Pancreatology 19 (2019) 429–435

Contents lists available at ScienceDirect

Pancreatology

journal homepage: www.elsevier.com/locate/pan

Over-expression of low-density lipoprotein receptor-related Protein-1 is associated with poor prognosis and invasion in pancreatic ductal adenocarcinoma

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ARTICLE INFO

Article history:
Received 1 December 2018
Received in revised form
16 February 2019
Accepted 23 February 2019
Available online 14 March 2019

Keywords:
PDAC
LRP-1
Prognosis
Invasion

ABSTRACT

Background: Low-density lipoprotein receptor-Related Protein-1 (LRP-1) has been reported to involve in tumor development. However, its role in pancreatic cancer has not been elucidated. The present study was designed to evaluate the expression of LRP-1 in Pancreatic Ductal Adenocarcinoma Cancer (PDAC) as well as its association with prognosis.

Methods: Here, 478 pancreatic cancers were screened for suitable primary PDAC tumors. The samples were analyzed using qRT-PCR, western blotting, and Immunohistochemistry (IHC) staining as well as LRP-1 expression in association with clinicopathological features.

Results: The relative LRP-1 mRNA expression was up-regulated in 82.3% (42/51) of the PDAC tumors and its expression (3.72 ± 1.25) was significantly higher than that in pancreatic normal margins (1.0 ± 0.23, $P < 0.05$). This up-regulation was stage dependent ($P < 0.05$). A similar pattern of LRP-1 protein expression was discovered ($P < 0.05$). The high expression of LRP-1 in the PDAC tissues was strongly correlated with the low survival time ($P = 0.001$), TNM classification ($P = 0.001$), low differentiations status ($P = 0.001$), lymphatic invasion ($P = 0.01$) and Perineural Invasion (PNI) status ($P = 0.001$).

Conclusions: Our finding for the first time revealed that LRP-1 expression inversely associated with poor prognosis and PNI in PDAC tumor.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the most lethal cancer that is responsible for more than 330,000 deaths per year worldwide; this cancer is also characterized as the fourth leading cause of cancer-related deaths in the USA [1]. It has been reported that only 10–20% of the diagnosed pancreatic cancer surgically resectable, and its 5-year survival rate is less than 5% [2]. Despite advances in this area, the early detection method for diagnosis and metastasis of this type of cancer is still poorly identified.

Perineural invasion (PNI) is the neoplastic invasion of cancer cells to nerves which is widely believed as the most important adverse feature of some malignancies, such as PDAC. It also has been indicated to have a relationship with a poor prognosis of PDAC. The exact matches mechanism underlying PNI is largely unclear, however, growing body of evidence has been shown that PNI pathogenesis has placed a significant emphasis on the active role of some protein expression [3].

Collectively, finding a potential biomarker for detection of prognosis and PNI is needed to improve early detection and taking the best decision for proper treatment of PDAC. Emerging studies showed that PNI, invasion, and metastasis of PDAC are partially regulated by the over and down-regulation of some proteins [4]. Using the study of genes which are related to prognosis and PNI, we hypothesized that LRP1 might be strongly involved in this area.

The Low-Density Lipoprotein (LDL) receptor-related protein (LRP) superfamily contains seven transmembrane proteins participating in a wide range of physiological processes [5]. LRP-1 has been shown to widely expressed in numerous tissues which have a vital role in several processes such as lipoprotein

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https://doi.org/10.1016/j.pan.2019.02.012
1424-3903/

