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ELUCIDATION OF STRUCTURE - FUNCTION RELATIONSHIP IN FAMILIES OF COPPER BINDING PROTEINS IN *HOMO SAPIENS* – A BIOINFORMATICS APPROACH

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ABSTRACT

Copper is one of the important trace metals used in a multitude of cellular activities including respiration, angiogenesis and generation of immune responses. Its homeostasis is strictly orchestrated by copper binding proteins which include structural membrane proteins, intracellular enzymes and copper chaperones. Apart from serving as an important co-factor in various enzymes, copper plays a vital role in triggering normal and tumor angiogenesis, the latter resulting in metastasis. Hence, curbing excess copper levels is the key to designing antiangiogenic, anticancer therapies. This evokes the need for a better understanding of copper binding proteins. This study presents a systematic classification of copper binding proteins. The binding domain within and across various copper binding protein families has been analysed. The importance of two highly conserved residues in the binding pocket namely cysteine and histidine have been discussed. The detailed study revealed the possibility of designing an antiangiogenic peptide based on conserved histidines present in the LOX family of enzymes.

KEYWORDS: Copper, copper binding proteins, angiogenesis, anticancer, peptide therapy.

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INTRODUCTION

Copper (Cu) is an essential trace element vital to the normal, healthy functioning of living organisms. By virtue of their ability to adopt discrete red-ox states namely the oxidized [Cu(II)] or reduced state [Cu(I)], Cu ions serve as critical catalytic co-factors for a multitude of cellular proteins and physiologically important enzymes. Some of the copper containing, copper binding and copper utilizing proteins are cytochrome c oxidase, lysyl oxidase (LOX), Cu/Zn superoxide dismutase (SOD), ceruloplasmin, matrix metalloproteinases, metallothioneins etc. These copper containing proteins are indispensable for fundamental, biological processes such as oxidative respiration, angiogenesis, neural development, collagen remodeling etc [1,2]. However, the uncontrolled red-ox reactivity and excess accumulation of copper can induce potent cytotoxic effects through the formation of reactive oxygen species (ROS) which thereby leads to cellular damage. Some of the direct effects of the generation of hydroxyl radicals include membrane lipid peroxidation, oxidation of essential enzymes, DNA and RNA cleavage. These events have serious implications in neurodegenerative disorders, aging and in the development of cancer [3,4,5]. Hence, copper homeostasis is a highly orchestrated process comprising of it's uptake, controlled transport across biological membranes by copper chaperones, distribution within different tissues to the specific proteins/enzymes which require copper followed by it's detoxification and excretion from the body [6].

The importance of achieving this crucial, delicate copper balance is amply illustrated by two well studied human genetic disorders, namely the Menke's disease and Wilson's disease [7,8]. Both these disorders arise due to the absence or dysfunctioning of copper transporting ATP-ases present in the trans-golgi network of all cells. The consequent impairment in copper transport and uptake culminates in excess copper deposition in the various tissues like liver and eye for instance, in Wilson's disease [9,10,11,12,13]. At the later stages, copper deposition occurs in the brain and eyes in the form of the Kayaer-Fleischer rings [14,15]. Likewise, various studies have demonstrated that copper mediated oxidative stress is associated with neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, prion diseases, and familial amyotrophic lateral sclerosis. In all these diseases, mitochondrial dysfunction was found to be a common feature. Copper deficiency had led to decreased cytochrome c oxidase activity consequently triggering the production of ROS. Therefore, deficit, excess or improper trafficking of copper was shown to be detrimental to neuronal cells which culminated in mitochondria mediated apoptotic neurodegeneration [16,17,18,19].

Cancer is a multistep, multifactorial process, one of the causative factors being copper. There are different modes by which copper mediates cancer growth. The pro-oxidant activity of copper is one of the important reasons for the development of cancer. Owing to it's mobilization and red-ox activity, copper initiates the formation of ROS like superoxide anion and hydroxyl radicals which bind to DNA or modify the bases inducing carcinogenesis [20,21]. Experimental studies have indicated elevated levels of copper in the serum of cancer patients [22,23]. Consistently, increased levels of copper have been observed in many kinds of human cancers [24,25,26]. In this regard, extensive research in the field by Folkman and his colleagues has revealed that tumor growth is dependent upon angiogenesis [27, 28]. Angiogenesis normally occurs during development and growth. Specifically, it remodels the existing vasculature in the embryo by the sprouting and branching of new blood vessels, thereby re-establishing the vascular network [28,29]. Angiogenesis also occurs in the adult during the ovarian cycle and is involved in the regeneration of tissues at the time of wound healing [30,31]. Tumor

