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Evaluation of a biomarker for the diagnosis of pancreas cancer using an animal model

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Short title: Evaluation of serum marker in rat model of pancreas cancer
Abstract

Many approaches have been taken to identify new biomarkers of pancreatic ductal carcinoma (PDC). Since animal models can be sampled under controlled conditions, better standardization is possible compared with heterogeneous human studies. Transgenic rats with conditional activation of oncogenic RAS in pancreatic tissue develop PDC that closely resembles the biological and histopathological features of human PDC. Using this model, we evaluated the usefulness of leucine-rich α2-glycoprotein-1 (LRG-1) as a serum marker. In this study, we found that LRG-1 was overexpressed in rat PDC compared with normal pancreas tissue of the control rats. Serum levels of LRG-1 were also significantly higher in rats bearing PDC than in controls. Importantly, chronic pancreatitis in male Wistar Bonn/Kobori rats, which is a widely accepted as a model of chronic pancreatitis, did not cause serum levels of LRG-1 to become elevated. These results strongly support serum LRG-1 as a candidate biomarker for noninvasive diagnosis of PDC. Our models of pancreas cancer provide a useful strategy for evaluation of candidate markers applicable to human cancer.

Key words: Pancreas cancer, serum marker, animal model, LRG-1

The abbreviations used are as follows: PDC, pancreatic ductal carcinoma; LRG, leucine-rich α2-glycoprotein; AxCANCre, Cre recombinase-carrying adenovirus; WBN/Kob, Wistar Bonn/Kobori
Introduction

Pancreatic ductal carcinoma (PDC) carries the most dismal prognosis of all solid tumors. PDC is one of the most lethal types of cancer, with a five-year survival rate of less than 10% and a mortality rate closely approaching the incidence rate. The survival rate of pancreas cancer patients can increase 6-fold with early detection; however, at present biomarkers have limited utility for detecting early-stage PDC. Currently, the best serum marker for pancreatic cancer is carbohydrate antigen 19-9 (CA19-9). The CA19-9 epitope is found on oligosaccharide sialylated Lewis A antigen and on multiple protein carriers including mucin core proteins. However, the utility of CA19-9 as a PDC biomarker is limited, as its performance varies with disease stage. Unfortunately, CA19-9 may also be positive in patients with nonmalignant diseases including chronic pancreatitis. Additionally, CA19-9 is not detectable in 5-10% of fucosyl transferase-deficient patients who are negative for the Lewis antigens.

Ras activation is thought to initiate focal lesions in the pancreatic ducts, which undergo graded histological progression to PDC. We have established transgenic rat lines carrying a human \( K_{ras}^{G12V} \) or a human \( H_{ras}^{G12V} \) oncogene in which the expression of the transgene is regulated by the Cre/loxP system. Targeted activation of the transgene is accomplished by injection of a Cre recombinase-carrying adenovirus (AxCANCre) into the pancreatic ducts through the common bile duct. Importantly, neoplastic lesions in the transgenic rats exhibit morphological and biological similarities to those observed in human pancreas lesions. Therefore, the transgenic PDC-rat model is suitable for screening for potential biomarkers of human PDC.

Leucine-rich \( \alpha 2 \)-glycoprotein-1 (LRG-1) was identified as a serum protein containing eight leucine-rich repeats. Increased LRG-1 expression has been
demonstrated in ovarian cancer\textsuperscript{14}, lung cancer\textsuperscript{15}, and pancreatic cancer tissue\textsuperscript{16}. In the present study, we present data that supports the use of LRG-1 as a serum marker for pancreas cancer.

Materials and Methods

Animals

Male \textit{Hras}\textsuperscript{G12V} or \textit{Kras}\textsuperscript{G12V} transgenic (Hras250, Kras301) rats were obtained from CLEA Japan (Tokyo, Japan); the establishment of these rats has been reported previously\textsuperscript{6-8}. Male Wistar Bonn/Kobori (WBN/Kob) rats were purchased from Japan SLC (Hamamatsu, Japan). The rats were maintained in plastic cages in an air-conditioned room with a 12-h light/12-h dark cycle. At the end of the experimental period, rats were euthanized by exsanguination from the abdominal aorta under deep anesthesia using isoflurane. All animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals of the Nagoya City University Graduate School of Medical Sciences and approved by the Institutional Animal Care and Use Committee (H25M-21).