metabolism, cellular trafficking, and regulation of cell surface protease activity. This protein is a large functional endocytic receptor which primarily synthesized as a 600-kDa precursor protein that cleaved to an extracellular, ligand-binding subunit of 515-kDa and a *trans*-membrane 85-kDa subunit. Dysregulation of LRP1-dependent signaling events is more likely to be associated with the development of pathophysiologic processes such as Parkinson's and Alzheimer's disease as well as atherosclerosis, inflammation, and coagulation. Furthermore, in an animal model, specific depletion of LRP1 in endothelial cells leads to abnormal development of new vessels suggesting its role in angiogenesis [6]. As indicated by Lee et al., LRP-1 is a receptor to internalize its ligand. They used LRP-expressing MEF-1 cells and LRP-deficient PEA13 to assess insulin-like growth factor binding protein 3 internalization. They found that the rate of IGFBP-3 internalization was significantly lower in PEA13 cells than in MEF-1 cells. Moreover, the internalization of IGFBP-3 in MEF-1 cells was blocked by the LRP-1 antagonist, suggesting that LRP1is responsible for the endocytosis of IGFBP-3 [7]. Additionally, it has been shown that after internalization, the LRP1 recycled back to the cell surface [8]. In this regard, it has the ability to bind with more than 30 different ligands from extracellular and deliver them to the cytoplasm [9]. In addition, it has been shown to interact with intracellular signaling proteins and acts as a co-receptor. These functions may emphasize the LRP1 roles in a wide variety human disease such as cancer and its related disorders. The role of LRP1 is not limited to ligand internalization. In vitro and in vivo evidence demonstrates that LRP1 has a vital role in many other processes such as tumorigenesis and tumor progression. For instance, LRP1 promotes tumor cell migration by regulating matrix metalloproteinase (MMP)-2 and MMP-9 expression. Additionally, it also inhibits cell apoptosis by regulating the insulin receptor [10].

In this study, we investigated the LRP1 expression (using qRT-PCR, western blotting, and IHC staining) in PDAC tumor in compared with paired (matched) adjacent normal tissue and analyzed its association with prognosis in patients with PDAC. To the best of our knowledge, the current study provides the first evidence that elevated LRP1 expression is related to PDAC prognosis, PNI and lymph node invasion.

Materials and methods

Patient information

The biological materials were provided by the Iran National Tumor Bank, which is founded by the Cancer Institute of Tehran University of Medical Sciences, for Cancer Research (Iran). More than 478 tissue sections of patients with pancreas cancer who underwent surgery at the Cancer Institute of Iran from 2003 to 2017 were examined to find suitable cases for this study. The histology data of patients, according to the pathology report include the histology, tumor size, grade, lymphovascular invasion and clinical TNM (Tumor, Node, Metastasis) staging were recorded by a pathologist of pathology laboratory of the Cancer Institute of Iran and rechecked and confirmed by second pathologist of Iran National Tumor Bank at the beginning of the study. Patients were diagnosed with primary pancreas cancer based on histopathological examination were included in our study. Patients with chronic or acute inflammatory diseases or second tumor, pancreatitis and other malignancies were excluded from the study. Subjects with chemotherapy or radiotherapy prior to surgery were also excluded from the study. None of the patients were smokers and none of them consumes alcohol. Based on exclusion and inclusion criteria, 51 paired of tissue samples including 11 fresh frozen and 40 FFPE tissue samples were selected to participate in our research project. The blocks then sectioned and examined to verify tissue and confirm tumor or normal tissue. The tumoral and normal morphology of all samples were performed by pathology expert using Hematoxylin and Eosin staining. Informed consent was obtained from all individual participants included in the study. All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee of the 2013 Helsinki declaration and also this study was approved by ethic committee of Isfahan University of Medical Science (Ethic number:395901). Overall survival was defined as the survival time after surgery. Patient characteristics are shown in Table 1.

Protein extraction and western blot analysis

FFPE tissue sections were deparaffinized, in brief, the paraffin of samples was removed by incubating the samples in two changes of xylene for 10 min each. After incubation, the samples were pelleted at 16,000×*g* for 3 min, and the incubation/centrifugation steps were repeated again. The samples were rehydrated through a series of graded alcohols for 10 min each: 100% ethanol, 85% ethanol, and 70% ethanol and then briefly air-dried in a fume hood. Then, the tissue pellets from FFPE section, fresh frozen tissues and cultured cells were homogenized by a Power Gen 125 Tissue Homogenizer (Thermo Fisher Scientific, Waltham, MA) in 100 vol of protein extraction buffer containing 500 mM Tris–HCl pH 8.0 and 2% SDS. The samples were incubated at 90 °C for 90 min. The extracts were centrifuged for 20 min at 16,000×*g* at 4 °C, and 4 μL of each supernatant was subjected to SDS-PAGE and finally, the supernatant was aliquoted and stored at −70 °C until further examination. The western blotting analysis was performed as described previously [11]. Equal concentration of each sample protein lysates (50 μg) was boiled for 10 min and loaded into a 10% SDS-PAGE gel followed by transferring into a PVDF membrane. Non-specific sites of the

Table 1
The relationship between the LRP-1 mRNA expression in the tumor tissues and the demographic variables of PDAC patients.

