JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2023; 7: 410-417
https://doi.org/10.30621/jbachs.1140895
RESEARCH ARTICLE

EXPRESSION OF THE LOW-DENSITY LIPOPROTEIN RECEPTOR (LDLR) GENE FAMILY IN CD133+/CD44+ PROSTATE CANCER STEM CELLS

Burak Cem Soner[1], Eda Acikgoz[2], Fahriye Duzagac[3], Cuneyd Parlayan[4]

[1] Izmir Democracy University, Faculty of Medicine, Department of Pharmacology, Izmir, Turkey
[2] Van Yuzuncu Yil University, Faculty of Medicine, Department of Histology and Embryology, Van, Turkey
[3] The University of Texas, M.D. Anderson Cancer Center, Department of Clinical Cancer Prevention, Houston, USA
[4] Bahcesehir University, Faculty of Medicine, Department of Biostatistics and Medical Informatics, Istanbul, Turkey

ORCID: B.C.S. 0000-0002-3712-3210; E.A. 0000-0002-6772-3081; F.D. 0000-0002-4130-2246; C.P. 0000-0002-6183-9489

Corresponding author: Burak Cem Soner, **E-mail:** burakcemsoner@gmail.com
Received: 05.07.2022; **Accepted:** 12.08.2022; **Available Online Date:** 31.01.2023

Cite this article as: Soner BC, Acikgoz E, Duzagac F, Parlayan C. Expression of the Low-Density Lipoprotein Receptor (LDLR) Gene Family in CD133+/CD44+ Prostate Cancer Stem Cells. J Basic Clin Health Sci 2023; 7: 410-417.

ABSTRACT

Purpose: The low-density lipoprotein receptor gene (LDLR) family plays a fundamental role in many malignancies and may have a putative cancer-boosting function. In our study, we have attempted to comparatively investigate the differential gene expressions of LDLR family in a normal prostate epithelial cell line (RWPE-1), prostate cancer cell line (DU145 cell line), prostate cancer stem cells (DU145 CSCs), and non-CSCs (DU145 non-CSCs, bulk population).
Material and Methods: Cancer stem cells in the DU-145 prostate cancer cell line were isolated by flow cytometry according to CD133 and CD44 cell surface properties. Whole transcriptome sequencing data was comprehensively analyzed for each group. The protein-protein interaction network was determined using the STRING protein database.
Results: Our data showed that the expression levels of Low-density lipoprotein receptor-related proteins (LRPs) such as LRP1, LRP3, LRP8 and, LRP11 were increased in the DU145 CSCs relative to the normal prostate epithelial cell line.
Conclusion: Overall, our data suggest that the LRP functions and/or the expression in prostate cancer may ultimately change the invasive phenotype of the CSCs.

Keywords: LDLR gen family, cancer stem cell, prostate cancer, transcriptional regulators

INTRODUCTION

New advanced targeted therapies are the cornerstone of precision medicine for diagnoses and treatment of cancer, but it is poorly understood for cancer stem cells (CSCs). These stem cell-like cells, known to be associated with poor prognosis, metastasis, and recurrence, have been found to be responsible for chemotherapy and radiotherapy resistance (1). Elimination of CSCs could be significant therapeutic potential in cancer. Previously unknown compounds or proteins appeared to be promising biomarkers or lead compounds in cancer diagnosis and treatments. LRDL family with its known physiological properties show characteristics of prognostic biomarkers for cancer (2,3).

First, the low-density lipoprotein receptor (LDL-R) was found to be a member of the family structurally closely related to transmembrane proteins (3,4).