angiogenesis is the process by which a network of blood vessels proliferate and penetrate into the tumor supplying it with nutrients and oxygen necessary for it's growth, followed by the removal of waste products. Hence, this process is significant to the formation of a tumor mass and consequently determines it's progression to a metastatic state [27,28,32,33]. Copper is the only transition metal which is an essential factor for the tumor angiogenic process [20,34,35]. The first step in tumor angiogenesis is the degradation of the extracellular matrix (ECM) combined with the activation and migration of endothelial cells from the pre-existing capillaries. Copper ions stimulate the proliferation and migration of endothelial cells [36,37]. They also act as molecular switches to activate several proangiogenic factors, e.g., vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), angiogenin, tumour necrosis factor alpha (TNF-α) and interleukin 1 (IL-1), among which VEGF assumes precedence over the others in the design of anti-angiogenic therapies [33,34,38,39,40,41]. Other factors like ceruloplasmin (copper carrying plasma protein), heparin, angiotropin, a monocyte-derived angiogenic factor are angiogenic when bound to copper [42,43,44]. Thus, copper is an indispensable metal co-factor for the initiation and subsequent progression of both normal and tumor angiogenesis. Copper binding proteins play an important role in the maintenance of copper homeostasis. Therefore, restricting copper levels in tumors while still maintaining its threshold for normal cellular processes is one of the promising and attractive approaches in designing antiangiogenic and anticancer therapies.

The use of copper chelators to diminish copper levels has been investigated in different animal models both *in vitro* and *in vivo*. As early as 1956, the first copper chelator D-pencillamine was developed by Walshe and administered successfully as an oral drug [45,46]. Penicillamine, a reductive chelator, when given to patients with Wilson's disease led to a massive excretion of copper in the urine. This therapy was found to be fairly effective except that it caused the onset of severe neurological symptoms. Another drawback was that it also induced hematologic and renal toxicities [45,46,47]. This attempt was succeeded by Brem and his co-workers who demonstrated that pencillamine induced copper deficiency coupled with a low copper diet limited the growth of tumors in rats and rabbits. The unsatisfactory aspect of the treatment was the low survival rate in the experimentally treated animals when compared to the controls [48,49]. Other agents developed to treat Wilson's disease include Trientine, zinc, and Tetrathiomolybdate (TM). Trientine was designed to treat patients who displayed intolerance towards pencillamine. Although this drug had more potent anti-cancer effects than pencillamine, due to its limited application and toxicity profile, its use was subsequently discouraged [50,51,52]. Zinc created copper deficiency by inducing the synthesis of hepatic and metallothioneins which bound to copper. The complexed copper was then excreted in the faeces. However, zinc too was unable to resolve the side effects caused due to neurological complications [52,53,54,55]. One of the most widely explored compounds for the treatment of Wilson's disease and cancer is tetrathiomolybdate. The therapies used for Wilson's disease are relevant and applicable in cancer treatment as well, since the same principle of antiangiogenesis has been employed. In particular, in the case of TM, both animal studies and clinical trials augment the copper deficiency theory as a potential chemotherapeutic strategy [56,57,58]. TM forms a stable, tripartite complex with copper and protein. When given with food, it complexes food copper with food protein and prevents absorption of copper from the GI tract. When given between meals, it is absorbed into the blood, and forms a tripartite complex with TM, albumin and the freely available serum copper. The complexed copper is not available for cellular uptake and is excreted through the bile and urine [59,60]. Although toxicity is quite rare with TM use but negative side effects includes elevation in the levels of liver function

enzymes, anemia and/or leucopenia are related to overtreatment [61]. Also, most of these drugs are not specific inhibitors of copper binding. Therefore, the various side effects and shortcomings of the current drugs evoke the necessity for safer alternative antiangiogenic therapies.

Our interest is in designing peptides (that bind to copper) as a therapeutic strategy to circumvent tumor angiogenesis. The advantages of peptides over proteins as therapeutics lie in their stability, ready solubility in water, easy bio-availability and lack of autoimmune responses [62]. To achieve this goal, we have attempted to gain an understanding of the different copper binding proteins present in humans. In particular, we have classified the families of copper binding proteins and assigned specific copper binding domains within each family and in between families. This consistent, exhaustive analysis has enabled us to implicate specific amino acid residues which bind to copper in a particular fashion based on the function that they perform. This study gives us a deeper insight into the copper binding domains of copper binding proteins which might be valuable while designing peptides that target and bind to copper thus favoring antiangiogenesis.

MATERIALS AND METHODS

(i) Identification of Copper Binding Proteins:

The different copper binding proteins in *Homo sapiens* were identified using the following databases and websites.