Parameter	LRP-1 mRNA expression[a]			P value [b]
	Number of cases	Low	High	
Age (year)				0.95
≥65	19	11	8	
<65	32	12	20	
Sex				0.97
Male	27	14	13	
Female	24	9	15	
Tumor Location				0.72
Head	30	12	18	
Body/tail	21	11	10	
Tumor size (cm)				0.47
≥2	30	10	20	
<2	21	13	8	
Lymphatic invasion				***0.01***
Positive	34	13	21	
Negative	17	10	7	
TNM stage				***0.001***
0 + I	19	11	8	
II	19	10	9	
III + IV	13	2	11	
Tumor differentiation (Grade)				***0.001***
Well	23	19	4	
Moderate	18	4	14	
Poor	10	0	10	
Perineural invasion(PNI)				***0.001***
Positive	32	16	16	
Negative	19	7	12	

[a] The LRP-1 mRNA Expression was quantified based on GAPDH in tumor and adjacent normal tissues using $2^{-\Delta\ \Delta CT}$ from at least 2 experiments.
[b] *P-value was calculated from Independent-samples T Test and One-Way Anova.

membrane were blocked with 5% bovine serum albumin in 0.2% Tween-20 in Tris-buffered saline (TBS-T) for 1 h at room temperature and incubated overnight with indicated primary antibodies (anti-LRP-1 and anti-GAPDH) followed by adding the horseradish peroxidase-conjugated secondary antibodies (1: 7000). The bound secondary antibodies were located with the ECL western blotting detection system (TaKaRa Inc., Kyoto, Japan) according to the manufacturer's instructions. Each membrane was exposed to the film for 2 min. All blots were stripped with GAPDH as a loading control. All bands were quantified using Scion Image software.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted from 10 μm thick sections of the FFPE samples of the pancreatic cancer tumors and paired normal adjacent pancreatic tissues, by RNeasy FFPE Kit (Qiagen, Hilden, Germany) according to the manufacturer instruction. Total RNA of fresh frozen samples and cultured cells were also extracted using TRIsol extraction reagent (Gibco, Life Technologies, and USA). Final extracted RNA was checked by Agarose gel electrophoresis. The RNA were used for cDNA synthesis using a TaKaRa cDNA synthesis kit (TaKaRa Inc., Kyoto, Japan) in a 10 μL reaction mixture (final volume) containing 3 μL RNA, 1 μL Prime-Script RT enzyme mix, 5 μL Prime Script buffer, and 0.5 μL random hexamer primer, 0.5 μL oligo dT primer. cDNA was generated by incubating this reaction mixture at 85 °C for 1 min and 37 °C for 15 min. Produced cDNA was subjected to SYBR Green (Qiagen, Hilden, Germany) based on standard quantitative real-time RT-PCR analysis using an ABI 7500 Sequence Detection System (Applied Biosystems). The sequences of the primers were as follows: LRP-1 forward, ACG AGT GCC AGA ACC TCA; LRP-1 reverse, GGC GTC AGA GAA GTA GAG C; GAPDH forward, CAG CCT CAA GAT CAT CAG C; GAPDH reverse, GGC AGT GAT GGC ATG GAC T. Based on manufacturer's instruction, the melting curve was generated at the end of each examination to verify that just a single product was amplified. The real-time PCR reaction conditions were set as follows: denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 50 °C for 30 s, and extension at 72 °C for 1 min. The gene expression was relatively quantified by $2^{-\triangle\triangle CT}$ method. All raw data was presented as the relative expression, normalized against the GAPDH as an internal control.

Immunohistochemistry

Immunohistochemical analysis for formalin-fixed, paraffin-embedded sections was performed. In brief, sections were stained with rabbit anti-LRP-1. After a series of washing with PBS and then horseradish peroxidase-linked secondary antibody was incubated for 1 h and visualized by chromogenic 3,3′-diaminobenzidine tetrachloride as a substrate. The hematoxylin was then used for counterstaining [12].

Cell culture

Human AsPc-1 pancreatic adenocarcinoma cells (Pasteur, Iran) were cultured in RPMI-1640 medium (Bioidiea, Iran) supplemented with 10% FBS, 1 mM of sodium pyruvate, 2.5 g/L glucose, penicillin (100 U/ml) and streptomycin (100 μg/ml). MiaPaCa-2 Cells (Pasteur, Iran) were grown in DMEM with 2 mM glutamine, 1 mM Na-pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin and 10% fetal bovine serum (FBS). The cells were incubated in a humidified incubator with 5% CO2 at 37 °C.