Recent studies indicated that the LDLR gene family (LRP3, LRP5, LRP6, LRP10, LRP11, LRP12) has key roles in different types of diseases (5). A large body of studies points to the important roles of changes in lipid metabolism in cancer development and progression (6). Considering the multiple functions of LRPs, the determination of the difference in expression levels in both normal cells and cancer cells contributes to revealing new therapeutic targets. A study by Furuya et al. demonstrated that the treatment of prostate cancer cells with simvastatin plays an important role in inhibiting prostate cancer cell growth by reducing cellular cholesterol through modulation of LDLR expression (7). The tumor mass consists of a heterogeneous cell population, and CSCs, which represent a small subpopulation, differ from their counterparts (non-CSCs, bulk populations) in their malignant potential and resistance to therapy. In this context, the differences in the expression levels of LRPs in CSCs and non-CSCs are quite significant. Studies have revealed that high lipid content is associated with tumorogenic and clonogenic potential due to CSCs when compared to the bulk population (8–10). In the literature, data related to the expression levels of the LDLR family in CD133+/CD44+ CSCs are not yet at a sufficient level. In the current study, our aim was to comprehensively investigate the differential gene expressions in the LDLR family and its sub-population in prostate cancer cells.

MATERIAL AND METHODS

Cell Culture

DU145 human prostate cancer cell line and RWPE-1 human prostate epithelial cells line were supplied by the American Type Culture Collection (ATCC) and were grown in RPMI 1640 (Lonza), culture medium containing 10% heat-inactivated fetal bovine serum (Gibco), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich) RWPE-1 cells were maintained in keratinocyte serum-free medium (Gibco) supplemented with 0.05 mg/ml bovine pituitary extract (BPE), 5 ng/ml human recombinant epidermal growth factor (EGF), 100 IU/mL penicillin, and 100 IU/mL streptomycin (Sigma, Aldrich). Cells were cultured in 25 cm2 polystyrene flasks (Corning Life Sciences) and incubated at 37°C in a humidified atmosphere in 5% CO2. When 80% of the flask was filled, the cells were harvested with 0.05% Trypsin-EDTA (Sigma-Aldrich). RPMI 1640 culture medium was added to the harvested cells for trypsin inactivation and centrifuged at 1000 rpm for 5 minutes.

Isolation Of CD133+/CD44+ Prostate Cancer Stem Cell by Fluorescence-Activated Cell Sorting (FACS)

The DU145 cells were subjected to flow cytometric analysis to detect the CD133+/CD44+ cell population in their attainment of the logarithmic proliferation phase. The antibody was diluted with PBS (1:100) and was added to the 1x106 cell pellet and incubated at 4°C for 15 minutes. Samples were labeled with PE-labeled CD133/1 (Clone AC133/1; Milteny Biotec Ltd.) and FITC-labeled CD44 (Clone G44-26; BD Biosciences) and eventually the cells were separated into CSCs and non-CSCs subpopulations with FACS device (BD Biosciences, San Jose, USA) and seeded in culture media.

Next-Generation Sequencing; Whole Transcriptome Analysis

RNA Isolation

RNA isolation was completed as instructed by the manufacturer manual (RNeasy Plus Mini Kit Qiagen, Lot No:148050825), shortly; genomic RNAs were cleared from the homogenized cells, and approximately 75ng/µl eluted. RNA quality was verified by UV absorbance (SpectraMax i3, Molecular Devices).

Library Preparation

Library preparation for sequencing was conducted by TruSeq Stranded RNA LT kit (Illumina, Ref: 15032612, Lot:10037008). Briefly, 750ng total RNA was fragmented by illumine RiboZero (Illumina Lot:10035196) in the first step, then cDNA synthesis was followed. 3’ ends were adenylated and all fragmented sequences were bound to the specifically designed adapters. The adapter bound sequences then were amplified by PCR. Each sample was pooled and normalized.

Library Quantification and Sequencing

The constructed library was prepared in 20 pM in 10 µl reaction volume and then quantified with qPCR with CFX Connect (BioRad) as described by Kappa library quantification (Illumina, Lot: KK4824) kit. As for the quality check each melting curve was checked and the average cycle quantification (Cq) score was set to 7.2. Each group was prepared in 3 technical replicates and sequenced in NextSeq500 (Illumina).

Clustering quality was set to minimum 300 bases as recommended by the manufacturer.

Bioinformatics Analysis

Raw data generated by NextSeq500 were extracted and converted to "fatsq" format by BioSpace (Illumina) database. Genetic mapping, clustering, sequencing, and other bioinformatics analysis were conducted by Genomics WorkBench (GBW) ver.8 (CLC bio,Qiagen) and GRCh 38 reference sequence was used in the analysis. The STRING database was used for integration of protein-protein association networks in genome-wide experimental datasets (https://string-db.org/) (11,12).

