Is Lysyl oxidase only a partial regulator in tumor progression and metastasis?

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Abstract

Lysyl oxidase (LOX) catalyzes the enzymatic cross-linking of collagen and elastin and thus, maintaining integrity of the extracellular matrix. The potential role of candidate biomarkers in cancer cell invasiveness has been evaluated by in vitro assays. In the present study we tested the hypothesis that whether or not, LOX alone will facilitate tumor progression through regulation of cell matrix adhesion formation. Furthermore, a dot blot assay using hyperimmune serum raised against recombinant LOX was carried out to study its efficacy in detecting circulating LOX in serum from canine mammary tumor case.

In silico characterization of the LOX protein along with its protein paralogs LOXL1, LOXL2, LOXL3 and LOXL4 was done to compare their physiochemical properties, predicted secondary structure and motifs wherein certain variations among LOX paralogs were observed, which may provide the insight into their functional myriads. Our study found that this alone is not responsible for tumor invasion and metastasis and it seems that a coordinated effect of LOX along with the other invasion associating factors like metalloproteases, hypoxia, hydrogen peroxide mediated mechanism etc. together contribute towards tumor progression.

Keywords: Lysyl oxidase, *in vitro* assays, mammary tumor, canine, cancer, extracellular matrix, *in silico*.

Introduction

Mammary gland tumors are the most commonly diagnosed neoplasm in female dogs. Dogs can serve as an imperative model for human breast cancer development study since they share their living environment with humans and are exposed to the same carcinogens. It has been estimated that one of four dogs aged 2 years and above probably dies due to cancer[21]. The compound interface between host and tumor microenvironment coupled with tumor progression and development provides exclusive insights into tumor biology.

Lysyl oxidase (LOX) and LOX-like 1 to 4 proteins act as an extracellular regulating enzymes, initiating covalent cross linking of collagen and elastin. LOX is synthesized as a pre-proenzyme and subsequently cleaved into a LOX propeptide and catalytically mature LOX. Increased activity of extracellular LOX has been reported to promote tumor cell invasion and metastasis by regulation of collagen cross-linking and stiffness[16]. A functional role for LOX in cancer has been reported in breast, colorectal, prostate, gastric, pancreatic cancer, head and neck squamous cell carcinoma (SCC), renal clear cell carcinoma, melanoma, oral and oropharyngeal SCC as well as basal and squamous cell skin carcinoma[27].

Over-expression of LOX mRNA or protein is associated with poor prognosis and decreased overall survival[22]. Further, inhibiting LOX activity has been found to be effective in preventing tumor initiation and metastatic colonization[20].

Extracellular matrix consists of a basement membrane and a stromal matrix which confers steric barrier to cell transmigration. Thus, matrix modelling and mobility from a non metastatic to a metastatic site are regarded as critical factors in tumor progression which involves forming a stable adhesion to the extracellular matrix[19]. ECM remodelling through increased collagen linearization has also been associated with stromal expression of LOX induced due to hypoxia. Increased invasion and metastatic potential of tumors have been associated with fluctuating oxygen levels. LOX expression and catalytic activity have been found to be upregulated driving poorly invasive breast cancer cells towards achieving metastatic competency following hypoxia/ reoxygenation[23]. Transmembrane MMPs and the gelatinases are reported as critical regulators of basement membrane remodelling[12].

The mammalian lysyl oxidase gene family consists of prototypic *lox* and additional four *lox*-like genes (*loxl* 1-4) encoding proteins that share a highly conserved C terminal domain but diverse at their N termini[10]. A catalytic domain (having a copper-binding motif and a unique lysyl-tyrosylquinone LTQ cofactor) and a cytokine receptor like domain is present at their C terminal[4]. Conserved LTQ cofactor is essential for the catalytic activity of LOX and LOX like proteins.

We have earlier reported the over-expression of LOX in canine mammary tumors through qPCR assay[24]. Here we studied the role of LOX in facilitation of tumor progression by investigating the invasive property conferred by LOX to MDCK cells using *in vitro* cell invasion assay.

In silico characterization of the LOX protein along with its protein paralogs LOXL1, LOXL2, LOXL3 and LOXL4 was also carried out using the available computational tools to study their physiochemical properties, predicted secondary structure and phylogenetic analysis.

Material and Methods

Purification of rLOX protein: The log phase grown culture of lysyl oxidase confirmed clones were prepared and were bulk cultured till log phase and induced with 1mM isopropyl-B-D-thiogalactopyranoside. Cultures were pelleted and re-suspended in lysis buffer (100mM Tris-Cl, 100mM Sodium phosphate, 8 M Urea; pH 8) for 20 min at 4°C. Subsequently, culture were sonicated at 15Hz with a pulse of 30 s for 20 cycles and centrifuged at 10000xg for 30 min. The supernatant was passed through Ni-NTA agarose column (Qiagen, Germany). Wash buffer (100mM Tris-Cl, 100mM sodium phosphate, 8 M Urea; pH 6.3) was used for washing the column followed by elution of the bound protein with increasing concentration of imidazole (10, 20, 40, 80 and 200 mM in elution buffer). Purified protein was stored at -20°C till further use.