Statistical analyses

A chi-square analysis was performed to detect the correlations of the categorical variables. The *t*-test was used to evaluate the significance of the differences in the tumor and normal adjacent tissue for western blotting and qRT PCR analysis. An Mc Nemar's test and the Mann–Whitney *U* test were carried out to compare LRP-1 staining between the tumor tissues and the adjacent non-tumor tissues. The Kaplan–Meier method was used to evaluate the survival analysis. SPSS (Statistical Package for the Social Sciences version 20; SPSS Inc., Chicago, IL, USA) was used for the statistical analyses. $P < 0.05$ was defined as being statistically significant.

Results

Comparison of mRNA expression level of LRP-1 between pancreatic cancer tissues and paired normal adjacent pancreatic tissues

LRP-1 expression was detected in all 51 pairs of pancreatic cancer and matched normal pancreatic tissue samples by qRT-PCR. The relative mRNA expression of LRP-1in pancreatic cancer tissues (3.72 ± 1.25) was significantly higher than that in matched adjacent normal pancreatic tissues (1.00 ± 0.23, $P < 0.05$; Fig. 1a). Further analysis on TNM staging revealed that up-regulation of LRP-1 mRNA expression was stage dependent ($P < 0.05$). For instance,

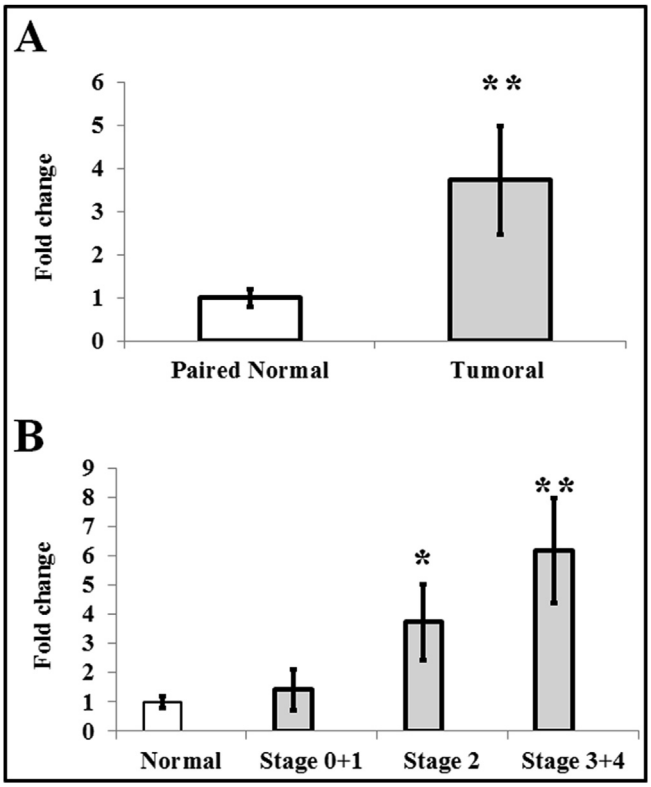

Fig. 1. The LRP-1 mRNA levels determination. **A**, The LRP-1 mRNA expression in tumor tissues and adjacent normal tissues of 51 patients with PDAC assessed with real-time PCR. **B**, mRNA relative expression of LRP-1 in a different stage of PDAC. The samples were classified based on TNM staging and the LRP-1 mRNA expression of each stage was reported. mRNA values of each sample were calculated using $2^{-\Delta\Delta CT}$ based on GAPDH. The tumoral and normal morphology (paired normal) of all samples were checked by pathology expert using Hematoxylin and Eosin staining. * stands for the statistical difference with normal tissues (*$P < 0.05$), ** is for $P < 0.01$, # is for the statistical difference with previous stages $P < 0.05$.

the mRNA expression increased from 1.0 ± 0.23 to 1.41 ± 0.71, 3.72 ± 1.25 and 6.15 ± 1.94, for Stage 0 + 1, Stage 2 and Stage 3 + 4, respectively. Statistical analysis showed that there was a significant difference between each stage in comparison with the previous stage ($P < 0.05$: Fig. 1b).

