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A serological marker of the N-terminal neoepitope generated during LOXL2 maturation is elevated in patients with cancer or idiopathic pulmonary fibrosis

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ABSTRACT

Objectives: Lysyl oxidase like 2 (LOXL2) is associated with poor prognosis in idiopathic pulmonary disease (IPF) and cancer. We developed an Enzyme-linked immunosorbent assay (ELISA) targeting the LOXL2 neo-epitope generated through the release of the signal peptide during LOXL2 maturation.

Design and methods: An ELISA targeting the N-terminal site of the human LOXL2 was developed including technical optimization and validation steps. Serum LOXL2 was measured in patients with breast, colorectal, lung, ovarian, pancreatic and prostate cancer, melanoma, IPF and in healthy controls (n = 16).

Results: A technically robust and specific assay was developed. LOXL2 was detectable in serum from healthy controls and showed reactivity towards recombinant LOXL2. Compared to controls, LOXL2 levels were significantly ($p < 0.001$–0.05) elevated in serum from patients with breast, colerectal, lung, ovarian and pancreatic cancer (mean range: 49–84 ng/mL), but not in prostate cancer (mean: 36 ng/mL) and malignant melanoma patients (41 ng/mL). Serum LOXL2 was elevated in IPF patients compared to healthy controls (mean: 76.5 vs 46.8 ng/mL; $p > 0.001$)

Conclusions: A specific ELISA towards the N-terminal neo-epitope site in LOXL2 was developed which detected significantly elevated serum levels from patients with above-mentioned cancer types or IPF compared to healthy controls.

1. Introduction

Post translation modifications of the extracellular matrix (ECM) are important in ECM maturation and cell signalling properties of proteins [1]. Collagen and elastin crosslinking by the family of lysyl oxidase (LOX) have particularly gained increasing attention in diseases leading to fibrosis in the pulmonary region and cancer [2–4]. *LOXL2* is a member of the lysyl oxidase (*LOX*) gene family in which five *LOX* family genes have been identified (LOXL1, LOXL2, LOXL3, and LOXL4) [5,6]. LOX family members are responsible for normal and pathology relevant crosslinking generating increased tissue stiffness, which in an uncontrolled state promotes resistance to fibrolysis in fibrotic tissue [7,8]. Members of the LOX family are overexpressed in the microenvironment of fibrotic lesions especially produced by activated fibroblasts [10]. LOXL2 is the most highly expressed of the LOX family [7,10,11]. LOX has the highest affinity for collagens precipitated in the form of fibrils and clustered forms of elastin [12].

LOXL2 activity and expression have been shown to be associated with cancer and fibrosis [8], which includes signalling roles in cancer proliferation, dedifferentiation, and increased stability of collagens accumulating during fibrosis [13–15]. The number of cross-links per collagen molecule has been shown to be elevated in fibrotic tissue compared to healthy state [8]. LOX is expressed in most tissues, however, LOXL2 appears to be more linked to tissue affected by fibrosis and

Abbreviations: AUROC, area under the receiver operating characteristics; LLOD, lower limit of detection; LLOQ, lower limit of quantification; ULOD, upper limit of detection

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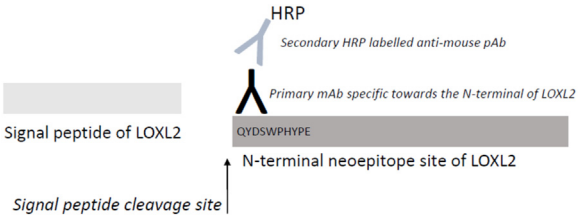

Fig. 1. Schematic overview of the principle behind the N-terminal LOXL2 neoepitope ELISA. The overview shows the activity of the monoclonal (mAb) primary antibody specific against the N-terminal neoepitope QYDSWPHYPE, which becomes exposed during LOXL2 maturation when the signal peptide is cleaved and released. A horseradish peroxidase (HRP) labelled anti-mouse polyclonal antibody (pAb) detects the N-terminal LOXL2 specific mAb.

cancer and is associated with worsening of tumour grade and fibrosis stage [16]. LOXL2 has been found to be over-expressed in lung tissue samples from IPF patients, especially in tissues with activated fibroblasts, reactive pneumocytes and vasculature in fibroblast foci [7,11,16].

