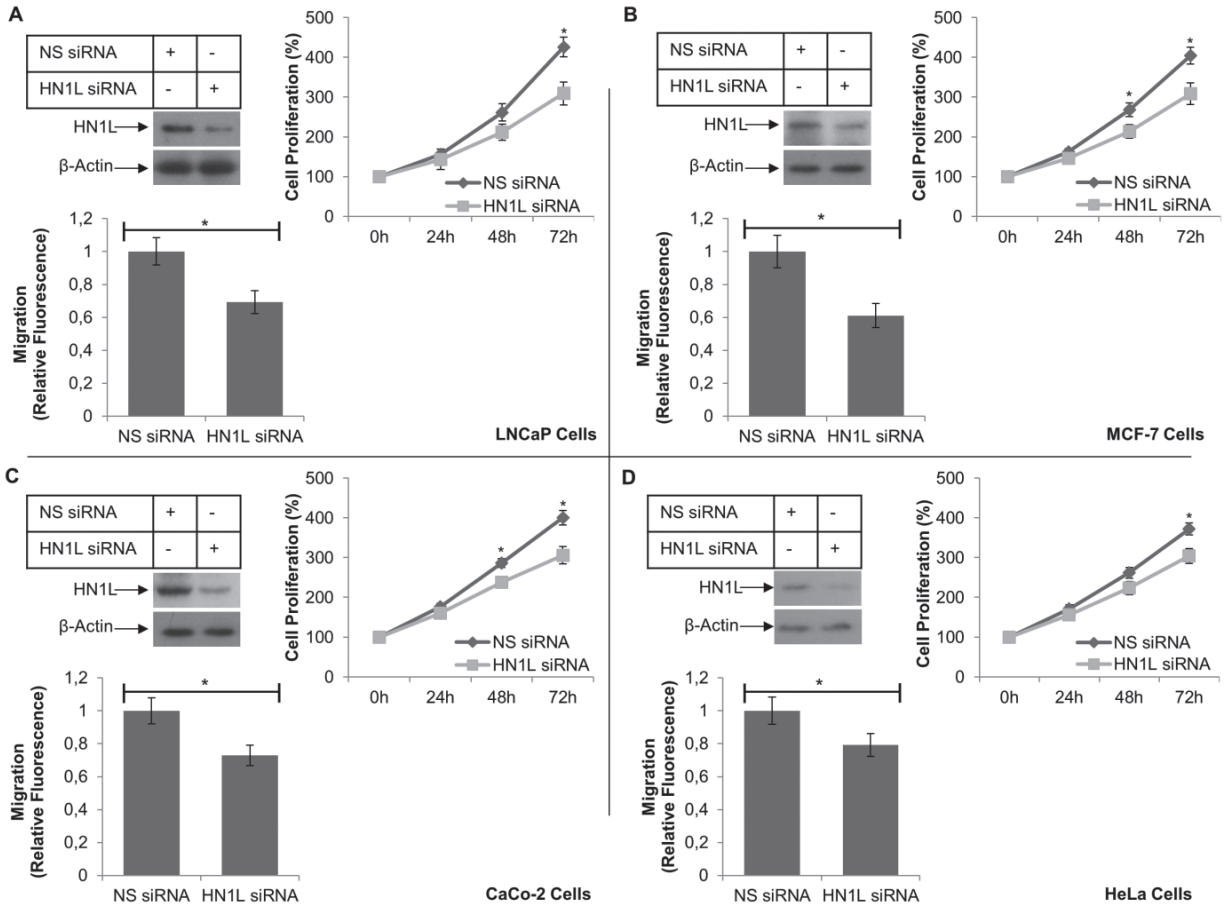

Figure 2. Silencing of HN1L represses proliferation and migration in PRAD, BRCA, COAD, and CESC cells. *Transfection of HN1L siRNA reduced endogenous HN1L level and significantly inhibited proliferative and migrative behaviors of LNCaP (a), MCF-7 (b), CaCo-2 (c), and HeLa (d) cells, which represent Prostate Adenocarcinoma (PRAD), Breast Cancer (BRCA), Colon Adenocarcinoma (COAD), and Cervical Squamous Cancer (CESC). The Student's t-test was used to evaluate the statistical significance and data in the graphs represent means ± standard deviation of three independent results. *<0.05*

or HN1L siRNA, and the proliferative and migrative abilities of the cells were compared. In this experiment, LNCaP, MCF-7, CaCo-2, and HeLa cells were used as the cell models of PRAD, BRCA, COAD, and CESC, respectively. Transfection of HN1L siRNA efficiently suppressed protein level of HN1L to 20-25%, compared to NS-siRNA transfected cells, in all investigated cells (Figure 2a, b, c, d). The cell proliferation results have shown that the proliferation rates of all investigated cells were markedly reduced in HN1L siRNA transfected cells compared to NS-siRNA transfected cells from 48-72 hours onwards ($p<0.05$) (Figure 2a, b, c, d). The results obtained from the cell migration assay showed that HN1L siRNA transfection represses the migrative capabilities of all investigated cells compared to NS-siRNA transfected cells ($p<0.05$) (Figure 2a, b, c, d).