Comparison of protein expression level of LRP-1 between pancreatic cancer tissues and adjacent normal pancreatic tissues

LRP-1 protein expression was similarly evaluated in all 51 pairs of pancreatic cancer and matched normal pancreatic tissue samples by western blotting analysis and IHC staining. The relative protein expression of LRP-1 (LRP-1 values of expression were divided to GAPDH values) in pancreatic cancer tissues was significantly lower than that in matched adjacent normal pancreatic tissues (1.42 ± 0.48 for stage 0 + 1, 2.24 ± 0.63 for stage 2 and, 3.3 ± 0.93 $P < 0.05$; Fig. 2). This result indicated that protein expression increased stage dependently. Further analysis of IHC scoring similarly indicated that LRP-1 level was increased in PDAC tumor tissue in a stage-dependent manner ($P < 0.05$; Fig. 3).

LRP-1 expression level in PDAC cell lines

We investigated the protein and mRNA of LRP-1 in two PDAC cell lines. The values of protein expression were quantified using GAPDH, as an internal control. The LRP-1 mRNA and protein expression was higher in AsPc-1 cell line as compared to MiaPaCa-2 cells (Fig. 4).

Correlation between the level of LRP-1 expression and survival time

The 51 pancreatic cancer patients were divided into a low expression group relative expression (lower than mean) and a high expression group (higher than mean), based on the median expression level of LRP-1. Survival analysis showed that month after surgery (survival) was significantly different among 51