Tumor induction and pathological examination

Adenovirus vector, AxCANCre, was purified after amplification in HEK293 cells, and pancreas tumors were induced as previously described\textsuperscript{6-8,17,18}. AxCANCre is a recombinant adenovirus vector that expresses Cre recombinase under the control of the CAG promoter. Infection of \textit{Hras}\textsuperscript{G12V} or \textit{Kras}\textsuperscript{G12V} transgenic (Hras250, Kras301) rats with AxCANCre results in expression of \textit{Hras}\textsuperscript{G12V} in infected cells in the Hras250 rat and expression of \textit{Kras}\textsuperscript{G12V} in infected cells in the Kras301 rat\textsuperscript{6,7}. AxCANCre was
introduced into the pancreatic ducts of Hras250 and Kras301 male rats via injection into the common bile duct. Four weeks after injection of AxCANCre, the rats were euthanized. After sacrifice, tumor nodules present in PDC-bearing pancreas tissue were isolated and frozen in liquid nitrogen for RNA assays or fixed in 4% paraformaldehyde and processed for histological observation. Pancreas tissue from control rats was also frozen in liquid nitrogen for RNA assays or fixed in 4% paraformaldehyde and processed for histological observation. The expression of the Kras\textsuperscript{G12V} transgene, which is expressed as an HA-fusion protein, was confirmed by immunohistochemistry using HA-tagged antibodies\textsuperscript{8}. The expression of the Hras\textsuperscript{G12V} transgene could not be determined directly because the transgene does not have a tag in the Hras250 rat by which it can be distinguished from endogenous Hras. Therefore, expression of total active Ras in the pancreas of these animals was determined using a Ras activation kit as previously described\textsuperscript{7}.

**RT-PCR**

RT-PCR was performed as previously described\textsuperscript{6}. Total RNA was isolated using ISOGEN (Nippon Gene, Toyama, Japan) and reverse-transcribed using PrimeScript RTase (Takara Bio Inc., Otsu, Japan) with Random Primers (Invitrogen, Carlsbad, CA, USA). The following primers were used for PCR: LRG-1, 5’-TTGGCAGCATCAAGGGAGAA -3’ and 5’-AGCATTGCGAGTCAGATCCA -3’; ribosome 18S, 5’-GTTGGTGGAGCGATTTGTCT-3’ and 5’-GGCCTCACTAAACCATCCAA-3’. The amplification protocol consisted of 32 (LRG-1) or 30 (18S) cycles of denaturation for 30 sec at 94°C, annealing for 30 sec at 60°C, and extension for 30 sec at 72°C.
Serum test

Blood was collected from the tail vein prior to terminal sacrifice and from the abdominal aorta at the time of terminal sacrifice; serum samples were stored at -80℃ until use. The serum levels of rat LRG-1 were quantified by ELISA (Code No. 27770, Rat LRG Assay Kit, IBL, Gunma, Japan). For all ELISA experiments, each sample was assayed in duplicate, and the absorbance was measured with a Model 680 microplate reader (Bio-Rad, Hercules, CA, USA).

Statistical analysis

All statistical analyses were done using JMP software (SAS Institute Japan, Tokyo, Japan). The data for the levels of LRG-1 in the serum were compared using the Wilcoxon test for nonparametric data. The data from the same rat were analyzed with the paired t test. Receiver operating characteristic (ROC) curves were constructed, and the area under the curve (AUC) was calculated to evaluate specificity and sensitivity. Cutoffs were defined for LRG-1 as the optimum point (Youden index) at which sensitivity and specificity were maximized. P-values < 0.05 were considered to be statistically significant.

Results

Expression of Kras<sup>G12V</sup> and Hras<sup>G12V</sup> in the rat pancreas

Four weeks after injection of AxCANCre, whitish nodules were observed throughout the pancreata of both types of ras transgenic rats, and histological examination showed that these nodules were adenocarinomas. Neoplastic lesions were
not found in other organs. The expression of the HA-tagged human Kras\textsuperscript{G12V} transgene was assessed by immunohistochemistry using HA-tag antibodies. The results were essentially identical to previous results (data not shown; see Tanaka et al.\textsuperscript{8}): the transgene was overexpressed in AxCANCre infected pancreas. The expression of the human Hras\textsuperscript{G12V} transgene was inferred by assessing the expression of activated Ras in control and AxCANCre-infected pancreas. The results were essentially identical to previous results (data not shown; see Ueda at al.\textsuperscript{7}): activated Ras was overexpressed in the AxCANCre-infected pancreas.