Statistical Analysis

RPKM (Reads per Kilobase of transcript per Millon mapped reads) was used as the "expression value" in the parametric statistical calculations. All groups were normalized by the "quantile" normalization algorithm provided by GBW software. T-test was used to identify relative significance and p <0.05 was considered as a "significant" value.

Ethical Approval

The study, by the Izmir Demokrasi University Clinical Researches Ethics Committee (Date: 02.07.2022, report number: 2022-08-01).

RESULTS

Isolation of CD133+/CD44+ Prostate CSCs

In previous studies by our colleagues, prostate CSCs were isolated based on their CD133+/CD44+ cell surface profiles (12–14). To maximize the isolation efficiency, firstly forward scatter and side scatter gating was used, and then CD133+/CD44+ subpopulation CSCs were isolated (Figure-1). We determined the ratio of CD133+/CD44+ cells to be approximately 2.3%.

Whole Transcriptome Analysis of LDLR Family Members

The expression level of LDLR family members is important for both cancer progression and prostate cancer (7). Our aim is to understand the expression levels of LDLR family members in the development of metastasis and survival of cancer cells and CSCs. For this purpose, we analyzed gene expression profiles of LDLR family members in prostate CSCs (DU145 CSCs) comparatively to non-CSCs, DU145) cells and prostate epithelial cells. LRPs associated with different biological processes are expressed in both normal and cancer cells. Furthermore, revealing the expression levels of LRPs in CSCs and non-CSCs is quite significant for new therapeutic strategies to specifically target CSCs. In this context, i) the expression level changes of LRPs between prostate cancer cells and normal prostate cells (G2/G1, G3/G1, G4/G1), and ii) the expression differences of LRPs in CSCs and non-CSCs (G4/G3) were investigated (Table-1).

Our data showed that a marked increase in low-density lipoprotein receptor-related protein-1 (LRP1) expression in the prostate CSCs compared to RWPE-1 cells ($p<0.05$). Furthermore, LRP1 expression in DU145 cell line and non-CSC group is significant compared to RWPE-1 (Figure-2 and Table-1)($p<0.01$). The expression levels of both low-density lipoprotein receptor-related protein-3 (LRP3) and low-density lipoprotein receptor-related protein-4 (LRP4) were increased in the DU145 prostate cancer cell line relative to RWPE-1 cells ($p<0.01$). Moreover, the LRP3 expression level was also found to be increased in both CSC and non-CSC compared to normal prostate epithelial cells ($p<0.05$). Yet comparisons to other group the expression levels of LRP3 and LRP4 were statistically insignificant ($p>0.05$). Similarly, the levels of low-density lipoprotein receptor-related protein5/6 (LRP5/6) gene expression in DU145 cell line, CSC, and non-CSC were found statistically insignificant (Table-1) ($p>0.05$).

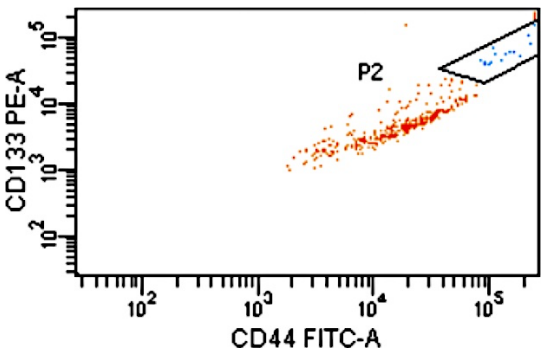

Figure 1. Isolation of CD133+/CD44+ CSCs subpopulation in DU-145 prostate cancer cell line by FACS.

