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Stromal LRP1 in lung adenocarcinoma predicts clinical outcome

He Meng^{1,+}, Guoan Chen^{2,+}, Xiaojie Zhang¹, Zhuwen Wang², Dafydd Thomas³, Thomas Giordano³, David G. Beer², and Michael M. Wang^{1,4,5,*}

¹ Department of Neurology, University of Michigan, Ann Arbor, MI 48109-5622

² Department of Surgery, University of Michigan, Ann Arbor, MI 48109-5622

³ Department of Pathology, University of Michigan, Ann Arbor, MI 48109-5622

⁴ Department of Molecular & Integrative Physiology, University of Michigan, Ann Arbor, MI 48109-5622

⁵ Neurology Service of the VA Ann Arbor Healthcare System, Ann Arbor, MI 48105

Abstract

Purpose—LRP1 is a broadly-expressed receptor that binds multiple extracellular ligands and participates in protein clearance. LRP1 is expressed numerous cancers, but its role in lung cancer has not been characterized. Here, we investigate the relationship between LRP1 and lung cancer.

Experimental Design—LRP1 mRNA levels were determined in lung tumors from several large, multicenter studies. LRP1 protein localization was determined by immunohistochemical analysis of lung tumor microarrays. Normal fibroblasts, fibroblasts treated with the LRP1 inhibitor RAP, and LRP1 null fibroblasts were co-cultured with three independent lung cancer cell lines to investigate the role of LRP1 on tumor cell proliferation.

Results—LRP1 mRNA levels are significantly decreased in lung tumors relative to non-tumorous lung tissue. Lower expression of LRP1 in lung adenocarcinomas correlates with less favorable clinical outcome in a cohort of 439 patients. Immunohistochemical analysis demonstrates that LRP1 is primarily expressed in stromal cells in 94/111 lung cancers, with very little protein found in cancer cells. A growth suppressive function of mouse embryonic fibroblast cells (MEF) was observed in three lung cancer cell lines tested (H460, H2347, and HCC4006 cells); growth suppression was blocked by the LRP1 inhibitor, RAP. LRP1 deletion in fibroblasts reduced the ability of MEF cells to suppress tumor cell mitosis. In a validation set of adenocarcinomas, we confirmed a significant positive correlation between both LRP1 mRNA and protein levels and favorable clinical outcomes.

Conclusions—LRP1 expression is associated with improved lung cancer outcomes. Mechanistically, stromal LRP1 may non-cell autonomously suppress lung tumor cell proliferation.

Introduction

The effects of the tumor stroma on the behavior of the cancer is known to be dual-natured (1). On one hand, early studies showed that reactive stroma in Rous sarcoma virus-infected chickens provides a receptive environment for cancer development (2). Factors, such as VEGF, are secreted into the stroma, providing an angiogenic environment with increased vascular permeability that facilitates matrix protein deposition and tumor propagation (3–5).

* Address Correspondence to: Michael M. Wang, 7629 Medical Science Building II Box 5622, 1137 Catherine St., Ann Arbor, MI 48109-5622, Tel. 734-763-5453; Fax 734-936-8813; micwang@umich.edu.

+These authors made equal contributions

Increased deposition of extracellular matrix components such as proteoglycans and tenascin C are also strongly predictive of poor clinical prognosis in bladder and breast cancer (6,7). On the other hand, other investigators have shown that stromal matrix components can repress cancer cells under specific circumstances. For example, inhibition of collagen fibril formation increases B16F10 melanoma tumor growth in a mouse model (8). Therefore, prior work supports the concept that tumor stroma exerts divergent and context-specific effects on cancer.

Newer data now suggests that the divergent effects of stroma on cancer progression could result from heterogeneity of the tumor stroma itself. In colon cancer, increased stromal myofibroblast content within the tumor predicts tumor recurrence (9). In a cohort of breast cancer patients, individuals whose tumors contained high levels of PDGF-B receptor within the stroma had less favorable outcomes (10). In non-small cell lung cancer, periostin expression in the stroma predicted poor clinical outcome (11). The association between stromal phenotypes and clinical outcomes has been further refined at the molecular level by recent studies that link breast cancer stromal gene expression patterns to patient outcomes (12) and tumor chemoresponsiveness (13).