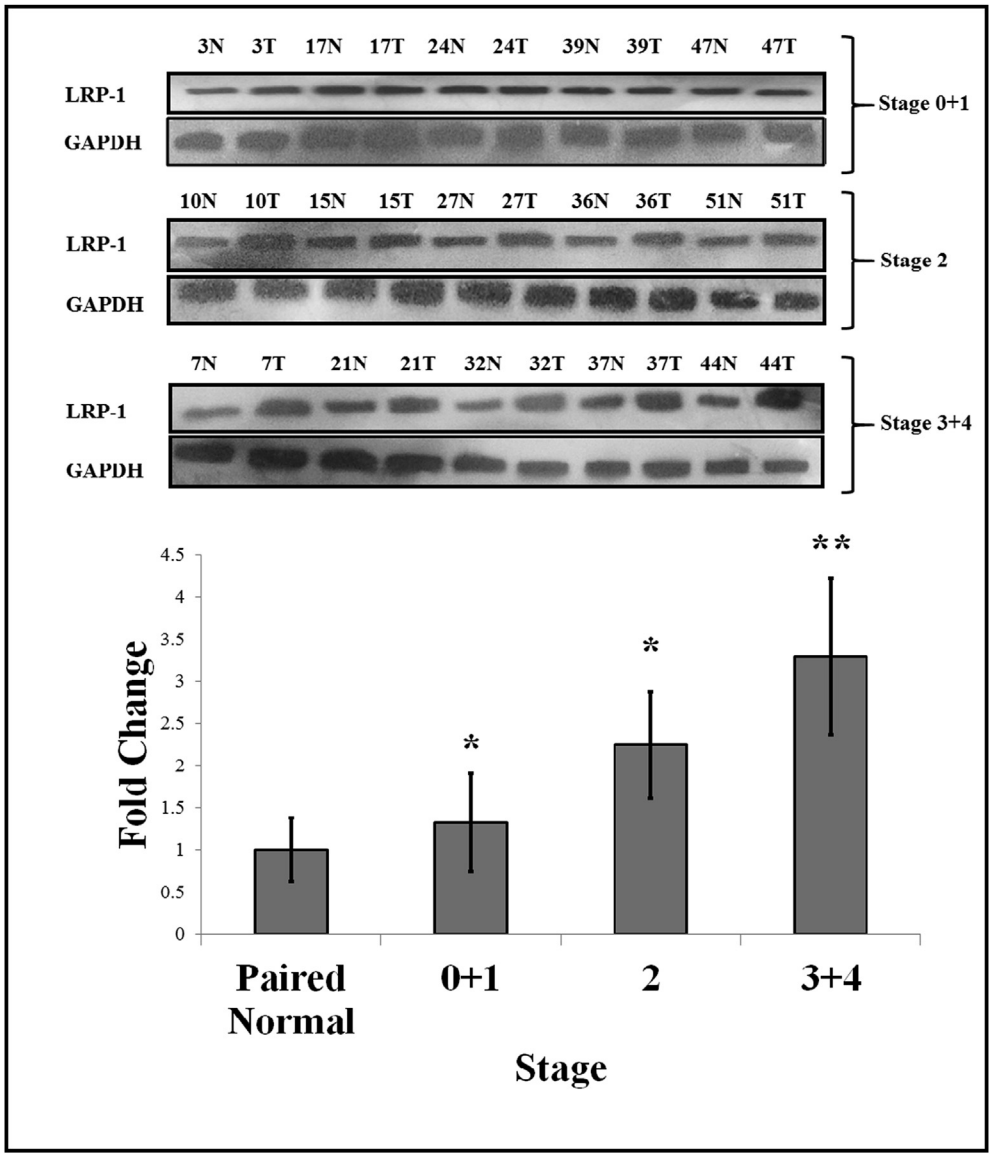

Fig. 2. Western blotting detection for LRP-1 protein expression. **A,** bands of western blotting in malignant tissues compared to adjacent normal pancreatic tissues. **B**, Protein relative level of LRP-1 was calculated by determining intensities of LRP-1 bands relative to GAPDH bands using ImageJ software. The tumoral and normal (paired normal) morphology of samples were evaluated by pathology expert using Hematoxylin and Eosin staining. **P < 0.01 is significant.

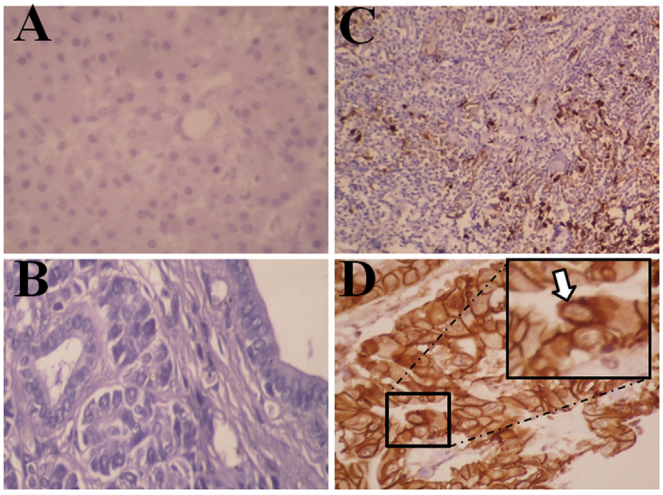

Fig. 3. Representative images of immunohistochemical staining of LRP-1 in adjacent normal tissue and PDAC tumor tissues. **A**, was for negative control of normal cells (× 100x). **B**, was for negative control of tumor cells (× 400x), **C,** was for low LRP-1 expression (× 400x) and D was for high LRP-1 expression (× 400x) the arrow indicated the LRP-1 location in cells.

patients according to LRP-1 expression status (P = 0.001) (Fig. 5) by using the Kaplan-Meier analysis [13]. Patients with low LRP-1 expression had a significantly longer survival than those with high LRP-1 expression (median survival, 17.75 ± 1.59.vs. 6.73 ± 0.85 months, respectively $P = 0.001$).

Correlation between LRP-1 expression and clinicopathological features

The association between LRP-1 expression and the clinicopathological variables in pancreatic cancer patients are summarized in Table 1. The high expression of LRP-1 in the pancreatic cancer tissues was strongly correlated with the TNM classification ($P = 0.001$), tumor differentiation ($P = 0.001$), lymphatic invasion ($P = 0.01$) and PNI status ($P = 0.001$). However, the level of LRP-1 expression in pancreatic cancer tissues was not associated with the age ($P = 0.95$), gender ($P = 0.97$), tumor location ($P = 0.72$) and tumor size ($P = 0.47$).

Discussion

Growing number of investigations showed that LRP1 expression levels are often impaired in cancer, while LRP1 roles are different from one tumor type to another. The LRP-1 expression has become a confusing question that is a tumor and metastasis promoter or suppressor. Studies have shown that low expression of LRP1 is closely associated with the aggressive phenotypes of cancer. For instance, it has been indicated that low expression of LRP1 was closely related to poor prognosis of hepatocellular carcinoma, colon, melanocytic tumors, Wilms [14] and lung tumors [15]. Conversely, LRP1 over-expression has been presented in several other types of cancer such as breast carcinoma, endometrial carcinomas [16], prostate cancer [17] and glioblastoma [18]. As an example of such finding, it has been reported that the up-regulation of LRP seems to correlate with the expression of the urokinase receptor (uPAR) and consequently induction of invasion astrocytomas [19]. Furthermore, the LRP 1 expression exhibited a correlation with the proliferation of Her-2/neu and triple-negative breast carcinoma [20].