We also observed higher expression of low-density lipoprotein receptor-related protein-8 (LRP8) in prostate cancer, especially in CSC, together with the DU145 cell line and non-CSC (Figure-2 and Table-1) ($p<0.05$) The expression of low-density lipoprotein

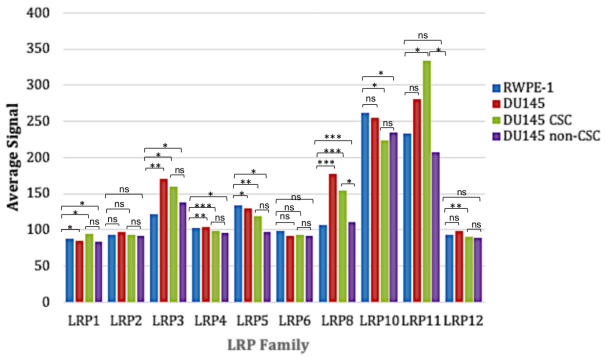

Figure 2. Histogram of whole transcriptome analysis of LDLR family members (Low-density lipoprotein receptor-related protein (LRP)-1,2,3,4,5,6,8,10,11,12) in prostate epithelial cell line (RWPE-1), prostate cancer cell line (DU-145), DU-145 prostate cancer stem cells (DU-145 CSCs) and bulk population (DU-145 non-CSCs) (ns: not significant, *p<0.05, **p<0.01, ***p<0.001).

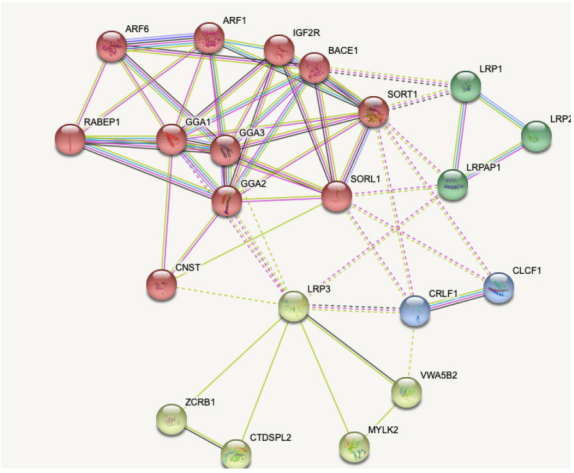

Figure 3. Protein-protein interaction networks of LRP3 using the STRING protein database (Von willebrand factor a domain-containing protein 5b2 (VWA5B2), Golgi associated, Gamma adaptin ear containing, arf binding protein 2 (GGA2), Golgi associated, gamma adaptin ear containing, arf binding protein 1 (GGA1), Alpha-2-macroglobulin receptor-associated protein (LRPAP1), Consortin (CNST), Cytokine receptor-like factor 1 (CRLF1), Golgi associated, gamma adaptin ear containing, arf binding protein 3 (GGA3), CTD small phosphatase-like protein 2 (CTDSPL2), Zinc finger cchc-type and rna-binding motif-containing protein 1 (ZCRB1), Myosin light chain kinase 2, skeletal/cardiac muscle (MYLK2)).

receptor-related protein-10 (LRP10) and low-density lipoprotein receptor-related protein-12 (LRP12) is not significant in DU145 CSC. However, the DU145 cell line shows a significant increase in low expression in LRP12 expression. Low-density lipoprotein receptor-related protein-11 (LRP11) gene expressions showed a statistically significant increase in CSCs (Figure-2 and Table-1) ($p<0.05$). Overall, the results of the study provide further support for the hypothesis that LRP-family in particularly LRP1, LRP3, LRP8, and LRP11 have an impact on CSCs.