Tumor fibroblasts have emerged as an important regulator within the stroma that may ultimately define whether the stroma promotes or inhibits cancer progression (14–17). Fibroblasts provide proteolytic enzymes that actively enhance growth and invasiveness (18) as well as increase metastatic tumor size (19). In several mouse models, cancer-associated fibroblasts (as compared to normal fibroblasts) accelerate invasiveness of tumors (20), tumor growth (21), metastasis (22), and angiogenesis within the tumor (23), while normal fibroblasts have been shown to inhibit cell growth and recruit inflammatory defense systems. Tumor fibroblasts frequently secrete growth factors such as TGF- β and PDGF, whose levels of expression can stimulate mitogenic activity in cancer cells (24–26). Specific molecules expressed in lung cancer stromal fibroblasts have not been functionally characterized.

Low density lipoprotein receptor-related protein 1 (LRP1) is a large transmembrane receptor that is abundantly produced by fibroblasts. LRP1 acts as both a signaling receptor and a clearance receptor. Its substrates and ligands include over 30 molecules with highly diverse function; therefore, LRP1 exerts multiple context-specific functions on normal cell physiology (27) (28).

Although early studies suggested that LRP1 was expressed in fibroblasts and excluded in cancers (29,30), LRP1 has been found in a wide range of human malignancies. The expression of LRP1 *in vitro* was lower in cell lines that exhibited increased invasiveness (31). But in other studies, increased LRP1 correlated with high levels of invasiveness and silencing of LRP1 prevented spread of malignant cells (32). In addition, a number of studies have suggested a role of LRP1 in regulation of tumor growth. The expression levels of LRP1 were observed to decrease during the progression of melanoma (33). In gliomas, the magnitude of LRP1 expression in tumors greatly exceeds its levels in normal brain (34); the protein is produced by glioma cells, and its expression correlates with aggressiveness of the cancer (35).

In lung cancer, little is known about LRP1 and its potential function. Yamamoto et al showed very low LRP1 mRNA expression in a small set of lung tumors, and the protein was present in the stroma in one of ten lung cancer samples (35). In this study, we performed a more comprehensive analysis of the level of expression and stromal distribution of LRP1; we determined whether LRP1 expression is linked to clinical outcomes in a large gene expression array study (36), localized LRP1 protein to the stroma within lung tumors using

tissue microarrays, and characterized the functional effects of LRP1 on lung cancer cell proliferation.

Materials and Methods

Microarray data and statistical analysis

Our previously described Affymetrix microarray data set representing 439 lung adenocarcinomas (36) was used to test the relation between LRP1 expression and clinical variables and also to define genes coregulated with LRP1 in lung cancer. Characteristics of this set of clinical samples are summarized in Supplementary Table 1. *t*-tests were used to identify statistically differences in mean gene-expression levels between different clinical variables. Survival curves were constructed using the method of Kaplan-Meier, and survival differences assessed using the log-rank test. The multivariate (adjusted by sex, age and stage) Cox proportional hazards model with continuous values of LRP1 mRNA was used to assess survival, censored at 5 years. *P* values < 0.05 were considered to indicate statistical significance. The Pearson correlation method was used to test the correlation between the expression of LRP1 and other genes in tumors. The hierarchical clustering with Treeview (37) was used for the representation of LRP1 correlated genes in 439 lung adenocarcinomas. Meta-analysis of LRP1 mRNA expression in multiple cancers was performed using the Oncomine website (<https://www.oncomine.org>).

Immunohistochemistry

Lung tumor tissues arrays (TMAs) were constructed at the University of Michigan Cancer Center. Sections were analyzed by immunohistochemical staining using standard techniques. Briefly, after deparaffinization and rehydration, antigen retrieval was performed using microwave-treatment; immunoperoxidase staining was conducted using a DAKO autostaining system. All sections were counterstained with hematoxylin. Digital images were captured using an Olympus system. Monoclonal antibody 8G1 (anti-LRP1; Santa Cruz) was applied at a 1:50 dilution.