Cell line: Madin-Darby Canine Kidney (MDCK) cell line was being maintained at College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) containing 2% or 10% fetal bovine serum (FBS), 100U/ml penicillin, 100 µg/ml streptomycin and incubated at 37°C in a humidified CO_2 incubator.

***In-vitro* cell invasion assay:** MDCK cells were sub-cultured in a cell culture flask in DMEM supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin and 2% FBS. For this assay, BioCoat™ Matrigel® Invasion Chambers (Corning, USA) were used. These chambers consist of cell culture inserts containing 8µm pore size membrane treated with Matrigel basement membrane matrix. About 2 ml of pre-warmed culture medium (DMEM with 2% FBS) was added to the interior of the inserts and bottom of wells. Plates were allowed to rehydrate for 2 h in humidified tissue culture incubator at 37°C in 5% CO_2 atmosphere. After rehydration, the medium was carefully removed without disturbing the layer of matrix on the membrane.

Chemo-attractant (DMEM with 10% FBS) was added to the bottom wells making sure that chemo-attractant liquid in the bottom well makes contact with the membrane on the lower side of the chamber to form a chemo-tactic gradient. Subsequently cell suspension in maintenance medium (2% FBS) was added to the top of the membrane so that the seeding density remains approximately 1 X 10^5 cells/ml. The plates were incubated for 2 h in a CO_2 incubator. The cells were treated with 50 ng/ml each of recombinant LOX or proteinase K (positive control) and incubated at 37°C for 36 h in a humidified CO_2 incubator. A negative control without any treatment was also kept.

Measurement of cell invasion: Trypsin harvesting method was used to quantify total cells that had invaded through each matrigel well. Excess media was aspirated followed by a brief rinse in PBS. Non invaded cells were swabbed from upper side of the membrane. The inserts were transferred to a fresh 6 well plate containing 500µl of 0.2% trypsin so that the invaded cells on the underside were released into the solution within 3 min. The solution was then centrifuged (2000 x g, 5 min) and this suspension was used for counting number of cells invaded through the membrane.

Cells were counted with Trypan Blue using a hemocytometer. Further matrices were visualized under inverted microscope (Nikon, Japan). We randomly selected 8 fields of view in the center as well as in the periphery of the membrane and images were captured.

Dot Blot Analysis: Briefly, 10 µl of recombinant LOX protein (positive antigen control), bovine serum albumin (negative control) and serum from mammary tumor subject were blotted onto nitrocellulose membrane. After incubation for 4 h at 4°C, the membrane was washed thrice with PBS-T. Blocking was done with 3% skim milk powder and membrane incubated overnight at 4°C.

Following incubation and washing, 10µl of primary antibody (mice hyperimmune serum raised against rLOX) diluted 1:100 in blocking buffer was added. Upon incubation at 37°C for 1 h and washing thrice with PBS-T, the secondary antibody (HRPO conjugated anti-mice IgG, Sigma, USA) (1:2000) was added and the membrane was again incubated at 37°C for 1 h. After washing, 50 µl of freshly prepared substrate/chromogen mixture (DAB-5mg, PBS-10ml, 30% H_2O_2-20 µl) was added to the membrane and kept at 37°C for approximately 5 min for color development.

***In silico* characterization**

Sequence retrieval: The protein sequences of the canine LOX, LOXL1, LOXL2, LOXL3, LOXL4 were retrieved from the NCBI protein database. Accession numbers ofthese proteins are presented in table I.

Table 1
Accession numbers of the proteins

Species name	Accession number
LOX	QBA55221.1
LOXL1	XP_022268408.1
LOXL2	XP_025318873.1
LOXL3	XP_025309468.1
LOXL4	XP_543959.2

Multiple sequence alignment (MSA): The sequences once retrieved in FASTA format were aligned using Clustal omega online tool using default parameters[25].

Amino acid composition: The amino acid composition of the proteins was computed using CLC Genomics Workbench 20.0 (https://digitalinsights.qiagen.com).

Physico-chemical properties: Physico-chemical properties of the proteins were determined using ProtParam tool from Expert Protein Analysis System (ExPASy)

(https://www.expasy.org/)[5]. Various biophysical and biochemical properties of the retrieved sequences were evaluated. Isoelectric point(pI), instability index (II-stability of proteins)[9], aliphatic index (AI-relative volume of protein occupied by aliphatic side chains)[13], molecular weight (Mw), number of positive and negative residues, extinction coefficients (EC-quantitative study of protein-protein and protein-ligand interactions)[7], grand average hydropathicity (GRAVY-sum of all hydropathicity values of all amino acids divided by number of residues in a sequence)[15] and half life[8] were calculated.

Secondary structure prediction: The secondary structure of the proteins was predicted using SOPMA server. This tool predicts the positional possibility and evaluates the percentage of the four states: alpha helix, β strands, turns and random coils using default parameters.

Conserved Motif Detection: Motif Elicitation (MEME) server[2] was used to identify motifs in profiles using multiple EM.

Phylogenetic analysis: To infer the evolutionary divergence between the LOX paralogs, a phylogenetic tree was constructed using unweighted pair group method (UPGMA) of the Molecular Evolutionary Genetics Analysis version 6.0 (MEGA)[14]. A bootstrap analysis of 1000 replications confirmed the consistency of the inferred phylogenetic tree. The gaps and missing data were eliminated.