**Expression of LRG-1 in the rat pancreas**

We previously performed transcriptomic analysis of pancreatic tissue by microarray analysis\textsuperscript{10}. We selected genes encoding upregulated secretory proteins because the secreted proteins, including LRG-1, are a potential source of serum biomarkers. Our array analysis indicated that LRG-1 was overexpressed in PDC compared with the pancreata of control rats. Previous reports revealed overexpression of LRG-1 in pancreas carcinomas in humans\textsuperscript{16}. Therefore, we focused on the expression of LRG-1. In the present study, the expression level of the LRG-1 gene was assessed by RT-PCR and found to be higher in PDC compared with normal pancreas tissue of the controls rats in both the Kras301 and Hras250 groups (Fig. 1).

**Serum levels of LRG-1 in rats with pancreas carcinoma**

To examine whether LRG-1 can be used as a surrogate marker of pancreas carcinoma, we used an ELISA to measure the serum LRG-1 levels in rats with PDC and their controls. The level of LRG-1 in PDC-bearing Kras301 rats was 706.2 ± 35.03
ng/ml (mean ± SE; n = 17), and that of the empty adenovirus vector-treated control Kras301 rats was 458.37 ± 28.74 ng/ml (n = 14; P<0.001; Fig. 2A). The level of LRG-1 in PDC-bearing Hras250 rats was 599.9 ± 76.83 ng/ml (n = 9), and that of the control Hras250 rats was 368.4 ± 28.18 ng/ml (n = 10; P<0.001; Fig. 2B).

**ROC curve analysis**

To determine if the changes in the serum levels of LRG-1 could significantly differentiate between pancreatic cancer and controls, ROC curves were constructed (Fig. 3). The AUCs for LRG-1 were 0.895 (95% confidence interval [CI], 0.71-0.97) for Kras301 and 0.833 (95% CI, 0.48-0.96) for Hras250 (Table 1).

ROC curves were used to evaluate the sensitivities and specificities of serum LRG-1 levels used to distinguish PDC-bearing from PDC-free rats. Using the Youden index-based optimal cut-point of 660.2 ng/ml, the sensitivity and specificity were 76.5% and 100% for LRG-1 in Kras301 rats, and using the Youden index-based optimal cut-point of 499.6 ng/ml, the sensitivity and specificity were 77.8% and 100% for LRG-1 in Hras250 rats. The positive predictive value, negative predictive value, and accuracy of the LRG-1 serum-level cutoff of 660.2 ng/ml in distinguishing rats with pancreatic cancer from controls were 100, 77.8, and 87.1%, respectively, in Kras301 rats (Table 1). The corresponding values in Hras250 rats for an LRG-1 serum-level cutoff of 499.6 ng/ml were 100, 83.3, and 89.5%, respectively (Table 1). These results demonstrate that the serum level of LRG-1 has reasonable capability to differentiate rats with pancreatic cancer from controls.

**Serum levels of LRG-1 in WBN/Kob rats**
It is reported that LRG-1 is overexpressed during inflammation\textsuperscript{19}. Therefore we evaluated serum levels of LRG-1 in a rat model of chronic pancreatitis. The male Wistar Bonn/Kobori (WBN/Kob) rat is a widely accepted rodent model of chronic pancreatitis\textsuperscript{20}. Chronic pancreatitis-like lesions are observed in 100\% of male WBN/Kob rats, which commonly develop chronic pancreatitis by the age of 3 months and diabetes mellitus by 9 months\textsuperscript{21}. In the present study, fibrosis was observed in small areas of the pancreas of 10-week-old WBN/Kob rats, and pancreatitis with marked fibrosis was observed in 17-week-old rats. LRG-1 serum levels were 375.0 ± 11.65 ng/ml in 10-week-old rats ($n=4$) and did not change significantly in these rats when they were 17 weeks old (348.4 ± 18.35 ng/ml, $n=4$; Fig. 4A). Because the 10-week-old rats had begun to develop pancreatitis, we examined rats when they were 8 weeks old and 20 weeks old. The pancreata showed no abnormality in 8-week-old male WBN/Kob rats, but there was pancreatitis with marked fibrosis when the rats were 20 weeks old. In the 8-week-old rats, the LRG-1 serum levels were 397.8 ± 20.9 ng/ml ($n=4$), and these levels did not change significantly when these rats were 20 weeks old (420.6 ± 39.7 ng/ml, $n=4$; Fig. 4B), indicating that there was no significant difference in LRG-1 serum levels in WBN/Kob rats before and after developing chronic pancreatitis.