Cell culture

Mouse embryonic fibroblasts and PEA13 cells have been described before; these cells were propagated in DMEM with 10% fetal bovine serum (FBS) (Invitrogen). H460, H2347, and HCC4006 lung cancer lines were selected for studies since they demonstrated very low LRP1 mRNA expression levels. Lung cancer cells were propagated in RPMI1640 with 10% FBS. Cocultures were performed in DMEM complete media. Co-cultures were grown for 24 hours before the addition of BrdU; after an additional 24 hours, cells were double stained for BrdU and TRA1-85 (70ng/ml) which reacts against human (but not mouse) CD147. Double-stained cells were counted as human cancer cells that had undergone mitosis. Unless noted, each experiment was repeated at least three times.

Western Blots

Cell monolayers were rinsed with PBS, harvested, and sonicated in lysis buffer (50 mM Tris-HCl, pH 8; 200 mM NaCl; 0.5% Nonidet-40; protease inhibitor cocktail [Pierce]). Cell lysates were centrifuged at 14,000 rpm for 10 min at 4°C, and supernatants were used for western blot analysis with the monoclonal antibody 11H4, which detects the 85 kDa B-chain of LRP1. IRDye-labeled secondary antibodies (Rockland, 1:10,000) were incubated with filters and then detected with an Odyssey imaging system (LI-COR Biosciences).

LRP1 mRNA expression and DNA copy number changes in validation set of adenocarcinomas

An independent set of adenocarcinomas were analyzed for mRNA expression, DNA copy number change and protein expression from patients at the University of Michigan. The characteristics of this set of clinical variables are summarized in Supplementary Table 1.

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Cat. no. 217004, Valencia, CA) according to the manufacturer's instructions. cDNA was prepared from RNA samples using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems) according to manufacturer's instructions.

qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems, PN.4367659) on an ABI Prism 7900HT Sequence Detection System using a two-temperature cycling protocol: 95° C for 10 min, followed by 40 cycles of 97° C for 30 sec and 65° C for 1 min. The oligonucleotide primers for LRP1 mRNA expression are shown in Supplementary Table 2. Beta actin expression was used to normalize total RNA content. Relative mRNA levels were assessed using the 2delta Ct method.

Genomic DNA from frozen lung tissues was extracted using UltraPure buffer-saturated phenol (Invitrogen, cat. No. 15513-047). Primers for LRP1 DNA amplification was are shown in Supplementary Table 2. The qPCR was performed using the same conditions as qRT-PCR.

Results

LRP1 expression in multiple cancers

LRP1 expression in normal and neoplastic tissue was examined using the Oncomine database that contains gene expression studies of multiple types of cancers. Organ-specific differences in LRP1 expression were observed (Supplementary Fig. 1). In some tissues, such as brain cancers, a marked increase in LRP1 expression is observed, suggesting that LRP1 plays a role in the promotion of glioma growth (Supplementary Fig. 2, right panel); these findings are consistent with smaller, individual pathological studies (34, 35). In contrast, multiple cancers, including lung cancer, exhibit reduced expression of LRP1 as compared to normal tissues, suggesting that LRP1 could exert context-specific functions within tumors. Notably, 8 of 8 lung cancer studies in this database demonstrated significantly reduced expression of LRP1 transcript levels in lung cancers relative to normal lung tissue (Supplementary Fig. 2, left panel).

LRP1 mRNA expression and lung adenocarcinoma outcomes

We examined the relationship between LRP1 mRNA expression and survival in lung adenocarcinomas, the most common subtype of lung cancer. We analyzed individual tumors for LRP1 expression along with clinical outcome measures and discovered a significant positive correlation between preserved LRP1 expression and patient survival (Fig. 1 and Table 1) which was independent of tumor stage, age and sex by using multivariate Cox model analysis (Table 1). LRP1 mRNA expression was not related to other variables, including age, sex, tumor stage, p53 status, smoking, and tumor differentiation. LRP1 mRNA levels did not correlate with survival in lung squamous cell carcinomas (not shown, samples described in (38)).