The NCBI website (www.ncbi.nlm.nih.gov)
The UniProt database (www.uniprot.org)
GO database at EMBL-EBI (www.ebi.ac.uk)
KEGG database (http://www.genome.jp/kegg/)
PDB (www.rcsb.org)
HighWire Press
(http://highwire.stanford.edu/lists/freeart.dtl)

The NCBI website was searched thoroughly for the copper binding proteins deposited under *Homo sapiens*. It yielded 178 hits for the query “copper binding proteins” AND *Homo sapiens*. A similar search was performed in the UniProt database using the keyword search term “molecular function” which gave 74 results for the query “copper” AND *Homo sapiens* AND “reviewed”. Similarly, the KEGG database revealed 357 hits for copper binding proteins but since these included copper binding proteins under all organisms, manual searching and filtering was done to obtain only the copper binding proteins present in the human. The gene ontology (GO) option at the EMBL-EBI database was explored using the query term “copper”. The ontology covers three domains; cellular component, molecular function viz. the fundamental activities of a gene product at the molecular level, and biological process, which include sets of molecular events with a distinct beginning and end, pertinent to the functioning of integrated living units eg. Cells, tissues, organs, and organisms. A total of 53 hits for copper were obtained with 23 under biological process and 30 under molecular function. The known structures of copper binding proteins deposited in the protein data bank (PDB) were also considered for this study. The advance search query option specifying Copper as the ligand name was utilized for the same. About 64 hits were procured which included repetitive proteins and mutant varities. The mutant varities were manually filtered and eliminated. In the case of repetitive protein-ligand complexes, the ones with the least resolution were chosen for further analyses. To avoid the posssibility of overlooking certain proteins and to make the quest more comprehensive, the available literature on copper binding proteins was also studied carefully. This study gave us a few hints on some copper binding proteins whose functions are yet to be ascertained. These proteins were listed under the unknown category. Redundancy was eliminated from the entire data set obtained from the seven sources by manual verification and checking. This resulted in 78 unique copper binding proteins identified in the *Homo sapiens*.

(ii) ***Classification of Copper Binding Proteins***

The 78 unique proteins identified were then subjected to classification. The classification was performed using the gene ontology concept primarily based on the molecular function of copper in the various copper binding proteins. This resulted in 6 main categories of classification and one unknown category where the function of copper in these proteins was still uncertain. The total list of 7 categories is mentioned below.

1. Copper transporting membrane proteins.
2. Intracellular copper transporters.
3. Copper binding enzymes.
4. Copper storing proteins.
5. Copper binding intracellular proteins.
6. Structural proteins.
7. Unknown classification.

(iii) ***Analysis of the Copper Binding Domain within Families***

Two approaches were employed to identify the metal binding domain in each of the above mentioned 7 categories. In the first approach, the information obtained during the identification of copper binding proteins from the UniProt database was used. Each hit in the UniProt database was supplemented with information about the protein, it's amino acid sequence, metal binding domain and the structure, if known. The PDB ID was then used to further inspect the metal binding domain. In the case where the structure was unknown, a closely related protein with a similar structure was mentioned. This was then used as a template to get an idea about the metal binding domain of the copper binding protein in query. In the second approach, the Pfam (Protein families) database was used to identify the domains which bound to copper.

(iv) ***Analysis of the Copper Binding Domain between Families***

After locating the copper binding domains within each of the 7 families, the functional, Cu binding domains between these families were then examined manually and the results tabulated.

(v) ***Identification of conserved residues present in the copper binding domain between families***

The tabulated data was scrutinized thoroughly to obtain the residues conserved across all the families of copper binding proteins in *Homo sapiens*.

RESULTS

An attempt to classify the copper binding proteins and elucidate the structure-function relationships based on the copper binding domains in *Homo sapiens* has been made in this study. 78 unique copper binding proteins were obtained from 6 different sources, verified manually to eradicate redundancy and classified into 7 categories based on molecular function. The results are represented in Table 1 & 2

The number of proteins present in each category is mentioned below:

1. Copper transporting membrane proteins (9).
2. Intracellular copper transporters (6).
3. Copper binding enzymes (36).
4. Copper storing proteins (12).
5. Copper binding extra cellular proteins (6).
6. Structural proteins (2).
7. Unknown classification (7).

Table 1. This Table shows the entire classification of 78 unique copper binding proteins in humans into 7 categories.>

Table1
CLASSIFICATION OF COPPER BINDING PROTEINS

COPPER TRANSPORTING MEMBRANE PROTEINS (9)

O15431 High affinity copper uptake protein 1 (Copper transporter 1)
O15432 Probable low affinity copper uptake protein 2(Copper transporter 2)
Q99571 P2X purinoceptor 4 (P2X4) (ATP receptor)
P35670 Copper-transporting ATPase 2
Q04656 Copper-transporting ATPase 1 (Menkes disease-associated protein)
Q9NRU3 Metal transporter CNNM1 (Cyclin-M1)
(Ancient conserved domain-containing protein 1)
Q9H8M5 Metal transporter CNNM2 (Cyclin-M2)
(Ancient conserved domain-containing protein 2)
Q8NE01 Metal transporter CNNM3 (Cyclin-M3)
(Ancient conserved domain-containing protein 3)
Q6P4Q7 Metal transporter CNNM4 (Cyclin-M4)
(Ancient conserved domain-containing protein 4)

INTRACELLULAR COPPER TRANSPORTERS (6)

O75880 Protein SCO1 homolog, mitochondrial
O43819 Protein SCO2 homolog, mitochondrial
Q14061 Cytochrome c oxidase copper chaperone
O00244 Copper transport protein ATOX1
O14618 Copper chaperone for superoxide dismutase
Q9NTM9 Copper homeostasis protein cutC homolog