We designed and aimed to develop a novel competitive enzyme linked immunosorbent assay (ELISA) using a monoclononal antibody against the N-terminal neoepitope site generated through the release of the signal peptide during LOXL2 maturation (Fig. 1) to show its biological relevance in patients with malignant melanoma, breast-, colorectal-, lung-, ovarian-, pancreatic-, prostate cancer, or IPF versus healthy controls.

2. Materials and methods

2.1. Selection of peptides

In the “Uniprot.org” database the N-terminal cleavage site separating the signal peptide from LOXL2 (Human LOXL2, UniProtKB Q9Y4K0) is predicted at amino acid position 25’. A ten amino acid sequence adjacent to the signal peptide cleavage site (↓) was chosen as the target for monoclonal antibody development: 26’↓QYDSWPHYPE’35. This sequence was used for immunization of mice and used as the standard peptide in assay development. The sequence was blasted for species homology and homology to other human secreted extracellular matrix proteins using the Prabi-Lyon-Gerland “NPS@: Network Protein Sequence Analysis with the UniprotKB/Swiss-prot database” software online [19].

Synthetic peptides used for monoclonal antibody production and validation of the ELISA assay were purchased from Chinese Peptide Company (China) and Genscript (Piscataway, NJ, USA). A biotinylated peptide (QYDSWPHYPE-biotin) was included as a coating peptide on streptavidin-coated ELISA plates. The specificity of the antibody was tested by including an elongated selection peptide with an additional amino acid added to the N-terminal of the target peptide sequence (AQYDSWPHYPE), as well as a non-sense selection peptide (IKAPKLPGGY) and a non-sense biotinylated coating peptide (biotin-IKAPKLPGGY) in the assay validation. The immunogenic peptide (QYDSWPHYPE-KLH) was generated by covalently cross-linking the selection peptide to Keyhole Limpet Hempcyanin (KLH) carrier protein using Succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate, SMCC (Thermo Fisher Scientific, Waltham, MA, USA, cat.no. 22336).

2.2. Monoclonal antibody production

Four to six weeks old Balb/C mice were immunized by subcutaneous injection of 200 µL emulsified antigen and 50 µg immunogenic peptide (QYDSWPHYPE-KLH) mixed with Freund's incomplete adjuvant (Sigma-Aldrich, St. Louis, MO, USA). Consecutive immunizations were performed at 2-weeks interval until stable sera titre levels were reached. The mouse with the highest titre rested for four weeks and was then boosted with 50 µg immunogenic peptide in 100 µL 0.9% NaCl solution intravenously. Hybridoma cells were produced by fusing spleen cells with SP2/0 myeloma cells as previously described (Gefter mL, Margulies DH, Scharff MD. A simple method for polyethylene glycol-promoted hybridization of mouse myeloma cells. Somatic Cell Genet. 1977;3:231–6). The selected hybridoma cells were cultured in 96-well microtiter plates and standard limited dilution was used to secure monoclonal growth. The supernatants were screened for reactivity using the biotinylated coater peptide (QYDSWPHYPE-biotin). All work performed using mice was approved by Beijing laboratory animal administration office and animal ethics committee of Nordic Bioscience (Beijing).

2.3. Monoclonal antibody characterization

Native reactivity and peptide affinity of the monoclonal antibodies were evaluated by displacement using human serum samples and the selection/standard peptide (QYDSWPHYPE) in a preliminary ELISA using 10 ng/mL biotinylated coating peptide on streptavidin-coated microtiter plates (Roche, Basel, Switzerland, cat. #11940279) and the supernatant from the antibody producing monoclonal hybridoma cells. The clones with the best peptide and native reactivity were purified using protein-G-columns according to the manufacturer's instructions (GE Healthcare Life Sciences, Little Chalfont, UK, cat. #17–0404–01). The final selection of the monoclonal antibody for assay development and validation was based on high reactivity towards the selection/standard peptide, relevant native samples including human serum samples and recombinant LOXL2, homolog2 (RnD system, Cat no. 2639-AO).