DISCUSSION

HN1L is a member of the HN1 gene family and encodes a small protein that has a Jupiter microtubule-binding domain. In previous studies, we and others have cloned and characterized HN1 which is another member of the HN1 family, in various cell models *(18-21,30-33)*. We have shown that HN1 has critical roles in normal cellular physiology and its expression increases, in many types of cancer. Furthermore, we have shown that increased HN1 level is associated with the malignant behaviors of cancer cells *(18,34-38)*. It was shown that HN1 is an androgen and EGF-regulated gene *(18,19)*. Besides, it was proven that it has inhibitory roles in the PI3K/AKT and androgen signaling in the PRAD cells *(18,19)*. Furthermore, it physically interacts with APC/β-Catenin/GSK3β complex and thereby is involved in the controlling of β-catenin degradation mechanism *(21)*. HN1 also interacts with gamma-tubulin and is involved in the regulation of centrosome organization, in advanced PRAD *(20)*.

Recent reports have shown that HN1L may have similar roles with HN1 in normal and cancer cell physiology. In the previous studies Nong et al. (2021), and Jiao et al. (2021), have shown the association between decreased HN1L level and repressing of proliferation and migration, in prostate and breast cancers *(3,4)*. The results that we presented here are in accordance with their results, and we showed that silencing of HN1L represses malignant behaviors not only in prostate and breast cancers but also in colon and cervix cancer cells. Wang et al. (2021), and Lei et al. (2019) reported a negative association between HN1L level and apoptosis *(2,7)*. In contrast, Li et al. (2017) and Li et al. (2019) reported that HN1L doesn't affect apoptotic processes *(5,6)*. We couldn't see an association between HN1L depletion and apoptotic induction in LNCaP cells (data not shown), in accordance with Li et al. (2017), and Li et al. (2019). On the other hand, it was reported that silencing of HN1L causes cell cycle arrest *(6,7)*. These reports were important to explain the effect of HN1L depletion on the inhibition of cell proliferation. It seems that HN1L, like HN1, is involved in the progression of the cell cycle and the inhibition of cell proliferation caused by HN1L silencing may rely on cell cycle arrest. On the other hand, we have seen that HN1L is co-expressed with the genes associated with DNA metabolism-related mechanisms such as DNA replication, DNA damage response, and repair. Indeed, increased expressions of many genes related to these mechanisms have been shown in various cancers and their association with malignant behaviors have already been reported *(39-42)*. Therefore, although the results of co-expression analysis generally need comprehensive experiments, our analysis has provided a strong starting point to uncover the cellular functions of HN1L in the cells. The report of Jiao et al., which showed HN1L interacts with HSPA9 and up-regulates the expression of HMGB1 was also significant to explain the effects of HN1L on migration, at least partly, since HMGB1 is a cytokine and has crucial roles in epithelial to mesenchymal transition (EMT) and metastasis *(3)*. Therefore, HN1L depletion-dependent inhibition of cell migration may be related to the down-regulation of HMGB1, at least partly. On the other hand, Nong et al., have shown that HN1L level is positively associated with TGF-β activity in PRAD cells, and increased HN1L level promotes stem cell-like properties and cancer progression by targeting FOXP2 in a TGF-β dependent manner *(4)*. Indeed, although TGF-β signaling promotes cell cycle arrest in the normal prostate cells, it induces survival, migration, and invasion mechanisms in the PCa cells *(43)*. However, it seems that these events are independent of EMT-inducing transcription factors such as Slug, Twist1, and Snail which are mostly under the control of TGF-β signaling *(3)*. Furthermore, the role of HN1L as a signal transducer in the NAADP signaling has been reported *(9)*. It was shown that HN1L interacts with Ryanodine Receptor (RYR) and thereby connects NAADP to Ca^{2+} microdomain formation on the RYR, and consequently enables NAADP to activate Ca^{2+} release from the endoplasmic reticulum through RYR *(9,10,44)*. Although the effects of abnormal Ca^{2+} signaling have been shown in the development and progression of cancer, the molecular basis of these effects are not fully understood *(45,46)*. Therefore, increased HN1L dependent promoting of malignant behaviors in cancer cells may be also related to increased activation of Ca^{2+} signaling.

CONCLUSIONS

HN1L seems to be involved in the DNA replication and DNA damage response/repair mechanisms, in addition to its previously identified functions such as regulation of TGF-β, LEPR-STAT3, and Ca^{2+} signaling. Furthermore, the results presented in this study indicated that HN1L expression increases in many types of cancer, and elevated HN1L level is associated with malignant behaviors in cancer cells.

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