COPPER STORING PROTEINS (12)

P04731 Metallothionein-1A (MT-1A)
P07438 Metallothionein-1B (MT-1B)
P04732 Metallothionein-1E (MT-1E)
P04733 Metallothionein-1F (MT-1F)
P13640 Metallothionein-1G (MT-1G)
(Metallothionein-1K) (MT-1K)
P80294 Metallothionein-1H (MT-1H)
Q93083 Metallothionein-1L (MT-1L)
Q8N339 Metallothionein-1M (MT-1M)
P80297 Metallothionein-1X (MT-1X)
P25713 Metallothionein-3 (MT-3)
P47944 Metallothionein-4 (MT-4)
P02795 Metallothinein-2 (MT-2)

STRUCTURAL PROTEINS (2)

P05067 Amyloid beta A4 protein
P51693 Amyloid -like protein 1(APLP-1)

COPPER BINDING ENZYMES (36)

P00395 Cytochrome c oxidase subunit 1
P00403 Cytochrome c oxidase subunit 2
Q9Y6N1 Cytochrome c oxidase assembly protein COX11, mitochondrial
O75106 Retina-specific copper amine oxidase (RAO)
Q9UHE8 Metalloreductase STEAP1
Q8NFT2 Metalloreductase STEAP2
Q658P3 Metalloreductase STEAP3
Q687X5 Metalloreductase STEAP4
P14679 Tyrosinase (EC 1.14.18.1)
P17643 5,6-dihydroxyindole-2-carboxylic acid oxidase
P40126 L-dopachrome tautomerase
P00441 Superoxide dismutase [Cu-Zn]
P08294 Extracellular superoxide dismutase
P10323 Acrosin
Q08397 Lysyl oxidase homolog 1
Q9Y4K0 Lysyl oxidase homolog 2
P58215 Lysyl oxidase homolog 3
Q96JB6 Lysyl oxidase homolog 4
P28300 Protein-lysine 6-oxidase
P19801 Amiloride-sensitive amine oxidase
Q6UVY6 DBH-like monooxygenase protein 1
A6NHM9 Putative DBH-like monooxygenase protein 2
P09172 Dopamine beta-hydroxylase (EC 1.14.17.1)
Q8TC92 Ecto-NOX disulfide-thiol exchanger 1
Q16206 Ecto-NOX disulfide-thiol exchanger 2
(Tumor-associated hydroquinone oxidase)
Q16853 Membrane primary amine oxidase
P00450 Ceruloplasmin
P19021 Peptidyl-glycine alpha-amidating monooxygenase
P03950 Angiogenin
Q7Z7K0 COX assembly mitochondrial protein homolog
A5PKW2 Copper amine oxidase
Q9BQS7 Hephaestin
Q6MZM0 Hephaestin-like protein 1

Q9H7H0 Protein RSM22 homolog, mitochondrial
P23526 Adenosylhomocysteinase (AdoHcyase)
(S-adenosyl-L-homocysteine hydrolase)
Q9NYA1 Sphingosine kinase 1 (SPK 1) (SK 1)

COPPER BINDING EXTRACELLULAR PROTEINS (6)

P09486 SPARC (Secreted protein acidic and rich in cysteine)
(Osteonectin)
P12259 Coagulation factor V
P00451 Coagulation factor VIII
P02771 Alpha-fetoprotein
P02768 Serum albumin
P06727 Apolipoprotein A-IV

UNKNOWN CLASSIFICATION (7)

P37840 Alpha-synuclein (Non-A beta component of AD
amyloid) (Non-A4 component of amyloid precursor)
Q8N668 COMM domain-containing protein 1
(Protein Murr1)-(Copper Metabolism in MURR1 domain)
P98170 Baculoviral IAP repeat-containing protein 4
(EC 6.3.2.-)
Q99584 S100A13 S100 calcium-binding protein A13
P14210 Hepatocyte growth factor (Scatter factor) (SF)
(Hepatopoeitin-A)
P04637 Cellular tumor antigen p53 (Tumor suppressor p53)
(Phosphoprotein p53) (Antigen NY-CO-13)
Q9UBF6 RING-box protein 2 (Rbx2) (RING finger protein 7)
(Regulator of cullins 2)