Results

***In vitro* cell invasion assay:** In cell invasion assay, there were not many cells that had invaded through the Matrigel membrane in case of rLOX treated cells when compared to non-treated control (Data not shown). Further microscopy of the invasion chambers upon treatment with rLOX showed no significant change in the membrane when compared to untreated control (Figure I). However, treatment with proteinase K (positive control) led to a distinct damage to cells and extra cellular matrix. This indicates that LOX on its own does not cause ECM degradation and rather, it might depend on the proteolytic activity of metalloproteases or a hydrogen peroxide mediated pathway for its role in enhanced metastasis and tumor progression.

Dot Blot analysis: In the present study, antibodies raised against the recombinant protein in mice were detected circulating LOX protein in the positive serum (serum from mammary tumor subject) in dot blot assay (Figure II). There was no reaction with negative antigen control. This detection of circulating serum LOX by dot blot assay could be considerably correlated with detection of tumor in dogs with mammary tumors.

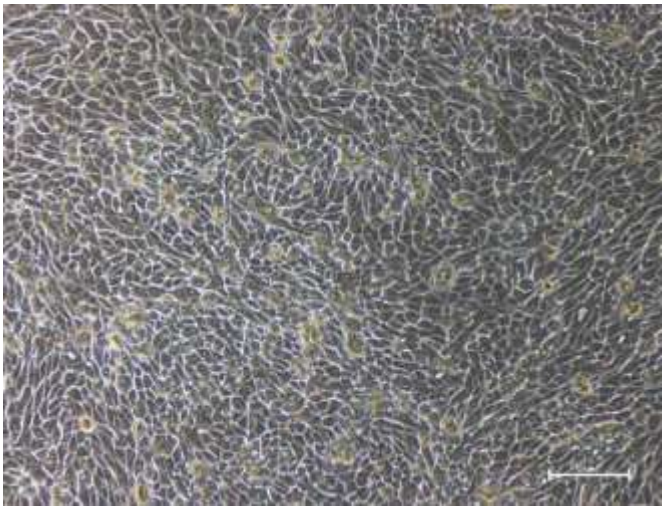
***In silico* characterization**

Multiple sequence alignment (MSA): Multiple sequence alignment using Clustal omega software produces sequence alignments of divergent sequences (Figure III). Percent similarity of the canine lysyl oxidase protein with other lysyl oxidase homologs was determined.

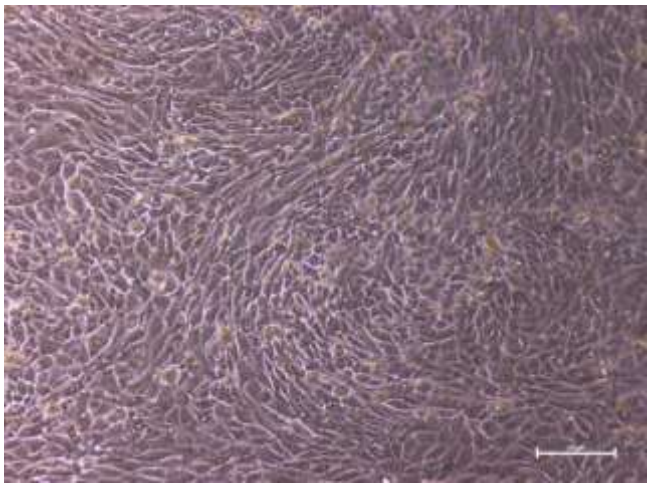
Table II
Amino acid composition computed using CLC workbench

Amino Acid	LOX	LOXL1	LOXL2	LOXL3	LOXL4
Ala (A)	11%	7.3%	6.7%	7.1%	7.0%
Arg (R)	9.5%	4.6%	5.4%	7.6%	7.9%
Asn (N)	4.2%	5.0%	5.1%	3.2%	4.4%
Asp(D)	6.1%	7.3%	5.0%	4.5%	4.1%
Cys (C)	2.7%	5.0%	4.5%	4.8%	4.7%
Gln (Q)	5.6%	4.6%	4.2%	4.4%	5.9%
Glu (E)	2.9%	4.1%	7.1%	6.3%	6.3%
Gly (G)	6.8%	4.1%	9.4%	10.2%	9.5%
His (H)	2.9%	5.0%	3.2%	4.0%	4.0%
Ile (I)	2.2%	3.2%	4.4%	3.5%	2.0%
Leu (L)	6.8%	7.3%	6.3%	7.8%	9.2%
Lys (K)	1.5%	4.6%	5.0%	4.0%	3.2%
Met (M)	1.2%	0.9%	2.1%	1.3%	2.0%
Phe (F)	2.2%	3.2%	3.1%	2.7%	2.2%
Pro (P)	9.0%	5.0%	4.1%	4.7%	4.4%
Ser (S)	7.1%	6.4%	6.3%	7.2%	6.6%
Thr (T)	4.6%	6.9%	4.4%	5.2%	3.4%
Trp (W)	1.7%	1.8%	2.4%	2.7%	2.6%
Tyr (Y)	8.1%	6.4%	4.1%	2.1%	2.6%
Val (V)	3.7%	6.9%	7.4%	6.7%	7.9%

Amino acid composition: The amino acid composition of the proteins was computed using CLC Genomics Workbench. It was observed that alanine was the most abundant amino acid present in LOX and LOXL1 while glycine was the most abundant in LOXL2, LOXL3 and LOXL4. The composition of methionine was least when compared to other amino acids for all the paralogs (Table II).