**Discussion**

In this study, we demonstrated that LRG-1 gene expression in PDC-bearing pancreata was higher than in controls (Fig. 1) and that serum levels of LRG-1 were significantly higher in PDC-bearing rats compared with control rats (Fig. 2). Serum and
plasma LRG-1 levels have been reported to be elevated in pancreas cancer patients\textsuperscript{22,23}, and our data strongly support the postulation that serum LRG-1 is a useful marker for cancer detection in pancreas cancer patients.

In humans, the sensitivity of serum LRG-1 for predicting PDC is slightly lower than the specificity of serum LRG-1 for predicting PDC\textsuperscript{22,23}. In the PDC-rat model, we obtained a similar result: the optimal predictive serum level of LRG-1 was 100\% specific with a sensitivity of approximately 76\% to 78\%. This suggests that there is potential for false negative results, and therefore, the diagnostic results using LRG-1 should be interpreted with caution. Detection of PDC will be improved by combination with another marker with high sensitivity. For example, it is reported that a three-marker panel (LRG-1, TIMP1, and CA19-9) improved detection of early-stage PDC in humans compared with CA19-9 alone\textsuperscript{23}.

The basal level of LRG-1 in serum was significantly different between Kras301 and Hras250 rats. In this study, we used homozygous Kras301 rats and heterozygous Hras250 rats. The Hras250 rats were maintained by breeding heterozygous male Hras250 rats with female SD rats. In contrast, the Kras301 rats were maintained by breeding homozygous male with homozygous female rats. It is likely that Kras301 rats with higher levels of LRG-1 were unintentionally selected in the course of establishing the homozygous rats. Another possible cause of the difference is the integration site of the ras transgene. Integration sites in the genome may affect the expression of LRG-1.

We also investigated the expression of LRG-1 in the WBN/Kob rat, which develops spontaneous chronic pancreatitis\textsuperscript{20}. The pancreatitis that develops in this rat mimics the pathophysiological processes of chronic inflammation and fibrosis in
humans\textsuperscript{20,21}. In our study, 8-week-old rats had no discernable pancreatic pathologies; the first pathologic changes in the pancreas were inflammatory cell infiltration and fibrosis in 10-week-old rats, and extensive fibrosis and parenchymal destruction were present in 17- and 20-week-old rats. Importantly, there were no significant differences in serum LRG-1 in these rats between before and after developing pancreatitis, indicating that elevated LRG-1 is specific to malignancy.

LRG-1 was first identified as a highly conserved member of the leucine-rich repeat (LRR) family of proteins\textsuperscript{12,13}. The LRR structural motif has been identified in a wide variety of proteins and participates in processes such as ligand–receptor interactions, enzyme inhibition, and cell adhesion\textsuperscript{24,25}. It has been suggested that the major function of the LRR motif is to provide a structural framework that enables protein–protein interactions\textsuperscript{24,25}. LRR proteins are overexpressed in several types of cancer, where they are proposed to have roles in processes such as cell signaling and metastasis\textsuperscript{26–34}.

LRG-1 has been proposed as a possible serum and/or plasma biomarker of pancreatic cancer\textsuperscript{22}. Serum/plasma LRG-1 levels are also elevated in patients with several other types of cancer, including lung\textsuperscript{15,35}, ovarian\textsuperscript{14,36}, gastric\textsuperscript{37}, colon\textsuperscript{38}, brain\textsuperscript{39}, and biliary tract cancers\textsuperscript{40}. LRG-1 is also detected and upregulated in urine samples from lung\textsuperscript{41,42} and ovarian\textsuperscript{43} cancer patients. Therefore, it is difficult to identify the tumor location using LRG-1 in humans. Animal models can be induced to specifically develop only pancreas tumors. Hence, the elevated serum levels of LRG-1 in the present study were derived from PDC. The serum level of LRG-1 was higher in rats with relatively large pancreas tumors. Although quantitative analysis was not done in this study, it seems likely that there is a relationship between the serum level of LRG-1 and
tumor size, and it is possible that the relative tumor size could be estimated from the serum level of LRG-1. Consequently, it is possible that LRG-1 may also be able to be used to screen for candidate chemotherapeutic agents, which could be evaluated for human use. Further studies need to be performed to assess whether LRG-1 can be used for evaluation of the effectiveness of chemotherapy.