Table 2.
COPPER BINDING DOMAIN IN PROTEINS

PROTEIN	BINDING REGION
COPPER BINDING ENZYMES	
Protein lysine 6 oxidase(LOX)	SC**H**Q**H**Y**H**SM
Lysyl oxidase homolog 1	SC**H**Q**H**Y**H**SM
Lysyl oxidase homolog 2	DC**H**R**H**Y**H**SM
Lysyl oxidase homolog 3	EC**H**G**H**Y**H**SM
LOX 4	QC**H**R**H**Y**H**SI
5,6-dihydroxyindole-2-carboxylic acid oxidase	WT**H**YYSVKKTFLGVGQESFGEVDFS**H**EGPAFLTW**H**RY
Tyrosinase	SL**H**NLA**H**LFLNGTGGQTHLSPNDPIFVLL**H**TF WM**H**YYVSMDALLGGSEIWRDIDFA**H**EAPAFLPW**H**RL SSM**H**NAL**H**IYMNGTMSQVQGSANDPIFLLH**H**AF
Peptidyl glycine alpha amidating monooxygenase	TV**HH**MLLFGCNMPSSTGSYWFCDEGTCTDKANILYAWARNAPPTRLPKGVGFRVGGET GSKYFVLQV**H**YG
Putative DBH-like monooxygenase protein 2	RV**H**T**H**HLGKVVSGYRVRNGQWTLIGRQSPQLPQAFYPVGHPVDVSFGDLLAARCVFTGEGRTEATHIGGTSSDE**M**CN TV**HH**ILVYACGNASVLPTGISDCYGADPAFSLCSQVIVGSAVGGTSYQFPDDVGVSIGTPLDPQWILEI**H**YS LL**H**T**H**LAGRALQAVQYRNGTQLRKICKDDSYDFNLQETRDLPSRVEIKPGDELLVECHYQTLDRDSMTFGGPSTINE**M**CL
DBH-like monooxygenase protein 1	LV**HH**ILLYQCSNNFNDSVLESGHECYHPNMPDAFLTCETVIFAWAIGGEGFSYPPHVGLSLGTPLDPHYVLLEV**H**YD
Dopamine beta hydroxylase	LL**HA**HLAGRGIRLRHFRKGKEMKLLAYDDDFDFNFQEFQYLKEEQTILPGDNLITECRYNTKDRAEMTWGGLSTRSE**M**CL LV**HH**MEVFQCAPEMDSVPHFSGPCDSKMKPDRLNYCRHVLAAWALGAKAFYYPEEAGLAFGGPGSSRYLRLEV**H**YH QL**H**T**H**LTGRKVVTVLVRDGREWEIVNQDNHYSPHFQEIRMLKKVVSVHPGDVLITSCTYNTEDRELATVGGFGILEE**M**CV
Superoxide dismutase [Cu-Zn]	GF**H**V**H**EFGDNTAGCTSAGP**H**FNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLSGDHCIIGRTLVV**H**EK
Extracellular superoxide dismutase(SOD3)	AI**H**V**H**QFGDLSQGCESTGP**H**YNPLAVPHPQHPGDFGNFAVRDGSLWRYRAGLAASLAGPHSIVGRAVVV**H**AG
Cytochrome c oxidase subunit 1	FG**H**PEV**Y**ILILPGFGMISHIVTYYSGKKEPFGYMGMVWAMMSIGFLGFIVWA**HH**MF
Cytochrome c oxidase subunit 2	VL**H**SWAVPTLGLKTDAIPGRLNQTTFTATRPGVYYGQ**C**SEI**C**GAN**H**SF
Amiloride sensitive amine oxidase	NI**H**T**H**LV,FL**H**IP
Membrane primary amine oxidase	TV**H**T**H**SA,FL**H**IP
Retina specific copper amine oxidase	TV**H**T**H**AF,FL**H**IP
Ceruloplasmin	TF**H**SH, TV**H**FH HS**H**GITYYKEHEGAIYPDNTTDFQRADDKVYPGEQYTYMLLATEEQSPGEGDGNCVTRI Y**H**SH, HC**H**VT HS**H**ID, HF**H**GHSFQYKHRGVYSSDVFDIFPGTYQTLEMFPRTPGIWLL**H**CH DV**H**AAFFHGQALTNKNYRIDTINLFPATLFDAYMVAQNPGEWMLS**C**QNLN**H**LK DV**H**GIYFSGNTYLWRGERRDTANLFPQTSLTLHMWPDTEGTFNVE**C**LTTD**H**YTGG**M**KQ DL**H**TVHFHGHSFQYKHRGVYSSDVFDIFPGTYQTLEMFPRTPGIWLLH**C**HVTD**H**IHAG**M**ET
Hephaestin like protein 1	SL**H**PH, TI**H**YH HP**H**GVFYNKDSEGALYPDGTSGRNKNDDMVPPGKNYTYVWPVREEYAPTPADANCLT WVY**H**SH, HC**H**VS HS**H**ID, HY**H**AESFLFKIDKSYREDVYDLFPGTFQTIELFADHPGTWLL**H**CH DI**H**SIYFYGNTFISRGHRTDVVNLFPATFLTTEMIAENPGKWMIT**C**QVSD**H**LQ DM**H**GIVFQGNTIHLRGTHRDSLALFPHMATTAFMQPDHAGIFRVF**C**ATMP**H**LSRG**M**GQ DI**H**TIHYHAESFLFKIDKSYREDVYDLFPGTFQTIELFADHPGTWLLH**C**HVSD**H**IHAG**M**ET
STRUCTURAL PROTEINS	
Amyloid beta A4 protein	FL**H**QERMDVCET**H**L**H**W**H**TV FR**H**DSG**Y**EV**HH**QK
COPPER BINDING INTRACELLULAR PROTEINS	
Alpha fetoprotein precursor	TL**H**RN
Coagulation factor V	VV**H**F**H**GQ
Serum albumin	DA**H**KS
INTRACELLULAR COPPER TRANSPORTERS	
Copper chaperone for superoxide dismutase	MT**C**QS**C**VD QI**C**S**C**DG
Copper transport protein ATOX1	MT**C**GG**C**AE
Cytochrome C oxidase copper chaperone	KP**CC**AC
Protein SCO1 homolog	TH**C**PDV**C**PE,VD**H**TI
Protein SCO2 homolog	TH**C**PDI**C**PD,VD**H**SI
COPPER STORING PROTEINS	
Metallothionein (All) [copper,cadmium,zinc etc)	KS**CC**S**CC**PMS**C**AK**C**AQG**C**I**C**KGASEK**C**S**CC**A PN**C**S**C**ATGGS**C**T**C**TGS**C**K**C**KE**C**K**C**NS**C**KK