Tissue distribution of LRP1 in lung tumors

One interpretation of these data is that LRP1 acts within neoplastic cells to cell-autonomously regulate growth. If this were true, LRP1 should be expressed by tumor cells

as observed in gliomas (34,35). Surprisingly, however, immunohistochemistry using lung tumor tissue microarrays demonstrated either no or very low expression of LRP1 within neoplastic lung tumor cells of 68 adenocarcinomas and 43 squamous lung cancer samples. Rather, LRP1 was abundantly expressed in stromal cells of 37% of the tumors, while it was only strongly expressed in 5% of neoplastic tumor cells (Fig. 2; Supplementary Table 3).

LRP1 and fibroblast gene expression in lung cancer

A major component of the tumor stroma includes fibroblasts, which are known to express high levels of LRP1 (29). One possible explanation for our observations that link LRP1 in lung cancer to clinical outcome could therefore be that LRP1 is simply a marker for increased tumor fibroblast quantity which may then determine outcome. To test this, we examined whether other fibroblast-expressed genes correlated with LRP1 expression and whether expression of these genes was linked to clinical outcome. Five genes associated with tumor stromal fibroblasts were modestly correlated with LRP1, but six other genes were not correlated (39) (40) (Supplementary Fig. 3 and Supplementary Table 4), suggesting that LRP1 is not simply a marker of overall stromal fibroblast content. Rather, the lack of association between LRP1 and most fibroblast markers suggests molecular diversity of tumor stromal fibroblasts, which has been suggested by Sugimoto who examined murine cancer models (40). Among all the fibroblast genes examined, only LRP1 levels predicted clinical outcome (Supplementary Table 4).

Direct role of LRP1 expression in suppressing cancer cell proliferation

The findings that increased LRP1 levels correlate with more favorable outcomes and that LRP1 is expressed in the stroma of tumors suggests that LRP1 may regulate tumor cell proliferation via a non-cell autonomous mechanism. To test this, we used an *in vitro* system to study the effects of LRP1 on proliferation of lung cancer cells. We co-cultured normal mouse embryonic fibroblasts (MEF) or PEA13 cells (LRP1 knockout fibroblasts; (41)) with H460, H2347, and HCC4006 lung tumor cells lines (that express very low levels of LRP1; Fig. 3A) and determined the number of lung cancer cells undergoing mitosis using BrdU incorporation (Fig. 3B–D). MEF cells significantly suppressed proliferation of H460 cells compared to LRP1 knockout fibroblasts (PEA13). This growth suppressive effect of MEF was blocked by incubation with RAP, which binds and inactivates LRP1. In contrast, RAP did not affect the division of any of the lung cancer cells cultured with PEA13 cells. In sum, LRP1, expressed in non-tumor cells, efficiently inhibited the proliferation of three lung cancer cell lines.

Genes co-regulated in LRP1 in lung cancer

In order to identify additional markers that may be co-regulated with LRP1, Pearson correlation analysis between LRP1 and other genes was performed; the top 100 most significant correlated genes are graphically displayed in Supplementary Fig. 4 and listed in Supplementary Table 5. A fraction of these coregulated genes (35%) also demonstrated significant favorable clinical outcomes, which are highlighted in Supplementary Table 5.

Validation of findings in an independent cohort of patients

To test the durability of our findings, we examined a validation set of 101 lung adenocarcinomas (clinical characteristics shown in Supplementary Table 1) and 39 normal lung samples. We confirmed that tumors in the validation set contained significantly less LRP1 mRNA than normal tissue (Supplementary Fig. 5). Moreover, the levels of LRP1 mRNA in this cohort were statistically associated with favorable outcome (Fig. 4A and Table 2). DNA amplification or deletion of the LRP1 gene was not common (Supplementary

Fig. 6A), and DNA copy number did not correlate with mRNA expression in these samples (Supplementary Fig. 6B).