(a)



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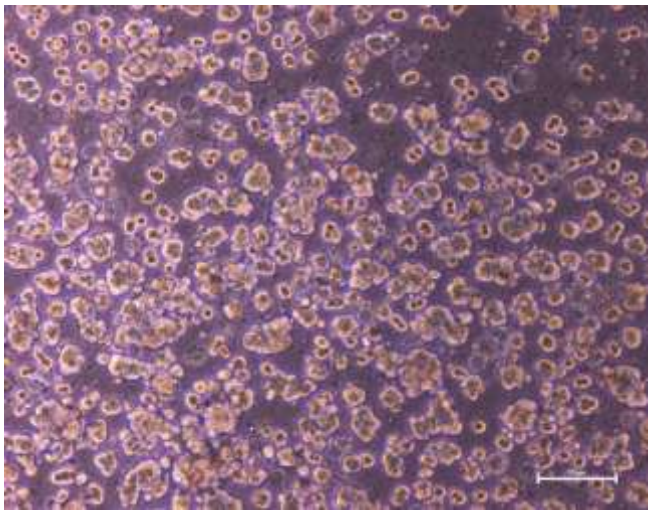

(c)

Fig. I: Microscopic changes in the matrigel matrix upon different treatments (10X) (a) control; (b) cells treated with lysyl oxidase; (c) cells treated with proteinase K

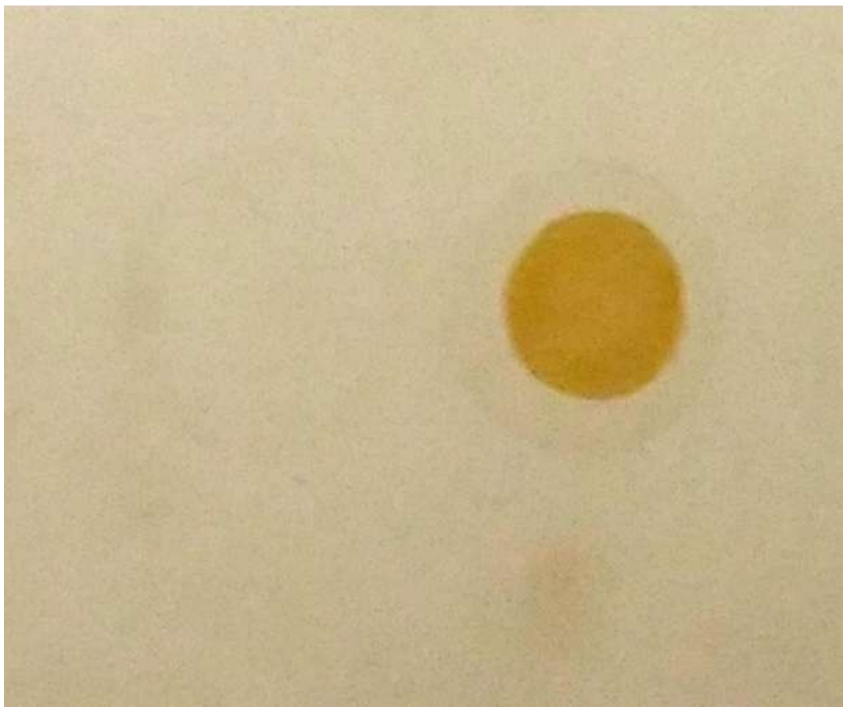

Fig. II: Dot Blot Analysis

Fig. III: Multiple sequence alignment (MSA) of Canine LOX with its paralogs (LOXL1, LOXL2, LOXL3, LOXL4)

Physico-chemical properties: Table III shows the different physico-chemical properties of the proteins computed using ProtParam tool. The properties like half life, aliphatic index, grand average of hydropathicity (GRAVY), molecular weight, theoretical pI, amino atomic composition, extinction coefficient, instability index etc. were computed as shown in the table. Computed value of pI of LOX, LOXL3 and LOXL4 was >7, while for LOXL1 and LOXL2 it was <7. Extinction coefficient aids in the quantitative study of protein-ligand and protein-protein interactions in solution. Maximum EC at 280nm was reported for LOXL2 at 1.769 M^{-1} cm^{-1}. The overall stability of the proteins can be studied by the index of instability, aliphatic index and GRAVY index. The stability of a protein *in vitro* and *in vivo* estimates its instability index.

The study shows that the instability index of all the proteins is >40 indicating that these proteins are unstable. In our study, all the proteins are thermostable owing to the higher aliphatic index. The grand average of hydropathy (GRAVY) index was found to be negative. The estimated half life of the proteins was around 30 h. Positively charged residues are calculated by the total number of Arginine (Arg) and Lysine (Lys) while the negatively charged residues are calculated by the total number of Aspartic acid (Asp) and Glutamic acid (Glu). They help in resolving the topology of protein.