The copper binding domains within each family of copper binding proteins were identified using the UniProt database and Pfam. The results are tabulated in **Table. 2.**

Figure - 1

The pie chart is a graphical representation of the same depicting the distribution of the different families of copper binding proteins.

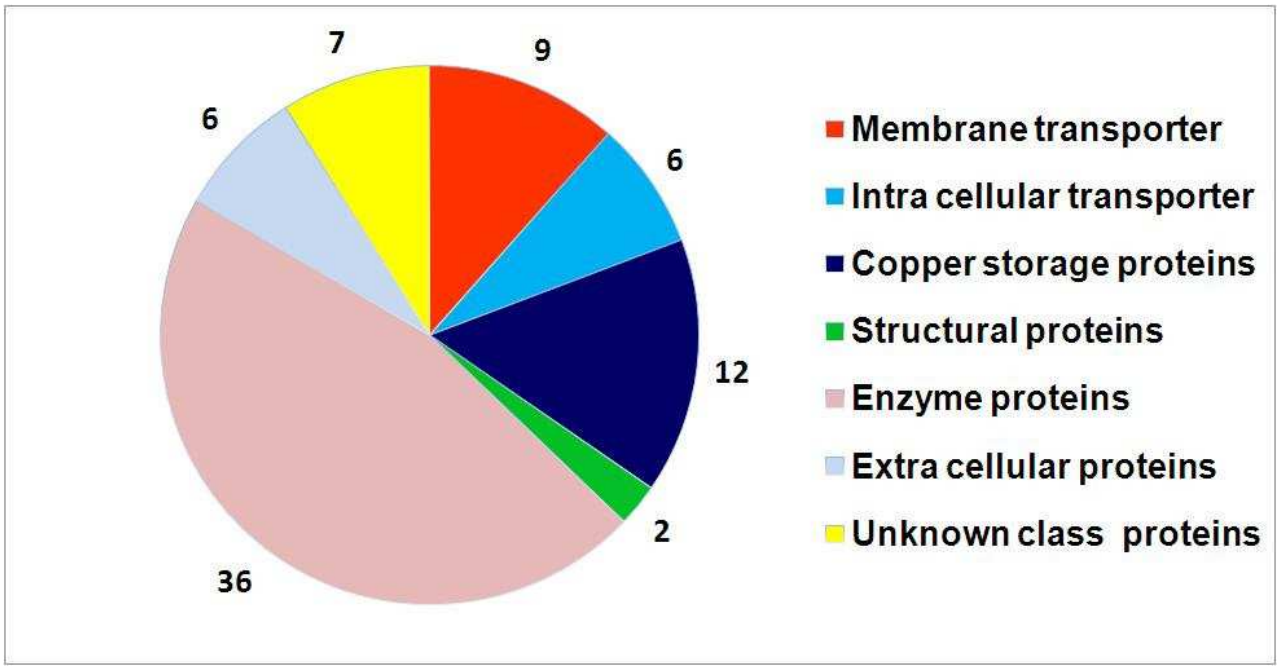

Table.3

ANALYSIS OF CONSERVED COPPER BINDING DOMAINS IN COPPER BINDING PROTEIN FAMILIES

FAMILY	DOMAIN
LOX	C**H**x**H**Y**H**S
TYROSINASE	Copper A- Wx**H**Yxxxxxxxxxxxxxxxxxxxxx**H**xxxxxxxx**H**R
	Copper B- Sx**H**Nxx**H**xxxxxxxxxxxxxxxxxxxxxLx**H**
MONOOXYGENASE	Copper A- V**HH**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**H**Y
	Copper B- **H**x**H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**M**C
SUPEROXIDE DISMUTASE	**H**V**H**xxxxxxxxxxxxxxP**H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxV**H**
CYTOCHROME C OXIDASE SUBUNIT 1	Copper B- **H**xxx**Y**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**HH**
CYTOCHROME C OXIDASE SUBUNIT 2	Copper A- **H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxx**C**xxx**H**
AMINE OXIDASE	**2H+H**
CERULOPLASMIN / HEPHAESTIN	Copper1,type2- **H+H**
	Copper2,type3**- 2H+H**
	Copper3,type3**- H**+2**H**
	Copper1,type1**- H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxxx**H**
	Copper1,type1- **H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxxx**H**xxxx**M**
	Copper1,type1- **H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxxx**H**xxxx**M**
AMYLOID BETA A4 PROTEIN	Copper- **H**xxxxxxxxx**H**x**H**x**H**
	Copper or Zinc- **H**xxx**Y**xx**HH**
COPPER BINDING INTRACELLULAR PROTEINS	**ONLY H**
COPPER CHAPERONES	**ONLY C**
SCO	**C and H**

METALLOTHIONEIN	**ONLY C**
FAMILY	**DOMAIN**
LOX	C**H**x**H**Y**H**S
TYROSINASE	Copper A- Wx**H**Yxxxxxxxxxxxxxxxxxxxxx**H**xxxxxxxx**H**R
	Copper B- Sx**H**Nxx**H**xxxxxxxxxxxxxxxxxxxxxLx**H**
MONOOXYGENASE	Copper A- V**HH**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**H**Y
	Copper B- **H**x**H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**M**C
SUPEROXIDE DISMUTASE	**H**V**H**xxxxxxxxxxxxxxP**H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxV**H**
CYTOCHROME C OXIDASE SUBUNIT 1	Copper B- **H**xxx**Y**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**HH**
CYTOCHROME C OXIDASE SUBUNIT 2	Copper A- **H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxx**C**xxx**H**
AMINE OXIDASE	**2H**+**H**
CERULOPLASMIN / HEPHAESTIN	Copper1,type2- **H**+**H**
	Copper2,type3**- 2H**+**H**
	Copper3,type3**- H**+2**H**
	Copper1,type1**- H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxxx**H**
	Copper1,type1- **H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxxx**H**xxxx**M**
	Copper1,type1- **H**xxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxxx**C**xxxx**H**xxxx**M**
AMYLOID BETA A4 PROTEIN	Copper- **H**xxxxxxxxx**H**x**H**x**H**
	Copper or Zinc- **H**xxx**Y**xx**HH**
COPPER BINDING INTRACELLULAR PROTEINS	**ONLY H**
COPPER CHAPERONES	**ONLY C**
SCO	**C and H**