2.4. LOXL2 ELISA protocol

Optimal incubation-buffer, -time and –temperature, as well as the optimal concentrations of antibody and coating peptide were determined and the finalized LOXL2 competitive ELISA protocol was developed to the following protocol:

A 96-well streptavidin-coated microtiter plate was coated with 0.5 ng/mL biotinylated coating peptide diluted in assay buffer (25 mM TBS-BTB, 4 g/L NaCl, pH 7.4) and incubated for 30 min at 20 °C shaking (300 rpm) in darkness. 20 µL standard peptide (500 ng/mL) or pre-diluted serum sample (1:2) were added to appropriate wells, followed by the addition of 100 µL monoclonal antibody dissolved in assay buffer to a concentration of 14 ng/mL to each well and incubated 1 h at 20 °C shaking (300 rpm) in darkness. 100 µL of rabbit POD-conjugated anti-mouse IgG antibody (Jackson Immunoresearch Laboratories, PA, USA, Cat no. 119936) diluted 1:5000 in assay buffer was added to each well and incubated 1 h at 20 °C shaking (300 rpm) in darkness. All incubation steps were followed by five washes in washing buffer (20 mM Tris, 50 mM NaCl, pH 7.2). Finally, 100 µL tetramethylbenzinidine (TMB) (cat. 438OH, Kem-En-Tec Diagnostics, Denmark) was added to each well, and the plate was incubated for 15 min at 20 °C in darkness shaking (300 rpm). The enzymatic reaction was stopped by adding 0.18 M H2SO4 and absorbance was measured at 450 nm with 650 nm as reference. A calibration curve was plotted using a 4-parameter logistic curve fit. Data were analysed using the SoftMax Pro v.6.3 software.

2.5. Technical evaluation of the LOXL2 ELISA

The inter- and intra-assay variation was determined by ten independent runs including eight quality control samples and two internal controls covering the detection range, with each run consisting of double-determinations of the samples. Five quality control samples consisted of four human serum samples of which two where spiked with the synthetic specific peptide, and one sample of buffer spiked with the synthetic specific peptide. Intra-assay variation was calculated as the mean coefficient of variance (CV %) within plates and the inter-assay

variation was calculated as the mean CV % between the ten individual runs. Two-fold dilutions of three human serum samples were used to calculate linearity. Recovery percentages were calculated with the undiluted, 1:2 or 1:4 sample as a reference value. The lower limit of detection (LLOD) was determined from 21 measurements of the zero sample (assay buffer) and was calculated as the mean + three standard deviations. The upper limit of detection (ULOD) was determined from ten independent runs of the highest standard peptide concentration and was calculated as the mean back-calibration calculation + three standard deviations. The lower limit of quantification (LLOQ) was determined from three independent runs of a serum sample diluted stepwise and calculated as the highest LOXL2 level quantifiable in serum with a coefficient of variation below 30%. Analyte stability was first determined by the effect of repeated freeze/thaw of serum samples by measuring the LOXL2 level in three human serum samples in four freeze/thaw cycles. The freeze/thaw recovery was calculated with the zero cycle as a reference. Second, analyte stability was determined at different time points and temperatures by measuring LOXL2 level in three human serum samples after 0, 2, 4, 24 and 48 h of storage at either 4 °C or 20 °C. Recovery was calculated with 0 h as a reference. Interference was determined by adding a low/high content of haemoglobin (0.155/0.310 mM), lipemia/lipids (4.83/10.98 mM) and biotin (3/9 ng/mL) to a serum sample of known concentration. Recovery percentage was calculated with the normal serum sample as reference.