Secondary structure prediction: The secondary structure of the proteins was predicted using SOPMA server (Table IV). The computed percentage of each confirmation revealed the abundance of the random coil followed by other secondary structures of alpha helix, extended strand and beta turn respectively. Presence of hydrophobic proline and flexible glycine has been attributed to the high coiled structural content of the proteins. Proline disrupts the secondary structure and creates kinks in the polypeptide structures.

Conserved Motif Detection: Motif Elicitation (MEME) server was used to identify motifs in profiles using multiple EM. MEME discovers ungapped, novel motifs in the sequence. Five significant motifs were identified (Figure IV). The motif matches shown have a position P<0.0001 and sequences with an E<10 are shown.

Phylogenetic analysis: The phylogenetic tree constructed using MEGA software showed that the canine LOX was phylogenetically more similar to LOXL2 sequence while the LOXL3 was more similar to LOXL4 (Figure V). LOXL1 formed an independent clad away from other homologs. In a previous study, broad selections of genomes were surveyed to infer the evolutionary history of LOX. LOX proteins have been identified in eukaryotes, archaea as well as in bacteria[10]. A preluding phylogenetic analysis of the human LOX genes revealed a common ancestry between LOX and LOXL1 while LOXL2, LOXL3 and LOXL4 formed an independent group[11].

Table III
Physicochemical properties computed using ProtParm tool

S.N.	Biophysical and Biochemical Properties	LOX	LOXL1	LOXL2	LOXL3	LOXL4
1.	No of amino acids	409	218	780	792	758
2.	Molecular weight	46150.29	24910.89	87203.29	87563.94	84675.86
3.	Isoelectric point	8.84	6.09	6.05	8.14	8.07
4.	Negatively charged residues (Asp + Glu)	37	25	94	86	79
5.	Positively charged residues (Arg + Lys)	45	20	81	92	84
6.	Extinction coefficients	88295	43485	154305	143205	142050
7.	Abs 0.1%	1.913	1.746	1.769	1.635	1.678
8.	Instability index:	50.34 (Unstable)	43.09 (Unstable)	42.91 (Unstable)	47.80 (Unstable)	43.55 (Unstable)
9.	Aliphatic index:	56.92	68.44	69.73	70.80	73.68
10.	(GRAVY)	-0.757	-0.504	-0.444	-0.451	-0.456
11.	Half life	30 hrs	30hrs	30hrs	30hrs	30hrs
12.	Atomic composition					
	Carbon	2034	1101	3823	3805	3673
	Hydrogen	3057	1657	5867	5943	5736
	Nitrogen	603	305	1087	1149	1120
	Oxygen	605	333	1157	1140	1091
	Sulphur	16	13	51	48	51

Table IV
Secondary structure prediction using SOPMA server

Secondary Structure	LOX	LOXL1	LOXL2	LOXL3	LOXL4
Alpha helix	23.96%	20.64%	21.92%	19.70%	22.03%
3_{10} helix	0.00%	0.00%	0.00%	0.00%	0.00%
Pi helix	0.00%	0.00%	0.00%	0.00%	0.00%
Beta bridge	0.00%	0.00%	0.00%	0.00%	0.00%
Extended strand	16.63%	24.31%	20.38%	21.21%	22.30%
Beta turn	6.36%	5.05%	6.54%	6.94%	6.33%
Bend region	0.00%	0.00%	0.00%	0.00%	0.00%
Random coil	53.06%	50.00%	51.15%	52.15%	49.34%
Ambigous states	0.00%	0.00%	0.00%	0.00%	0.00%
Other states	0.00%	0.00%	0.00%	0.00%	0.00%