METALLOTHIONEIN	**ONLY C**

<Supplementary Table.2 This table shows the list of copper binding protein domain identified through Uniprot and Pfam>

The residues lining the binding pocket in each family are listed in the binding region. Copper A and B mentioned in the binding site column refer to the two bound copper ions for each subunit. The metal co-ordination centres in copper binding proteins are classified into different types based on the manner in which they bind to distinct residues [63]. The types of Copper mentioned refer to the manner in which copper co-ordinates with the residues bound to it like Cysteine or Histidine. Overall, Histidines and Cysteines are predominantly conserved within and among the different families.

Finally, an analysis of the conserved residues in the copper binding region within and across different protein families was done. The results are depicted in **Table.3.** It is a notable feature that Histidine and Cysteine residues exhibit remarkable conservation both, within a single, specific family and in general across all families. While comparing the binding region between families, a “pattern” of similarity was found to exist, although certain residues did differ. This variability in the residues lining the binding pocket could be attributed to the distinct functions that the different families of copper binding proteins perform. By and large, the important residues that were involved in copper binding were Histidine and Cysteine. The copper binding enzymes, structural proteins and intracellular proteins had mainly histidine influencing the copper binding to them. The exception was Cytochrome c oxidase where cysteine was also present along with histidine. Copper transporters, storage proteins had primarily cysteine which determined the binding of copper. The exception was the SCO homolog protein where histidine was also found along with cysteine.

DISCUSSION

A bioinformatics approach has been undertaken in this study to comprehend the structure-function relationships of copper binding proteins in *Homo sapiens* with particular emphasis on the metal binding domain. Copper binding proteins play vital roles in the regulation of copper ion homeostasis and accordingly assume significance in both, maintenance of normal cellular processes as well as the onset and progression of disease [16,64]. Therefore, copper binding proteins carry a wealth of information which can be exploited to target copper for the development of various therapeutic strategies. This necessitates a thorough understanding of the different families of copper binding proteins based on their molecular function. A systematic mode of classification performed in this study enabled elimination of the redundant proteins and served to focus only on the unique, individual ones that were subsequently grouped into 7 different families. Among the different families examined, two amino acids namely histidine and cysteine exhibited a consistent pattern of conservation in the copper binding domain.