2.6. Clinical validation of the N-terminal LOXL2 ELISA

Cancer patient serum samples included patients with primary malignant melanoma, breast-, colon-, lung-, ovarian-, pancreas-, prostate cancer and colonoscopy-negative controls (Table 1). The cohort was obtained from the commercial vendors Proteogenex (Culver City, CA, USA) and Asterand Bioscience (Hertfordshire, UK). The IPF cohort included serum samples from 123 patients diagnosed with IPF and enrolled into a clinical trial (baseline samples, CTgov reg. NCT00786201) and healthy control serum samples acquired from the commercial vendor Valley Biomedical (Winchester, VA, USA) (Table 2). Informed consent from all patients was obtained by each commercial vendor prior to sample collection. Sample collections were approved by the local Ethical Committee. The IPF study CTgov reg. NCT00786201 was approved by the Sterling Institutional review board (Atlanta, US). All experiments were performed in accordance with relevant guidelines and regulations.

2.7. Statistical analysis

The level of LOXL2 in serum samples was compared using one-way ANOVA adjusted for Tukey's multiple comparisons test (parametric data), Kruskal-Wallis adjusted for Dunn's multiple comparisons test (non-parametric data) or unpaired, two-tailed Mann-Whitney test. The diagnostic power to identify cancer patients or IPF patients was investigated by the area under the receiver operating characteristics (AUROC). Sensitivity and specificity were determined for optimal cut-off values based on the ROC curves. P-values < 0.05 were considered significant. Graphs and statistical analyses were performed using GraphPad Prism version 6 (GraphPad Software, Inc., CA, USA) and MedCalc Statistical Software version 12 (MedCalc Software, Ostend, Belgium).

3. Results

3.1. Selection and specificity of the antibody utilized in the LOXL2 assay

The N-terminal site of the human target sequence, $\downarrow^{26}$'QYDSWPHYPE'^{35}, was targeted for antibody and sequence blasting showed a 100% homology to Pongo abelii and 80% homology to Bos taurus. The protein blast did not indicate any other similar sequences.

A hybridoma clone producing a monoclonal antibody with optimal native reactivity towards human serum samples and affinity towards the selection peptide was selected (Fig. 2A). The antibody reacted towards the selection peptide generating a standard curve following a five parametric curve. No signal was observed when adding a non-sense biotinylated coating peptide, elongated peptide or non-sense peptide to the assay. These data suggest that the selected antibody was specific towards the target neoepitope. Furthermore, the antibody was able to react towards recombinant LOXL2 protein which includes the N-terminal cleavage site sequence (Fig. 2B).

3.2. Development and technical evaluation of the LOXL2 ELISA assay

A panel of technical validations was performed to evaluate the LOXL2 ELISA assay (Table 3). The measuring range (LLOD to ULOD) of the assay was determined to be 5.7–401.5 ng/mL and the LLOQ was 12.9 ng/mL. Intra- and inter-assay variation was 8% and 12%, respectively, and within our acceptance criteria $< 10\%$ for the intra-assay variation and $< 15\%$ for the inter-assay variation. The recommended human serum dilution was 1:2 and the mean linearity was within 100 ± 20%. A mean analyte recovery in three serum was 106% after 4 freeze/thaw cycles and after storage at 4 °C for 2–48 h the recovery was between 83% and 99% or at 20 °C for 2–48 h the recovery was between 84% and 97%. Finally, the acceptance criterion was a recovery within 100% ± 20%. These data indicate that the analyte in serum is highly stable at 4 and 20 °C. No interference was detected from either low or high content of lipids or haemoglobin with recoveries ranging from 85% to 112%. The acceptance criterion was a recovery within 100% ± 20%. The high level of biotin reduced the levels of LOXL2, whereas low levels (30 ng/mL) did not affect the assay. Thus, the cut-off level should be biotin below 30 ng/mL.

Table 1
Overview of clinical and patients demographics of cohort 1 and 2. SD: Standard deviation. SCLC: small-cell lung cancer; NSCLC: non-SCLC, IPF: Idiopathic pulmonary fibrosis.