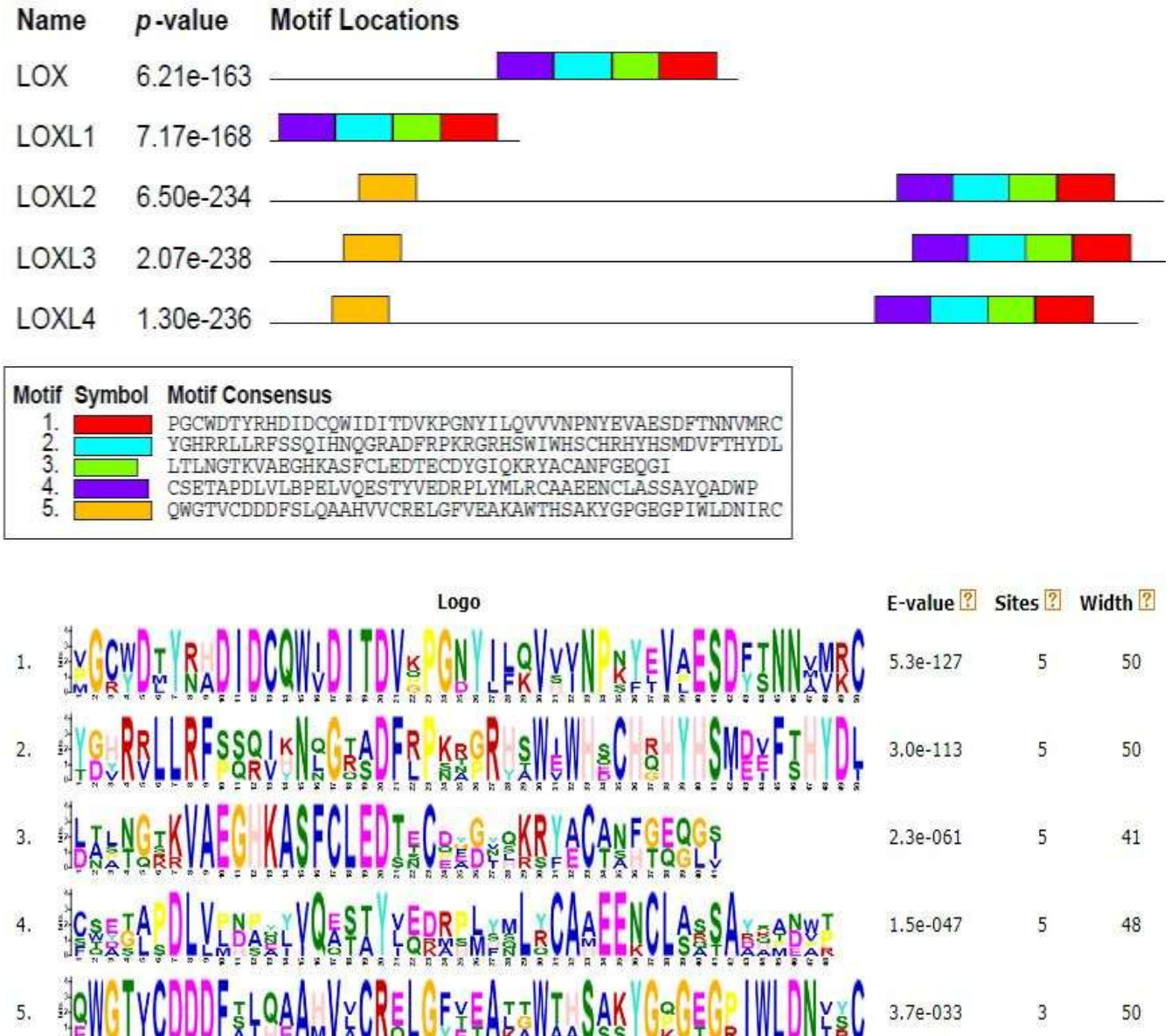

Fig. IV: Conserved Motif Detection using Motif Elicitation (MEME) server

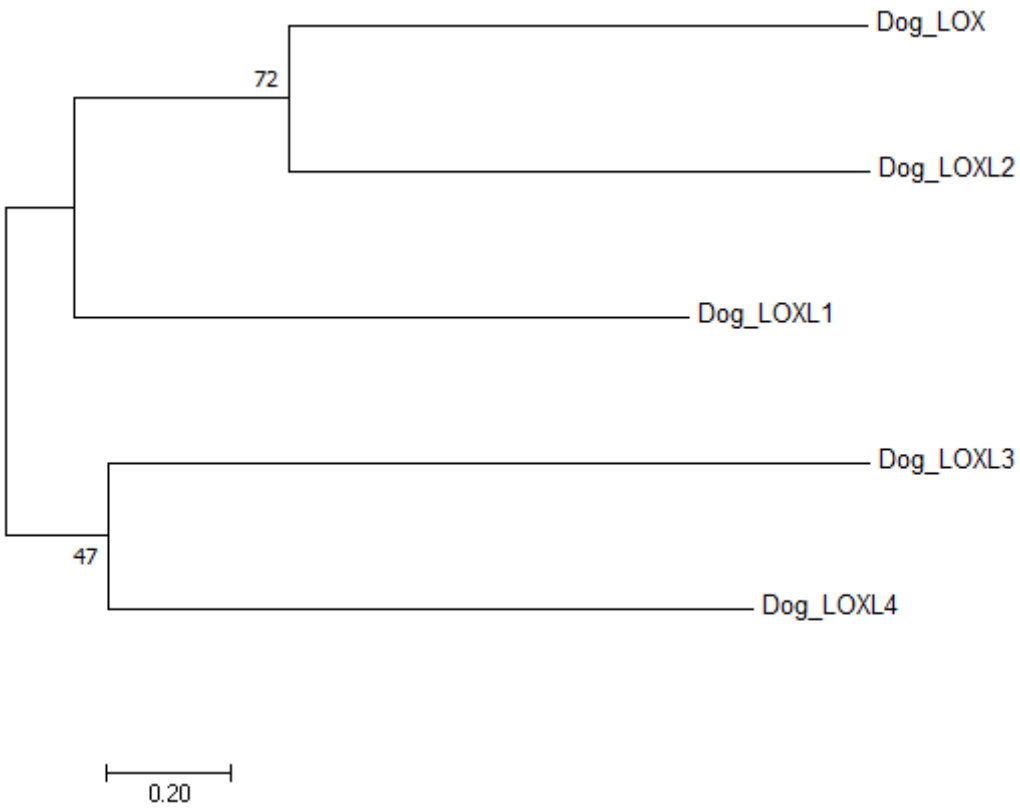

Fig. V: Phylogenetic analysis showing evolutionary relationship among different homologs using MEGA 6.0 software (Boot-straps: 1000 replicates)

Discussion

In vitro assays are valuable for evaluating the potential role of candidate biomarkers in their role in cancer cell invasiveness[1,12]. For patients diagnosed with advanced disease, devising effective strategies to prevent further metastatic spread is instrumental for improving their survival. Cancer disease progression involves cell-cell and cell-matrix adhesion followed by migration and invasion[6]. Matrigel is assumed to resemble the basement membrane matrices and thus, used for evaluating *in vitro* invasion within short duration of time[1].