In the present study, our finding clearly indicated that LRP-1 was overexpressed in PDAC tumor in a stage-dependent manner (Figs. 1–4). In parallel with such observation, we showed that over-expression of LRP-1 conversely related to survival time (Fig. 5). This finding in line with other facts about PDAC might be providing an opportunity for effective diagnosis and treatment. As an example of such observation, HER-2/neu has been produced as a prognostic factor and a therapeutic target as well [5]. In addition, due to ligand internalization ability of LRP-1, more recently it has been used targeted delivery [21].

Our data indicated that there is a close relationship between LRP-1 expression and both PNI and lymph node invasion (Table 1). Another related study also indicated that LRP-1 effects on invasion in various types of cancer cells may be closely related to the abundance of LRP-1 and its ligands concentration as well as other cytoplasmic proteins and *trans*-membrane receptors interacting with LRP1. In this regard, LRP1 Knocking down or genetically deletion, decreased invasiveness of glioblastoma cells, and thyroid carcinoma cells [22]. With a glance to LRP-1 roles in the mechanism underlying PNI and lymph node invasion, it is well established that MMP2 and MMP-9, are extremely involved in the invasion and metastasis of several types of human cancers. A large body of experiments also indicated that MMP2 and MMP9 are both over-expressed in down-stream of LRP-1 activation through an ERK-dependent pathway [23]. In addition, it has been reviewed that collagens which are a substrate of MMPs are known to be present in

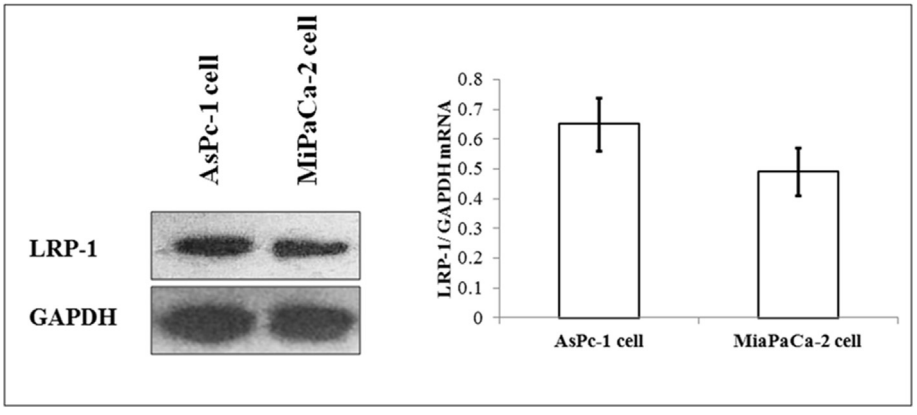

Fig. 4. The LRP-1 expression in PDAC cell lines. The LRP-1 expression in both AsPc-1 and MiaPaCa-2 cell lines were evaluated by western blotting and Real-time PCR based on GAPDH as an internal control.