We evaluated LRP1 by immunohistochemistry in the validation set of lung tumors (Supplementary Table 6) and again identified strong LRP1 protein expression in the stroma in a significant fraction of the samples (98%). Strong neoplastic cell expression of LRP1 was only seen in 13% of the tumors. LRP1 protein staining levels in the validation set demonstrated a strong correlation between LRP1 protein expression and favorable clinical outcomes (Fig. 4B and Table 2).

Discussion

Our results provide new information supporting a role of LRP1 in lung cancer. Preservation of LRP1 expression correlates with improved clinical outcomes; importantly, expression of LRP1 protein localizes predominantly to the tumor stroma, suggesting that it may modulate tumor cells via a non-cell autonomous mechanism. *In vitro* studies support a model in which stromal fibroblasts expressing LRP1 repress tumor cell growth or proliferation. As such, we propose that LRP1 may function as a stromal tumor growth inhibitory gene product.

The well-established expression of LRP1 in fibroblasts and its central role in extracellular protein catabolism logically positions it as a functionally important protein in the tumor stroma; we show that indeed, LRP1 is expressed at low levels in transformed epithelial cancer cells yet is strongly expressed in stromal cells of most cancers. In this report, also we provide three pieces of evidence in support of a functional role of LRP1 in the stroma. First, there is a significant correlation between LRP1 expression and favorable lung cancer outcomes, suggesting that LRP1 function in stromal fibroblasts may be a key factor in defining the effects of stroma on tumor cell growth. Second, cell culture experiments strongly suggest a direct functional role of LRP1 in growth suppression. And third, we emphasize that LRP1 is a unique determinant of stromal character, since our analysis of gene expression of other markers of cancer associated fibroblasts showed no statistical association between clinical outcomes and expression of 11 other established cancer fibroblast genes.

The stromal expression pattern of LRP1 within lung tumors indicates that it may serve as a potential biomarker for clinically favorable tumors. Gene expression patterns within the stroma that influence outcome have been elegantly described in breast cancer (12,13) and have suggested that clusters of co-regulated genes underlying specific cellular programs could influence tumor growth and behavior. Because fewer than half of the cancer-associated fibroblast markers analyzed were co-regulated with LRP1, it is tempting to speculate, in analogy, that a small subset of co-regulated genes in lung tumor stroma (which include LRP1) determines a favorable stroma in lung cancers; however, in opposition to this, among the cancer fibroblast genes, only LRP1 was linked to clinical outcome.

LRP1 binds to over 30 different ligands and has both signaling properties and endocytic functions (27,28). Therefore, a large array of mechanisms could account for its activity. Two specific examples should be mentioned. First, LRP1 functions in concert with thrombospondin 2 (TSP2; THBS2), whose expression is also significantly co-regulated with LRP1 expression (Supplementary Table 5; last row), to clear matrix metalloproteinases (MMP2/9) from the extracellular space (42). Since MMPs have been shown to participate in cancer cell growth (20) and in metastasis (43), it is tempting to speculate that increased LRP1 and TSP2 in stromal cells enhances the clearance of pro-growth MMP expression. Additionally, recent studies show that LRP1, together with TSP2, regulates the strength of Notch3 signaling in tumor cells, which inhibits lung cancer cell growth *in vitro* (44,45).

Second, alpha-2-macroglobulin (A2M), a protein that targets proteases for catabolism by LRP1, is also co-regulated with LRP1 in lung cancers. Notably, increased levels of A2M are strongly associated with improved clinical outcomes in our initial patient cohort (439 tumors; Supplementary Fig. 7). Thus, A2M-LRP1 cooperative activity is a potentially a very potent mechanism by which the tumor stroma may regulate patient outcomes.

Notably, a majority of the clinically-significant genes co-regulated with LRP1 (Supplementary Table 5) are molecules that are expressed in the extracellular space, where they may functionally interact with LRP1; of the 35 genes co-regulated with LRP1 and positively associated with clinical outcome, 22 (63%) could potentially interact with LRP1 (17 encoded extracellular proteins and five encoded transmembrane proteins). This is consistent with the possibility that tumor microenvironment is important in modulation of cancer outcome (non-cell autonomous factors) and are perhaps as influential as intracellular gene products (that are more likely to mediate cell autonomous functions within transformed cells). More work will be needed to determine whether these gene products physically and functionally interact with LRP1 to influence tumor cells.