Histidine is the residue which was largely occurred in copper binding enzymes, structural and intracellular proteins. A classic example of a copper binding enzyme is cytochrome c oxidase. It is an integral membrane protein and a key mitochondrial enzyme in the respiratory chain. In mammals, it is composed of 13 subunits and many metal prosthetic sites. The high resolution three-dimensional x-ray crystal structure of bovine heart cytochrome c oxidase has been elucidated [65,66]. According to this crystallographic structure, Histidine (His 240) co-ordinates with Tyrosine (Tyr 244) to enable the Cu_B binuclear center to accept four

electrons during the reduction of molecular oxygen to water. Thus, it aids in an important step in electron transfer [65,66]. Various studies have reported that histidine residues are commonly found in consensus copper-binding motifs [67]. Histidines play a significant role in the modulation of copper binding. This has been well illustrated in two independent data sets, in amphibians (*Xenopus laevis*) and rats [68,69]. Both these studies were aimed at identifying structural determinants for the binding of copper. Results from these researches have shown that Histidines at specific positions are critical for the modulatory action of copper in the ion channel family of P2X receptors. P2X receptors are a group of membrane purinoreceptors that are widely distributed in the central nervous system and play a role in synaptic transmission. Briefly, the experimental results indicated that mutation of histidines to arginines and threonine drastically altered the conformation of the receptor thus abrogating it's normal function due to lack of copper co-ordination [68,69,70].

Apart from histidines, our results also show that cysteines are highly conserved in copper transport proteins. They appear to be vital for copper transport and storage, thereby assisting the copper-chaperone activity. The role of cysteines in copper transport has been well documented in several animal models, from yeast to mammals.[71,72]. Copper incorporation into yeast cytochrome c oxidase requires the SCO proteins SCO1 and SCO2, which are mitochondrial inner membrane proteins. SCO1 is homologous to subunit 2 of cytochrome oxidase and contains two conserved cysteines. It has been proposed that this region directly transfers copper to cytochrome oxidase [71,72,73]. In our data, we speculate that the exceptional occurrence of cysteine residues along with histidines in cytochrome c oxidase and vice versa in SCO proteins could possibly be due to a secondary function that the enzyme might perform. Especially, in the assembly of cytochrome c oxidase wherein it co-ordinates with copper chaperones that predominantly contain cysteine [71,72].

Since copper favours angiogenesis and tumor growth at various levels like initiation of endothelial cell migration, activation of proangiogenic factors like VEGF, bFGF, generation of ROS etc., targeting copper would be an effective approach to eliminate all the downstream effects which culminate in cancer. Using our preliminary data, our objective/ultimate goal is to achieve an antiangiogenic state by curbing copper levels such that only a threshold level of copper is available for normal, biological processes. We hereby propose that addition of small, exogenous peptides that bind specifically to copper is a safe, efficient strategy to attain an antiangiogenic condition.

From our detailed domain analyses of copper binding proteins, the results that were obtained for families other than that of Lysyl oxidase (LOX) could not be used to identify a peptide that could inhibit angiogenesis. This was because the conserved domains that were identified in such families were too big a peptide and had more than 30 amino acid residues hence raising the question of their bio-availability. Thus we focused only on the LOX family of enzymes for further research. The LOX family comprises of LOX and four LOX like homologues namely LOX1, LOX2, LOX3 and LOX4. Lysyl oxidase is an extracellular copper enzyme that catalyzes formation of aldehydes from lysine residues in collagen and elastin precursors.[74]. These aldehydes are highly reactive and undergo spontaneous chemical reactions with other lysyl oxidase-derived aldehyde residues or with unmodified lysine residues. This results in the cross-linking of collagen and elastin, which is essential for the stabilization of collagen fibrils and for the integrity, elasticity of mature elastin [75,76]. The secreted form of LOX is responsible for the invasive properties of hypoxic cancer cells through focal adhesion kinase activity and cell-to-matrix adhesion. It has been proposed that LOX may be required to create a niche

permissive for metastatic growth and thus may be essential for hypoxia-induced metastasis [77,78]. Also, small-molecule or antibody inhibitors of LOX were found to abolish metastasis in a rodent model of breast cancer [77,78]. Therefore, inhibitors of the LOX enzyme might be useful in preventing tumor progression and metastasis as well as treating other fibrotic diseases involving remodeling of the extracellular matrix viz. neurodegenerative disorders [79,80].

In this work, a consensus amino acid sequence containing three histidines was identified in the LOX family. The peptide sequence is C**H**x**H**Y**H**S where X could be glutamine, arginine or glycine, all of which are promising novel copper-binding peptides [67]. This peptide should first be subjected to laboratory analysis *in vitro* and then *in vivo* to test their efficacy and biological effects. If such a synthetic peptide could be made with a higher binding affinity to copper than the natural copper binding proteins and administered exogenously in the body, the excess copper that causes tumor angiogenesis would bind to this peptide, thus reducing tumor growth. This peptide-copper complex would then be excreted from the body. Therefore, this research of copper binding proteins is a useful tool that will aid in the development of antiangiogenic, anticancer therapeutics in the near future.

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