Patient type	No. of subjects	Mean age years (SD)	Gender (% females)	AJCC UICC stage (%)
Colonoscopy-negative controls	16	55.5 (5.6)	44	–
NSCLC patients	19	60.4 (9.3)	21	IA(11), IB(22), IIA(11), IIB(11),IIIA(33), IV(12)
SCLC patients	7	61.4 (12.6)	29	IA (14), IB(14), IIA(14), IIB(),IIIA(), IIIB() IIIC()
Breast cancer patients	20	55 (10.3)	95	IIA(30), IIB(35),IIIA(25), IIIB(5) IIIC(5)
Colorectal patients	7	61.9 (8.6)	86	IIA(43), IIIA(43), IIIB(14)
Gastric cancer patients	8	69.3 (9.2)	38	IA (12.5), IB(37.5), II(12.5) IIA(25), IV(12.5)
Melanoma patients	7	45.6 (14.4)	43	I(14), II(72), IIA(14)
Ovarian cancer	9	55.9 (11.2)	100	IA (11),II(11), IIA(11), III(34), IIIA(11), IIIB(11) IIIC(11)
Pancreatic cancer	5	68.4 (10.1)	60	IB(50), III(50)
Prostate cancer	14	63.7 (5.7)	0	I(7), II(64), IIA(22), IIB(7)
Healthy controls	51	35.9 (11.9)	19	–

Table 2
Overview of clinical and patients demographics patients with Idiopathic pulmonary fibrosis (IPF). NA: Not available.

Samples	No. of subjects	Age (SD)	BMI (SD)	Gender (%females)	FVC%	FEV1	DLCO%
IPF patients	123	65.0	30.5	20	68.6	2.3	39.7
Healthy controls	51	35.9 (11.9)	NA	19	NA	NA	NA

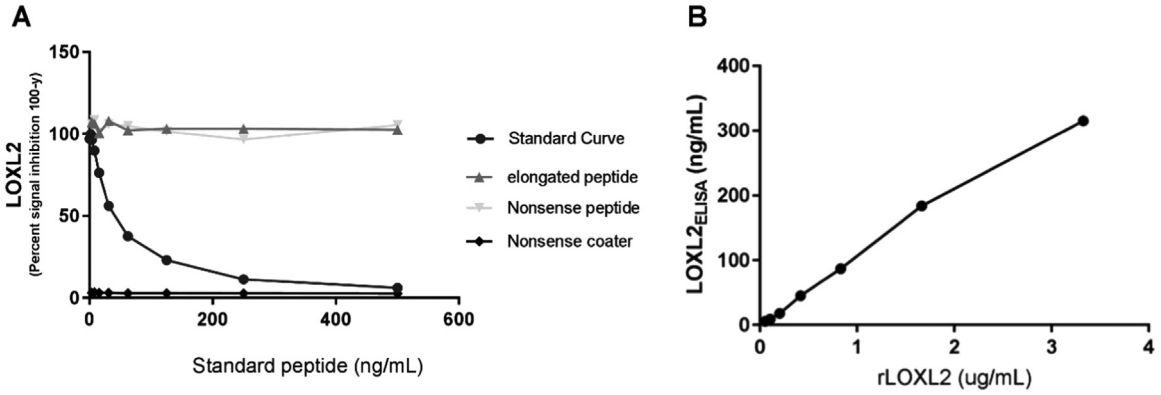

Fig. 2. Specificity data of the LOXL2 ELISA. A) The activity of the monoclonal antibody employed in the LOXL2 ELISA towards the target peptide (QYDSWPHYPE), the elongated peptide (AQYDSWPHYPE), a non-sense peptide (IKAPKLPGGY) and a non-sense coating peptide (biotin- IKAPKLPGGY). Reactivity was shown as percent inhibition of the zero sample (buffer) signal assessed as optical density (OD) at 450 nm (subtracted the background at 650 nm) and as a function of the peptide concentrations. B) Recombinant LOXL2 (rLOXL2) was added at the indicated concentrations to the LOXL2 ELISA. The LOXL2 ELISA data are shown as mean of double determinations for each rLOXL2 concentration.

Table 3
Summary of the technical details of the N-terminal LOXL2 ELISA.