The function of LRG-1 remains unknown. Expression of LRG-1 is regulated by IL-6 synergistically with either IL-1β or TNFα19, and a recent study identified LRG-1 as a direct downstream target of PPARβ/δ44. Due to its leucine-rich repeats, it is predicted that LRG-1 could have a role in cell adhesion13,25. A role for LRG-1 in granulocyte differentiation has also been suggested45. LRG-1 was reported to be coordinately expressed with the TGF-β type II receptor46, and LRG-1 activates the TGF-β angiogenic switch by binding directly to the TGF-β accessory receptor endoglin47. Furthermore, LRG-1 promotes TGF-β-mediated growth suppression of Lewis lung carcinoma cell lines48. In addition, overexpression of LRG-1 enhances tumor migration in gastric cancer37, and LRG-1 promotes proliferation and inhibits apoptosis of colorectal cancer cells via RUNX1 activation49. These reports suggest a potential role of LRG-1 in cancer progression.

A variety of approaches have been taken in an attempt to identify biomarkers of PDC in humans, including analysis of cell-free nucleic acids (e.g., mutant DNA, methylated DNA, and noncoding RNAs), metabolites, autoantibodies, glycosylated antigens, tumor-derived exosomes, and circulating tumor cells50-56. In contrast to human studies, animal models offer uniform environmental conditions, standardized blood and tissue sampling, and defined stages of tumor development, thereby reducing biological and nonbiological heterogeneity. The rat pancreas cancer model provides a controlled
model system for the preliminary identification of possible PDC biomarkers. We have identified several candidate biomarkers by proteome, metabolome, and transcriptome analysis using the rat pancreas cancer model\textsuperscript{9-11}, and in the present study, we confirmed LRG-1 as a strong candidate for a serum biomarker of PDC. LRG-1 is also upregulated in the serum of the rat colon cancer model $Apc^{Pirc/+}$ rat, which like humans develops adenomas and localized adenocarcinomas preferentially in the colon\textsuperscript{57}; increased serum LRG-1 has also been reported in human colon cancer patients\textsuperscript{38}. A final goal of cancer biomarker research is the development of noninvasive tests that enable early cancer detection. Our findings indicate that the rat models of pancreas cancer used in the present study provide a useful strategy to identify candidate markers applicable to human cancer.

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**Figure legends**

**Figure 1.** Overexpression of LRG-1 in PDC-bearing pancreas tissue. RT-PCR for LRG-1 in normal pancreas tissue (controls) and PDC-bearing pancreas tissue (carcinomas) in (A) Kras\(^{G12V}\) and (B) Hras\(^{G12V}\) transgenic rats. Each lane represents RNA prepared from an individual rat. 18S ribosome serves as an RNA control.

**Figure 2.** Serum levels of LRG-1 in ras\(^{G12V}\) transgenic rats. The serum levels of LRG-1 in (A) Kras\(^{G12V}\) and (B) Hras\(^{G12V}\) transgenic rats with PDC were significantly higher than in control rats (P<0.001). Open circles, control rat; closed circle, PDC-bearing rat.

**Figure 3.** Receiver operating characteristics (ROC) curve analysis of pancreas cancer-bearing rats versus control rats. ROC curves were constructed to evaluate LRG-1 as a marker of PDC. (A) The AUC was 0.895 for LRG-1 in Kras\(^{G12V}\) transgenic rats. (B) The AUC was 0.833 for LRG-1 in Hras\(^{G12V}\) transgenic rats.

**Figure 4.** Serum levels of LRG-1 in WBN/Kob rats. The WBN/Kob rat strain is an animal model of spontaneous chronic pancreatitis. Male WBN/Kob rats commonly develop chronic pancreatitis by the age of 3 months. (A) Serum levels of LRG-1 were measured in 10- and 17-week-old male WBN/Kob rats: blood was collected from the same rats at the 10- and 17-week time points. (B) Because fibrosis had begun to develop in the 10-week-old rats, serum levels of LRG-1 were also measured in 8- and 20-week-old male WBN/Kob rats: blood was collected from the same rats at the 8- and
20-week time points. The serum levels of LRG-1 showed little change during progression of chronic pancreatitis.
Table 1. Performance of LRG-1 in rat pancreas cancer models

|          | AUC  | Sensitivity | Specificity | Accuracy |
|----------|------|-------------|-------------|----------|
| Kras301  | 0.895| 76.50%      | 100%        | 87.10%   |
| Hras250  | 0.833| 77.80%      | 100%        | 89.50%   |
Figure 1

A

| Controls | Carcinomas |
|----------|------------|
| LRG-1    |            |
| 18S      |            |

B

| Controls | Carcinomas |
|----------|------------|
| LRG-1    |            |
| 18S      |            |

Figure 2

A

B

P<0.001
Figure 3

A

B

Figure 4

A

B