Our recent research indicated that LOX expression in tissues correlates positively with canine mammary tumors[24]. Also, we could demonstrate that canine mammary tumors can be distinguished from healthy subjects by detecting LOX in serum or gene expression profiling. The promising role of LOX in metastasis strongly suggests that altering LOX action might be a novel approach in treatment. However, invasion and metastasis mechanism of LOX still remain unclear. In our present study, the addition of exogenous rLOX could not confer any invasive character and cells were unable to invade the basement membrane, thus, indicating no invasion, or migration *in vitro.*

Erler and Giaccia[3] reported that cell-cell and cell-matrix adhesion are vital for metastasis and colonization in secondary organs. The invasive property of hypoxia induced over-expression of intracellular LOX had been reported[28]. Postovit et al[23] demonstrated that both hypoxia and re-oxygenation are necessary for LOX catalytic activity which drives poorly invasive breast cancer cells towards a more aggressive phenotype through a hydrogen peroxide-mediated mechanism suggesting a potential mechanism by which less invasive cells can obtain metastatic capability.

LOX has been proposed to change the ECM structure and over-express MMP's through some unknown signal as they both share a deep relation with ECM and basement membrane. LOX probably exerts a synergistic effect along with MMP-2 and MMP-9 on ECM remodeling and tumor cell invasion[18].

Dot blot is a faster, cheaper and easier technique to test for the presence of a protein of interest in a sample. In the dot blot assay carried out in present study, hyperimmune serum raised in mice detected circulating LOX in the serum from dog with mammary tumor. In human breast cancer, the serum HER2 levels were measured and evaluated by dot blot assay predicting its clinical value in tumor progression[26]. Previously, serum lysyl oxidase based enzyme-linked immunoabsorbent assay (ELISA) using hyperimmune serum has been shown to differentiate dogs with mammary tumor from healthy dogs[24].

Multiple sequence alignment helps us to compare and contrast query sequences based on their shared positions assuming to have an evolutionary relationship and

descending from a common ancestry. In the present study, LOX protein had the highest percent identity with LOXL1 and the least with LOXL2. LOXL1 has been reported as the closest mammal paralog of LOX[17].

Theoretical pI calculated using the pKa values of amino acids is crucial for defining the pH-dependent characteristics of a protein. Computed value of pI indicated LOX, LOXL3 and LOXL4 were found to be basic, while LOXL1 and LOXL2 were found to be acidic in nature. The relative volume of protein occupied by its aliphatic side chains (Alanine, isoleucine, leucine and valine) implies the thermostability of proteins. Aliphatic index values are propotional to the structural stability of a protein and higher values are reported to enhance the thermal stability of globular proteins[13]. The grand average of hydropathy (GRAVY) index deciphers the solubility of proteins wherein a positive value indicates a hydrophobic protein while a negative value indicates hydrophillic protein[15]. The negative GRAVY index of our proteins indicates a better interaction with water.

Conclusion

In conclusion, LOX alone is not responsible for tumor metastasis and its interaction with invasion associating factors like MMP-2, MMP-9, hypoxia and other such mechanisms might be responsible for this. Insights from their secondary structure prediction, motif detection and evolution of this family of proteins are also reported in the study. Further studies planned in future indicating the interactions of LOX protein with the various invasion factors will provide substantial insights into this area of research and might aid in treatment of this malady.

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References

1. Albini A., Benelli R., Noonan D.M. and Brigati C., The "chemoinvasion assay": a tool to study tumor and endothelial cell invasion of basement membranes, *Int J Dev Biol.*, **48(5-6)**, 563-571 **(2004)**

2. Bailey T.L. and Elkan C., Fitting a mixture model by expectation maximization to discover motifs in biopolymers, Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology, AAAI Press, Menlo Park, California, 28-36 **(1994)**

3. Erler J.T. and Giaccia A.J., Lysyl oxidase mediates hypoxic control of metastasis, *Cancer Res.*, **66(21)**, 10238-41 **(2006)**

4. Gacheru S.N., Trackman P.C., Shah M.A., O'Gara C.Y., Spacciapoli P., Greenaway F.T. and Kagan H.M., Structural and catalytic properties of copper in lysyl oxidase, *J Biol Chem*., **265**, 19022-7 **(1990)**

5. Gasteiger E., Hoogland C., Gattiker A., Duvaud S., Wilkins M.R., Appel R.D. and Bairoch A., Protein Identification and Analysis Tools on the ExPASy Server, The Proteomics Protocols Handbook, Humana Press, 571-607 **(2005)**

6. Ghosh S., Wu Y. and Stack M.S., Ovarian cancer-associated proteinases, *Cancer Treatment and Research*, **107**, 331–351 **(2002)**