Technical validation step	LOXL2 performance
Measuring range (LLOD-ULOD)	5.7 – 401.5 ng/mL
Lower limit of quantification (LLOQ)	12.9 ng/mL
Intra-assay variation	8%
Inter-assay variation	12%
Dilution of serum samples	1:2
Linearity[a]	102% (95–109%)
Freeze/thaw recovery (4 cycles)[a]	106% (104–108%)
Analyte stability up to 48 h, 4 °C[a]	84% (84–97%)
Analyte stability up to 48 h, 20 °C[a]	93% (83–99%)
Interference Lipids, low/high	106% / 85%
Interference Biotin, low/high	95% / 21%
Interference Haemoglobin, low/high	98% / 112%

[a] Percentages are reported as mean with range shown in brackets.

3.3. LOXL2 is elevated in patients with cancer and IPF

Serum LOXL2 levels were in elevated 218%, 227%, 375%, 213%, 225% and 325% in patients with breast cancer (BCa), colorectal cancer, non-small cell lung cancer (NSCLC), small-cell lung cancer (SCLS), ovarian cancer and pancreatic cancer respectively compared to healthy controls ($p = 0.05–0.0001$)(Fig. 3). Furthermore, it was seen that serum LOXL2 was increased + 163% in patients with IPF compared to healthy controls ($p < 0.0001$)(Fig. 4). The diagnostic value of LOXL2, assessed by the AUROC, was 0.89 for diagnosis of NSCLC ($p < 0.0001$), and 0.72 ($p = 0.02$) and 0.81 ($p < 0.0001$) for diagnosis of prostate cancer (PCa) and BCa, respectively (Table 4). The AUROC of LOXL2 for detection of IPF versus healthy controls was 0.72 ($p < 0.0001$)(Table 4).

4. Discussion

In the present work, we have demonstrated that a new technical assay towards LOXL2 maturation was developed using a highly specific monoclonal antibody towards the signal peptide cleavage site within LOXL2. Furthermore, it was shown that LOXL2 maturation is elevated

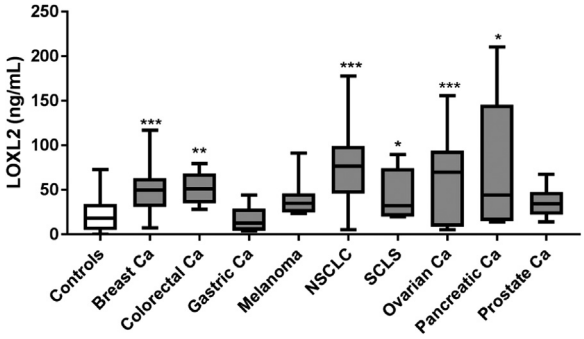

Fig. 3. Serum LOXL2 in patients with various cancer diseases LOXL2 was assessed in serum of healthy controls (n = 16) or patients with breast cancer (Ca) (n = 20), colorectal cancer (n = 7), gastric cancer (n = 8), melanoma (n = 19), non-small cell lung cancer (NSCLC) (n = 8), small cell lung cancer (SCLS) (n = 7), ovarian cancer (n = 9), pancreatic cancer (n = 5) or prostate cancer (n = 14). Data were compared using Kruskal-Willis comparison test for non-parametric data. Data are shown as box-whisker plots. Significance levels: * **: $p < 0.001$ and * ***: $p < 0.0001$.

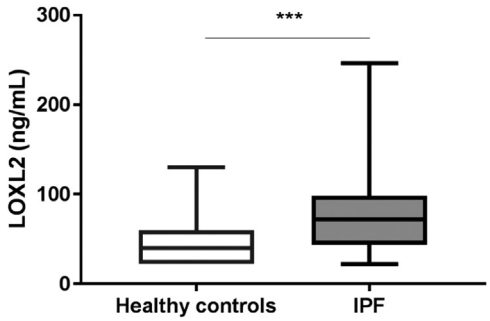

Fig. 4. Serum LOXL2 in patients with idiopathic pulmonary fibrosis (IPF) LOXL2 was assessed in serum of patients that included patients diagnosed with IPF (n = 123) or healthy controls (n = 51). Data including two groups were compared using unpaired, two-tailed Mann-Whitney test. Data are shown as box-whisker plots. Significance levels: * **: $p < 0.001$ and * ***: $p < 0.0001$.