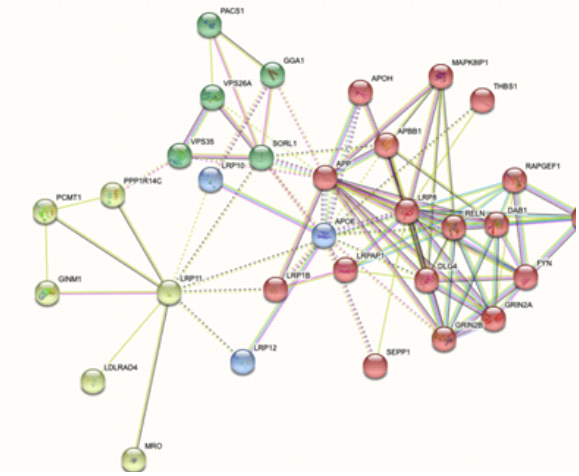

Figure 4. Protein-protein interaction networks of LRP11 using the STRING protein database (Protein phosphatase 1 regulatory subunit 14C (PPP1R14C), Low-density lipoprotein receptor class A domain-containing protein 4 (LDLRAD4), Low-density lipoprotein receptor-related protein 1B (LRP1B), Glycoprotein integral membrane 1 (GINM1), Low-density lipoprotein receptor-related protein 12 (LRP12), Low-density lipoprotein receptor-related protein 10 (LRP10), Protein maestro (MRO), Protein-L-isoaspartate(D-aspartate) O-methyltransferase (PCMT1), Low-density lipoprotein receptor-related protein 8 (LRP18), Sortilin-related receptor (SORL1)).

Protein-Protein Interactions Network of LRPs

The STRING protein database was used to analyze the interaction of LRPs both among themselves and with other proteins. The Markov Cluster Algprithm (MCL clustering) was preferred to simplify interactions in the protein network. In general, it has been found that LRP3 with increased expression level in prostate cancer cells (DU-145 cell line, DU-145 CSCs and DU-145 non-CSCs) compared to normal prostate cells, was associated with Von willebrand factor a domain-containing protein 5b2 (VWA5B2), Golgi associated, Gamma adaptin ear containing, arf binding protein 2 (GGA2), Golgi associated, Gamma adaptin ear containing, arf binding protein 1 (GGA1), Alpha-2-macroglobulin receptor-associated protein (LRPAP1), Consortin (CNST), Cytokine receptor-like factor 1 (CRLF1), Golgi associated, gamma adaptin ear containing, arf binding protein 3 (GGA3), CTD small phosphatase-like protein 2 (CTDSPL2), Zinc

Table 1. Whole transcriptome analysis of LDLR family members in prostate cancer cells RPKM were used as the "expression value" in the parametric statistical calculations

Gene	RWPE-1 (G1)	DU 145-cell line (G2)	DU145 CSC (G3)	DU145 non-CSC (G4)	Ratio G2/G1	p-value	Ratio G3/G1	p-value	Ratio G4/G1	p-value	Ratio G4/G3	p-value
LRP1	87,6	84,2	94,9	83,2	0,96	0,03	1,08	0,02	0,94	0,02	0,87	>0,05
LRP2	92,6	96,6	92,6	92,1	1,04	>0,05	0,96	>0,05	0,99	>0,05	0,99	>0,05
LRP3	121,9	171,1	160,2	137,5	1,4	0,006	1,31	0,02	1,13	0,01	0,86	>0,05
LRP4	102,7	104,1	99	96	1,01	0,01	0,96	0,001	0,93	0,007	0,97	>0,05
LRP5	133,8	129,8	118,9	96,5	0,97	0,02	0,89	0,0006	0,72	0,01	0,81	>0,05
LRP6	98,7	91,2	93,4	91,3	0,92	>0,05	0,94	>0,05	0,93	>0,05	0,98	>0,05
LRP8	106,3	177,7	154,4	111,3	1,67	<0,000	1,45	0,0004	1,05	0,0005	0,72	0,04
LRP10	261,2	254,3	224,3	234,5	0,97	>0,05	0,86	0,03	0,9	0,02	1,05	>0,05
LRP11	233,2	280,8	333,7	207,3	1,2	>0,05	1,43	0,03	0,89	>0,05	0,62	0,01
LRP12	92,7	97,9	90,8	88,9	1,05	>0,05	0,97	0,007	0,96	>0,05	0,98	>0,05

finger cchc-type and rna-binding motif-containing protein 1 (ZCRB1), Myosin light chain kinase 2, skeletal/cardiac muscle MYLK2 (Figure-3).