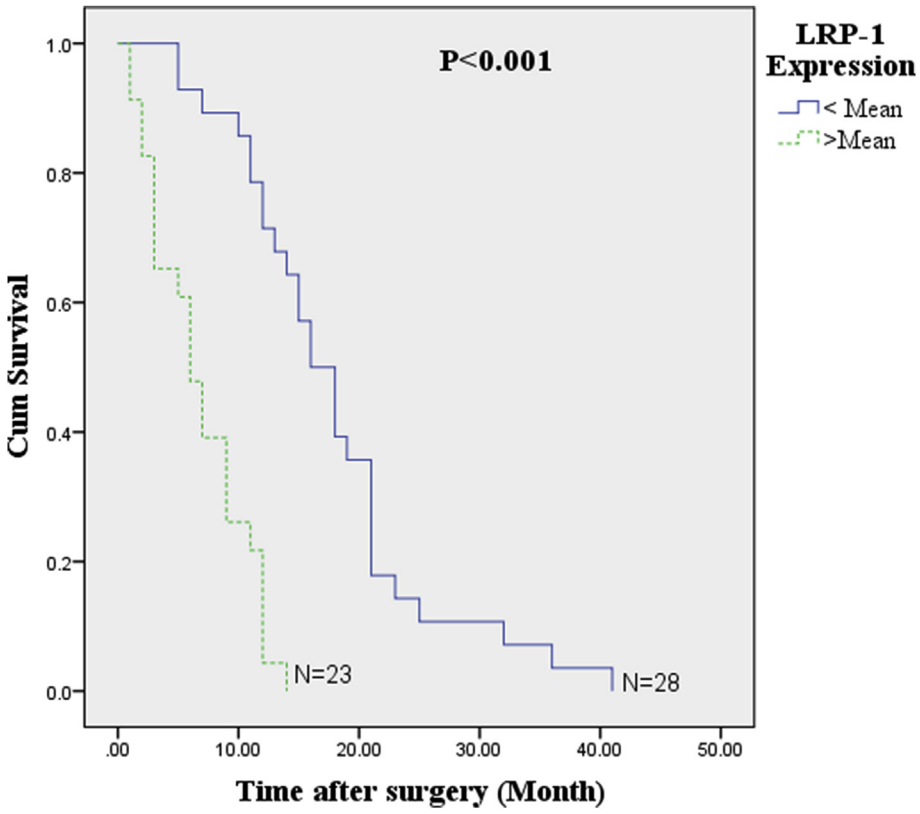

Fig. 5. Kaplan-Meier survival analyze by LRP-1 expression. The high expression level of LRP-1 was significantly correlated with the shorter overall survival time of pancreatic cancer patients (median survival, 17.75 ± 1.59.vs. 6.73 ± 0.85 months, respectively $P = 0.001$).

Schwann cell basement membrane. Further analyses have been revealed that both MMP-2 and MMP9 were over-expressed in pancreatic cancers [24]. Similarly, Chuang et al., have reported that over-expression of MMP-2 and MMP-9 promotes a more invasive cancer phenotype in human oral squamous cell carcinomas [25]. Therefore, over-expression of LRP-1 and MMPs leading to the higher motility of cancer cells which are resulted in PNI induction and lymph node invasion. As mentioned previously ERK activation and JNK inhibition are the main molecular signaling pathway located downstream of LRP-1 [26]. Moreover, these data are consistent with those of previous studies indicating that ERK activation is one of the main paradigms of PDAC [27]. JNK inhibition was also shown to be required for tumor cell invasion and metastases [26]. Growing body of studies has uncovered that hypoxia is one of the most important factors in tumor motility invasion and metastases. Emerging experiments have been revealed that LRP-1 and its ligand (Extracellular Hsp90 protein) are dramatically elevated in the cell surface of tumor cells. In such study which performed by Chen et al. a significant elevation of HSP90 levels was detected in the serum of the patients with PDAC. The extracellular Hsp90 protein-LRP1 complex was shown to regulate activation of the tyrosine kinase receptor stimulating cell motility and invasion [28]. Such observations indicated that a complex of extracellular Hsp90 protein LRP-1 promotes tumor invasion and metastases.

Our finding also highlighted a negative correlation between LRP-1 expression and differentiation (Table 1). The effects of LRP-1 over-expression on tumor differentiation have not been addressed to date, however, Kawata et al., reported its effects on chondrocyte differentiation [29]. More recently Leslie et al., have also tried to identify LRP-1 in tumorgenesis. They showed that LRP-1 is a p53 target gene. In addition, lethal doses of p53-activating stress such as Doxorubicin inhibit LRP1 translation through an miRNA 103 and miRNA 107-based translational repression resulting in suppression of LRP1 expression and cell death induction [30]. Moreover LRP-1 has been found to enhance exosome production to invade cancer cell [31]. Furthermore, LRP-1 might participate in differentiation through interaction with other signaling elements such as Protease Nexin-1 (PN-1), midkine and pro-cathepsin D [32]. Therefore, more data are required to investigate LRP-1 effects in Tumor differentiation.

In conclusion, this study in line with the mentioned study showed that LRP-1stongley overexpressed in PDAC tumors. This study and other related investigations may provide a physician to make better decisions about PDAC cancer treatment similar to breast cancer which classified based on HER2 status making a good experience in treatment with monoclonal antibodies. However, up to now, the current molecular approaches have not been used in routine clinical practice but such observation might be used in future diagnosis and treatment.

Conflicts of interest

No conflict of interest declared.

Acknowledgments

This work was supported by the Isfahan University of Medical Science as a funding source [grant numbers 2363589]. The authors appreciate *Dr. Erfan Sadeghi* and *Dr. Behzad Mahaki* for their technical support in statistical analysis.

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