Table 4
AUROC for diagnosis of idiopathic pulmonary fibrosis (IPF) or cancer by LOXL2, patient group with n > 10. NSCL: Non-small cell lung cancer, BCa: Breast cancer; PCa: Prostate cancer.

Disease	AUROC	Sensitivity	Specificity	P-value	Cut off ng/mL
NSCLC	0.89	73.7	93.7	< 0.0001	51.4
BCa	0.81	70.0	81.2	< 0.0001	34.3
PCa	0.72	85.7	62.5	0.02	19.8
IPF	0.72	52.5	88.2	< 0.0001	69.0

in patients with IPF as well as in patients with various primary cancer types. We concluded that this may be a new assay to be used for the assessment of the degree of LOX2 maturation rather than LOXL2 concentration, which other LOXL2 assays as a measure of. Furthermore, the levels of LOXL2 maturation were detectable in healthy individuals. Finally, the diagnostic value of LOXL2 for NCSLC and IPF was high with AUROC ranging from 0.72 to 0.89.

Several groups have shown that LOXL2 is elevated in patients with various cancers such as pancreatic, breast, lung, colorectal and prostate cancers, which may relate to progression when determined by immunohistochemistry, protein determination in tissue, and tissue array [24–30]. In IPF patients from the ARTEMIS-IPF study and GAP study high serum LOXL2 levels were associated with progression, were correlated to lung related parameters and in the GAP study LOXL2 was related to an increased risk for mortality [21]. These data are in alignment with the data for the novel LOXL2 maturation assay presented here, however we did not evaluate mortality.

The neoepitope nature of the novel LOXL2 maturation assay, reflects the degree of maturation LOXL2 rather than LOXL2 activity. Other assays targeting LOXL2 have been developed, however these do not to our knowledge reflect LOXL2 maturation [18,21]. The most well-described LOXL2 assay, developed by Chien and colleagues is a sandwich ELISA that detects LOXL2 in serum using a polyclonal antibody and a monoclonal antibody raised against full-length recombinant human LOXL2 and the C-terminal catalytic domain of LOXL2, respectively. This assay thus targets epitopes close to the C-terminal end reflecting LOXL2 concentration, in contrast to the LOXL2 maturation assay which targets the signal peptide cleavage site. The assay by Chien et al. was not able to detect LOXL2 levels in healthy individuals [18], in contrast to the LOXL2 maturation assay, which was detectable. The reason for this discrepancy may lie within the difference in analyte detection between each assay and that one targets a neoepitope reflecting LOXL2 maturation. It is noteworthy, that the LOXL2 assay developed by Chien and colleagues is not a commercially available assay, thus it cannot be assessed by other researchers or in a central laboratory. Also, for this reason a head-to-head comparison could not be made between these two assays.

Inhibitors of LOXL2 have been proposed in the literature as potential clinical trials of IPF patients or in patients with colorectal cancer, or pancreatic cancer, that a humanized monoclonal antibody against LOXL2, simtuzumab, did not reach the primary endpoint of progression-free survival [17,20]. However, the clinical design within these trials has been discussed extensively, debating whether a poor design or antibody may have had an impact on the validity of the results. A fair number of pharmaceutical companies have stayed engaged in testing small molecule inhibitors of LOXL2 as drug candidates.

5. Conclusion

In conclusion, we demonstrated that a technical robust assay for a neoepitope site in the N-terminal site of LOXL2 as a measure of protein maturation could be developed and showed that the level of LOXL2 maturation was elevated in patients with various cancer types and IPF compared to healthy controls. Thus, we demonstrated the feasibility and the potential biological value of this newly developed LOXL2 maturation assay to be used commercially.

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Appendix A. Transparency document

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbrep.2018.11.002.

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