LRP11 protein interactions highly expressed in CD133+/CD44+ CSCs compared to normal prostate cells were used as precursors in the analyses. The results revealed that LRP11 interacted with Protein phosphatase 1 regulatory subunit 14C (PPP1R14C), Glycoprotein integral membrane 1 (GINM1), Protein maestro (MRO), Protein-L-isoaspartate(D-aspartate) O-methyltransferase (PCMT1), Sortilin-related receptor (SORL1) as well as other LRDs (Low-density lipoprotein receptor class A domain-containing protein 4 (LDLRAD4), Low-density lipoprotein receptor-related protein 1B (LRP1B), LRP12, LRP10, LRP8) (Figure-4).

DISCUSSION

Since the revelation of the LDLR in the early 1980s, a group of fundamentally related proteins having Apolipoprotein-E (ApoE) as a common ligand, has been discovered, and the extensive functions of its members have been defined (4). Whereas a large number of multicellular receptors only exhibit signaling properties, the LDLR family further has endocytic capacities (15). It has been shown that this complex receptor family, with a particular focus on the multiple roles played by LRP1, LRP2, LRP5, LRP6, and LRP8 genes, is crucial in cancer progression (16,17). The results of our study revealed different expression levels of LRPs in both CSCs and non-CSCs compared to normal prostate cells. Furthermore, some LRPs were found to have similar expression patterns in all prostate cancer cell groups. In contrast, LRPs (such as LRP10 and LRP12) with lower expression levels were detected in CSCs subpopulations compared to other groups (DU-145 cell line and non-CSCs). Together, these results indicate that there are CD133+/CD44+ cell population-specific levels of therapeutically targetable LRPs expression.

LRP1, a member of the LDRL family, is involved in the clearance and cellular uptake of proteinases by endocytosis (18). LRP2, or megalin, is a member of this family and is an endocytic receptor expressed on the apical surface of several epithelial cells that internalizes a variety of ligands including signaling molecules, morphogens, and extracellular matrix proteins (4). It was determined that the expression level of LRP2 was especially high in DU145 prostate cancer cells. LRPs, particularly LRP2, effect genetic and epigenetic changes in cancer development by affecting endocytic ligands. Master developmental pathways, such as SHH, Wnt, and BMP, are widely active in the development of primary and metastatic tumors, as well as contributing to cancer initiation in many solid tumors (19) They may undergo LRP-mediated regulation, gradually increase the anti-cancer drug resistance and cause proliferation of cancer cells above all cancer stem cells (20,21). We analyzed the presence of this protease clearance factor in the studied prostate pathologies, given that both LRP1 and LRP2 modulate cell behavior, either as a cargo protein or as a signal transducer mediating endocytosis of a great variety of ligands.

Regulating cellular uptake via LRPs is deemed to be the crucial way of intracellular communication, and as

a consequence of all this, our findings showed a marked increase in LRP1 expression in prostate CSCs compared to normal prostate epithelial cells. LRP2 expression was significantly increased only in the DU145 cell line relative to RWPE-1. A striking adaptation of CSC to limited tumor microzone conditions such as hypoxia and nutritional deficiency has recently been shown (22). The carcinogenic effect of LRP2/Megalin on CSC has so far remained obscure. According to the results we have achieved, we may hypothesize that Megalin is showing its effect by associating on the bulk population. There would therefore seem to be a definite need for larger studies that include analyzes of genetic variation within LRP2 gene and alternative splicing during LRP2 gene expression are needed to increase the consistency of our results. Considering the results, we can assume that CSC used LRP1 more likely as a biomarker network hub due to its role as an extracellular ligand internalizer (18).

LRP3, an important member of the LRP family, is known to be expressed in various human tissues (23). LRP3 has been implicated in the modulation of cellular uptake of β-VLDL (23). Unlike LRP5, which has been shown to participate in the canonical WNT pathway (24), the biological function of LRP3 has not yet been elucidated. LRP4 is a type II transmembrane serine protease and acts as a pro-atrial natriuretic peptide-converting enzyme that regulates blood pressure (25). In our study, the expression levels of both LRP3 and LRP4 were increased in the DU145 prostate cancer cell line relative to the RWPE-1 normal prostate epithelial cell line. LRP3 expression level was also found to be increased in both CSC and non-CSC compared to normal prostate epithelial cells. Comparisons of other group expression levels for LRP3 and LRP4 were statistically insignificant.