7. Gill S.C. and Hippel P.H.V., Calculation of Protein Extinction Coefficient from Amino Acid Sequence Data, *Analytical Biochem.,* **182**, 319-326 **(1989)**

8. Gonda D.K., Bachmair A., Wunning I., Tobias J.W., Lane W.S. and Varshavsky A., A Universality and structure of the N-end rule. *J Biol Chem.,* **264**, 16700–16712 **(1989)**

9. Guruprasad K., Reddy B.V.B. and Pandit M.W., Correlation between stability of a protein and its dipeptide composition: a novel approach for predicting in vivo stability of a protein from its primary sequence, *Protein Engineering*, **4**, 55-161 **(1990)**

10. Grau-Bové X., Ruiz-Trillo I. and Rodriguez-Pascual F., Origin and evolution of lysyl oxidases, *Scientific Reports*, **5**, 10568 **(2015)**

11. Huxley-Jones J., Robertson D.L. and Boot-Handford R.P., On the origins of the extracellular matrix in vertebrates, *Matrix Biol.*, **26(1)**, 2-11 **(2007)**

12. Hotary K., Li X.Y., Allen E., Stevens S.L. and Weiss S.J., A cancer cell metalloprotease triad regulates the basement membrane transmigration program, *Genes Dev.*, **20(19)**, 2673-2686 **(2006)**

13. Ikai A., Thermostability and aliphatic index of globular proteins, *J Biochem.,* **88**, 1895-1898 **(1980)**

14. Kumar S., Stecher G., Li M., Knyaz C. and Tamura K., MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms, *Mol Biol and Evol.*, **35**, 1547-9 **(2018)**

15. Kyte J. and Doolittle R.F., A simple method for displaying the hydropathic character of a protein, *J Mol Biol.*, **157**, 105-132 **(1982)**

16. Levental K.R., Yu H., Kass L., Lakins J.N., Egeblad M., Erler J.T., Fong S.F., Csiszar K., Giaccia A., Weninger W., Yamauchi M., Gasser D.L. and Weaver V.M., Matrix crosslinking forces tumor progression by enhancing integrin signalling, *Cell*, **139**, 891-906 **(2009)**

17. Liu X. et al, Elastic fiber homeostasis requires lysyl oxidase–like 1 protein, *Nat Genet.*, **36**, 178–182 **(2004)**

18. Liu J.L., Wei W., Tang W., Jiang Y., Yang H.W., Li J.T. and Zhou X., Silencing of lysyl oxidase gene expression by RNA interference suppresses metastasis of breast cancer, *Asian Pac J Cancer Prev.*, **13(7)**, 3507-3511 **(2012)**

19. Manes S., Mira E., Gomez-Mouton C., Lacalle R.A. and Martinez C., Cells on the move: a dialogue between polarization and motility, *IUBMB Life*, **49**, 89–96 **(2000)**

20. Nilsson M., Adamo H., Bergh A. and Halin Bergström S., Inhibition of Lysyl Oxidase and Lysyl Oxidase-Like Enzymes Has

Tumour-Promoting and Tumour-Suppressing Roles in Experimental Prostate Cancer, *Sci Rep.*, **6**, 19608 **(2016)**

21. Olson P.N., Using the canine genome to cure cancer and other diseases, *Theriogenology*, **68(3)**, 378-81 **(2007)**

22. Payne S.L., Hendrix M.J.C. and Kirschmann D., Paradoxical roles for lysyl oxidases in cancer – A prospect, *J Cell Biochem*, **101(6)**, 1338-1354 **(2007)**

23. Postovit L.M., Abbott D.E., Payne S.L., Wheaton W.W., Margaryan N.V., Sullivan R., Jansen M.K., Csiszar K., Hendrix M.J. and Kirschmann D.A., Hypoxia/reoxygenation: a dynamic regulator of lysyl oxidase-facilitated breast cancer migration, *Journal of Cellular Biochemistry*, **103**, 1369-1378 **(2008)**

24. Saleem A., Singh S., Sunil Kumar B.V., Arora J.S. and Choudhary R.K., Analysis of lysyl oxidase as a marker for diagnosis of canine mammary tumors, *Mol Biol Rep.*, **46(5)**, 4909-4919 **(2019)**

25. Sievers F., Wilm A., Dineen D., Gibson T.J., Karplus K., Li W., Lopez R., McWilliam H., Remmert M., Söding J., Thompson J.D. and Higgins D.G., Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega, *Mol Syst Biol.*, **7**, 539 **(2011)**

26. Tan L.D., Xu Y.Y., Yu Y., Li X.Q., Chen Y. and Feng Y.M., Serum HER2 level measured by dot blot: a valid and inexpensive assay for monitoring breast cancer progression, *PLoS ONE*, **6(4)**, e18764 **(2011)**

27. Xiao Q. and Ge G., Lysyl oxidase, extracellular matrix remodeling and cancer metastasis, *Cancer Microenvironment*, **5**, 261-273 **(2012)**

28. Yang X., Li S., Li W., Chen J., Xiao X., Wang Y., Yan G. and Chen L., Inactivation of lysyl oxidase by β-aminopropionitrile inhibits hypoxia-induced invasion and migration of cervical cancer cells, *Oncology Reports*, **29**, 541-548 **(2013)**.

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