Finally, there are potential advantages of promoting LRP1 as part of cancer therapy. First, therapies activating LRP1 in the stroma may produce a durable effect on tumor behavior, since the tumor stroma could be more genetically stable (46) and less likely to evolve drug resistance during therapy (47); analysis of the LRP1 locus in the validation set of tumors failed to demonstrate a high rate of genetic amplification or deletion (Supplementary Fig. 6A). Second, concerted activation of a broad range of potential tumor cell extracellular pathways affected by a multifunctional molecule such as LRP1 may hold promise since neoplastic cells are not likely to simultaneously adapt to multiple antiproliferative pathways.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig 1.

Kaplan-Meier survival curve indicated that high levels of LRP1 mRNA was favorable for patient survival in 439 lung adenocarcinomas (higher 2/3 vs. lower 1/3, log-rank test, $P = 0.047$). We used a multivariable Cox model adjusted by age, gender and stage using continuous LRP1 mRNA values with likelihood test, $P = 0.024$, $\beta = -0.26$.

Fig 2. Immunohistochemistry on lung cancer tissue array including adenocarcinomas and squamous cell cancers indicated that positive LRP1 protein staining, when present, was predominantly in stroma, whereas malignant tumor cells failed to strongly stain.

Fig 3.

Non-cell autonomous inhibition of tumor cell line growth by LRP1 in fibroblasts. We co-cultured MEF cells or LRP1-null PEA13 cells with three lung cancer cell lines. Only MEF cells expressed detectable levels of LRP1 (A). Proliferation was quantified by double staining for human cancer cells (expressing human CD147) and BrdU incorporated into the nucleus after pulse labeling of the co-culture. Proliferation of all three lung cancer cell lines (B–D) was inhibited by cells containing functional LRP1 (MEF cells); inhibition was blocked by RAP or genetic inactivation of LRP1. Lung cancer cell lines that were growth without co-culture incorporated BrdU at more than eight and two times the level of cells co-cultured with MEF or PEA13 cells (not shown). Monocultures were not affected by RAP.

Fig 4.

Kaplan-Meier survival curve indicated that higher LRP1 mRNA and protein expression favored survival in a validation set of lung adenocarcinomas (higher 2/3 vs. lower 1/3 samples, log-rank test, $P = 0.04$ for mRNA, $n = 101$, Fig. A; $P = 0.009$ for protein, $n = 96$, Fig. B). The levels of LRP1 expression were determined by quantitative RT-PCR. In addition, protein expression scores were determined from TMA stained for LRP1 protein (the average of stroma, neoplastic cell, and macrophage scores were used for survival analysis). We also applied a multivariable Cox model adjusted by age, gender and stage using LRP1 values with likelihood test, $P = 0.04$, $\beta = -0.75$ for mRNA and $P = 0.01$, $\beta = -0.99$ for protein.

Table 1

LRP mRNA expression and patient survival in 439 adenocarcinomas

Group	<i>n</i>	HR	95% CI	<i>P</i> [*]
Low LRP	146	1		
High LRP	293	0.73	0.56 – 0.98	0.03

* log-rank test was used, high 2/3 vs low 1/3 samples.

Multivariable Cox model (age, gender and stage adjusted) using continuous LRP1 mRNA value: $P = 0.024$, $\beta = -0.26$

Table 2

Influence of LRP1 mRNA and protein expression on patient survival in a validation set of adenocarcinomas

Group	<i>n</i>	HR	95% CI	<i>P</i> [*]
Low LRP1 mRNA	34	1		
High LRP mRNA	67	0.54	0.297–0.989	0.043
Low LRP1 protein	34	1		
High LRP1 protein	62	0.45	0.24–0.833	0.009

* Log rank test was used, high 2/3 vs low 1/3 samples.

Protein scores were derived from the average of stroma, neoplastic cell, and macrophage scores.