The Wnt/ß-catenin pathway contributes to carcinogenesis and malignant behavior, besides that Wnt signal is required for the maintenance of cancer stem cells (26). The binding of Wnt proteins to Fz receptors and LRP5/6 co-receptors leads to the disaggregation of the β-catenin degradation complex, resulting in a decreased cytoplasmic accumulation of β-catenin. β-Catenin is stabilized in the cytoplasm and can translocate to the nucleus where it interacts with T-cell factor/lymphoid enhancer factor (TCT/LEF) thus activating gene-encoding transcription factors for epithelial-mesenchymal transition (EMT) survival, angiogenesis activity, and proteins in the invasion (27). Therefore, improper activation of the canonical pathway may lead to an increase in intracellular β-catenin levels. This may occur as a consequence of the overexpression of Wnt ligands and/or the loss of inhibitors and regulatory proteins or as a mutation in β-catenin and other proteins involved in the pathway (28). Controversially in our study, the levels of LRP5/6 gene expression in DU145 cell line, CSC and non-CSC were found statistically insignificant. This may be due to the fact that the Wnt receptor Frizzled (Frz) and the co-receptors LRP5 and LRP6 (LRP5/6) interact directly with each other, and this interaction may be regulated by the external domain of LRPs.

Proof of epigenetic alteration of miRNA expression is to promote angiogenesis (29). These miRNAs targeting ApoE, which binds to the LRP8 receptor in endothelial cells, have been shown to regulate micro vessel density, endothelial cell uptake, and metastasis, and their increased expression was associated with poor prognosis (30). Similar to the literature in our findings, we also observed the increased expression of LRP8 in prostate cancer, especially in CSC, together with the DU145 cell line and non-CSC. Overexpression of LRP8 in DU145 CSCs suggests that this receptor and its signaling function are important for in vitro survival of cells and that also initiates signaling events as well as being a passive transmembrane transporter of lipids (31). Moreover, like LRP5/6, LRP8 has been reported to interact with Wnt pathway, indicating that LRP8 might serve as a novel Wnt co-receptor (31). Since Wnt signaling plays a critical role in regulating the self-renewal of CSCs, these findings may help us to understand why CSCs express a high level of LRP8. In our data, LRP10 and LRP12 expressions are not significant in DU145 CSCs. On the other hand, LRP11 gene expressions showed a statistically significant increase in CSCs over normal epithelial cell line. In the study of Gan et al. in prostate cancer, the LRP11/β-catenin/PD-L1 regulatory network was investigated and it was found that LRP11 and PD-L1 expression was quite high compared to matched normal tissues and LRP11-dependent induction of PD-L1 was associated with the β-catenin pathway (32).

CONCLUSION

In concordance with the important role of the LRP family in many malignancies, we have attempted to determine the metabolic adaptivity of the LRP family in prostate cancer. Interestingly, one of the more

significant findings to emerge from this study is the possible effect of LRP1 and LRP8 on prostate cancer, especially in CSC. We can extrapolate that the LRP functions and/or the expression in prostate cancer may ultimately change the invasive phenotype of the CSC. New possibilities for eliminating the tumor-promoting and metabolic adaptive functions of CSC through LRP targeting can be considered as a therapeutic application.

Acknowledgement: None.

Author contribution: BC. Soner devised the project. BC. Soner, E. Acikgoz and F. Duzagac conceived and planned the experiments. E. Acikgoz and F. Duzagac carried out the experiments. BC. Soner, F. Duzagac, C. Parlayan and E. Acikgoz contributed to the interpretation of the results. C. Parlayan performed the analytic calculations. BC. Soner wrote the manuscript with support from E. Acikgoz, F. Duzagac. All authors discussed the results and contributed to the final manuscript.

Conflict of interests: None.

Ethical approval: The study, by the Izmir Demokrasi University Clinical Researches Ethics Committee (Date: 02.07.2022, report number: 2022-08-01).

Funding None.

Peer-review: Externally peer-reviewed.

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