COMPREHENSIVE IMMUNOPHENOTYPIC EXPRESSION ANALYSIS OF PHOSPHOLIPID BINDING PROTEINS IN RENAL ORGANOGENESIS AND IN KIDNEY CARCINOMA

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By

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DECLARATION BY THE CANDIDATE

I hereby declare that this thesis entitled "***Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma***" is a bonafide and genuine research work carried out by me under the guidance of Prof **Dr. B. M. Bannur**, Professor of Anatomy, BLDE (Deemed to be University)'s Shri B M Patil Medical College, Hospital & Research Centre, Vijayapura, and Prof **Dr. Praveen Kumar Shetty**, Professor of Biochemistry, K.S. Hegde Medical Academy, Nitte (Deemed to be University), Mangalore, Karnataka. No part of this thesis has been formed the bases for the award of any degree or fellowship previously. Shall have the rights to preserve, use and disseminate this dissertation/thesis in print or electronic format for academic/research purpose.

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With the blessings of Lord Almighty, I dedicate this work to

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"Thinking is the hardest work there is, which is probably the reason so few engage in it"

~ *Henry Ford*

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List of Abbrevations

PAX2- Paired box2

LIM1- Homeobox protein lim-1

WT1- Wilm's tumor suppressor gene 1

SHH- Sonic Hedgehog

NOTCH-Neurogenic locus notch homolog protein

WNT - wingless-type

AnxA1- Annexin A1

AnxA2- AnnexinA2

PI3 kinase- Phosphoinositide 3-kinase

HGF receptor-tyrosine kinase – Hepatocyte growth factor receptor tyrosine kinase

EGF- Epidermal growth factor

ERK MAP kinase glucocorticoid – Extracellular signal regulated kinases/ mitogen activated protein kinases

VEGF- Vascular endothelial growth factor

S100A10- S100 calcium binding protein A10

TRPV5- Transient receptor potential 5

TRPV6- Transient receptor potential 6

PI (4, 5) P2- Phosphotidyl-inositol 4, 5- bisphosphate

RCC- Renal cell carcinoma

PET- Positron emission tomography

CT- Computed tomography

pMRI- perfusion Magnetic Resonance Imaging

IL-6- Interleukin 6

NGAL- Neutrophil gelatinase associated lipocalin

KIM-1- Kidney injury molecule

PAX8- Paired box8

CK7- Cytokeratin

c-KIT- Stem cell factor receptor

INF-α- Interferon alpha

IL-2-Interleukin 2

PGDF-R- Platelet derived growth factor receptor

LOH- Loop of Henle

GDNF- Glial - derived neutrotrophic factor

HGF- Hepatocyte growth factor

FGF2-Fibroblast growth factor 2

Bmp7- Bone morphogenetic protein 7

WNT4- Wingless-related 4

MMP-9-Matrix metalloprotenases

ECM -Extracellular matrix

NCAM- Neural cell adhesion molecule

CRCC-Clear cell renal cell carcinoma

TNM- Tumor, Node, Metastasis

VHL gene- Von –Hippel-Lindau tumor suppressor gene

FHIT gene- Fragile histidine triad protein

MET oncogene- Tyrosine protein kinase Met/ hepatocyte growth factor

PRCC- Papillary renal cell carcinoma

CDC- Collecting duct RCC

CD10- Cluster of differentiation 10/ Neprilysin

HLRCC- Hereditary leiomyomatosis RCC syndrome associated renal cell carcinoma

ACDRCC- Acquired cystic disease associated renal cell carcinoma

CCPRCC-Clear cell (tubule) papillary renal cell carcinoma

TLFRCC-Thyroid like follicular renal cell carcinoma

ALK translocation RCC -Lymphoma kinase translocation and renal cell carcinoma

CAIX – Carbonic Anhydrase IX

PMA -phorbol 12-myristate 13-acetate

Rac1 –Ras related C3 botulinum toxin substrate 1

MDCK cell line- Madin- Darby Canine Kidney cells

ANOVA- Analysis of variance

PAS- Periodic Acid- Schiff stain

DAB- Diamino benzidine chromogen

ABC -Avidin biotin horse raddish peroxidase complex

IHC- Immunohistochemistry

H & E- Haematoxylin and eosin

rv - Renal vesicle

PCT- Proximal convoluted tubules

DCT- Distal convoluted tubules

CD- Collecting duct

ub - Ureteric bud

DAG -Diacylgycerol

IP_3- Inositol triphosphate

$cPLA_2$ -Cytosolic Phospholipase A_2

GFR- Glomerular filtration rate

FPR- Formyl peptide receptors

EGFR- Epidermal growth factor receptor-1

TNF α- Tumor necrosis factor alpha

IL-17- Interleukin 17

cAMP - Cyclic adenosine monophosphate

TAL - Thick ascending limb of Henle

NKCC2- Na^+-K^+-2Cl co-transporter

AHNAK- Neuroblast differentiation –associated protein

ROMK- Renal outer medullary potassium channel

MET- Mesenchymal epithelial transition

FgF8- Fibroblast growth factor 8

Osrl- Odd Skipped related transcription factor

Salll- Spalt like transcription factor 1

EYA- Eye absent homolog

SIX- Sine oculis homeobox homolog

ODD1- Odd-skipped related 1

PI3K- Phosphoinositide-3-kinase

AKT- Protein kinase B alpha

NF-Kb- nuclear factor of kappa light polypeptide gene enhancer in B-cells

Abstract

Background:

Several studies on the mechanism of renal development indicate a close relationship between renal embryonic cells and renal cancer cells contributing to the diversity of morphologic patterns, molecular and immunohistochemical phenotypes of renal cancers. The normal human kidney develops from three sources; the metanephric blastema, ureteric bud and angiogenic mesenchyme. The development of the collecting and secretory components of human kidney require a controlled cellular proliferation, apoptosis, differentiation, cell-cell contacts and cell matrix interactions. One of the causes in the etiology of renal neoplasms is the mutation of molecules or the reactivation of repressed genes which have important role in nephrogenesis.

Annexin A1 (AnxA1) and Annexin A2 (AnxA2) are multifuctional calcium regulated phospholipid binding proteins found in a subset of renal neoplasms. With varying concentration of Ca^{2+}, they are considered to regulate several biological processes such as cell proliferation, apoptosis and differentiation. On account of these features, we analyzed the expression of these proteins in fetal kidney at different gestational periods, mature kidney and in kidney cancer tissues in order to possibly understand their biological role during nephrogenesis, functions in cells of mature kidney and understand renal tumors from the perspective of developmental biology.

Objectives:

a) To demonstrate the expression pattern of AnxA1 and AnxA2 in various renal structures of developing human fetal kidneys at different gestational ages.
b) Expression analysis of AnxA1 and AnxA2 in renal carcinoma and normal renal tissue.
c) To compare the expression pattern of AnxA1 and AnxA2 in various renal structures of developing human fetal kidneys, adult kidneys with those observed in renal carcinomatous tissues.

Materials and Methods:

AnxA1 and AnxA2 expression was investigated by immunohistochemistry technique in "Paraffin-embedded" renal tissue sections from autopsied fetuses of gestational age ranging from 14 to 39 weeks, in mature kidneys (35-85yrs of age) and renal cancer tissues. Haematoxylin and eosin staining of renal tissues was performed to study the a) histogenesis of fetal kidney in various stages of fetal development b) normal histological architecture of mature kidney c) types of renal cell carcinomas and confirm the original diagnosis, before performing immunohistochemistry. Tumors were graded according to Fuhrman et al.

Results:

The current study data demonstrated that AnxA1 is expressed in the mesangial cells and podocytes of maturing glomeruli in the developing renal cortex of fetal kidneys at 14 to 19 weeks of gestational age. The expression in the mesangial cells declined at later weeks of gestation and persisted in adulthood. AnxA1 expression increased with the progression of clear cell renal cell carcinoma and other types of renal cell carcinoma indicating a potential role of the protein in tumorigenesis.

The study showed moderate membranous expression of AnxA2 in the ureteric bud and collecting tubules of fetal kidneys in all gestational ages and in the collecting ducts of adult normal renal tissues. It is not often expressed in the proximal convoluted tubules of normal adult kidney; however younger fetal kidneys show moderate expression in the proximal convoluted tubules (thought to be the origin of renal cell carcinoma) and reappearance of strong membranous expression in the clear cell carcinoma suggesting a deregulation of the gene during tumorigenesis.

Conclusion: Understanding the molecular expression pattern of AnxA1 and AnxA2 during development, later their specific function and deregulated expression in different renal carcinoma indicates the decisive role of these proteins in the cancer progression. These results and concepts provide a framework to further dissect biological properties of AnxA1 and AnxA2 and thereby develop diagnostic, prognostic and therapeutic strategies targeting the molecule in various renal pathologies.

Key words: Annexin, phospholipid binding, kidney, nephrogenesis marker, renal cell carcinoma.

Chapter 1

INTRODUCTION

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

1. Introduction

The definitive human kidney develops from nephrogenic cord, an unsegmented column of intermediate mesoderm situated behind the embryonic coelom in the dorsal body wall.[1,2] Three successive kidneys-pronephros (cervical rudimentary kidney), mesonephros (thoracic kidney) and metanephros (functional kidney) develop in the intermediate mesoderm.[2] The first two are transient; it is the metanephros that forms the permanent system of excretion. The mesonephros that functions for a brief period consists of a complex system of functional tubules that open into the mesonephric duct.[3] A metanephric diverticulum (ureteric bud) arises from the caudal end of the wolffian duct to invade the metanephric blastemal cells. Reciprocal induction of the diverticle and the blastemal cells induces the development of excretory units of adult kidney. However, the collecting units develop from repeated branching of ureteric bud.[1] This complex mechanism of renal development suggest that the various components of developing kidney is derived from different cell sources and such dissimilarities in the embryonic origins of renal cells and various function of the nephron may contribute to the diversity of morphologic patterns, molecular and immunohistochemical phenotypes of renal diseases or renal cancers.[4]

Cell proliferation, cell differentiation, apoptosis, angiogenesis and vasculogenesis are the key processes involved in human renal development.[5] This exciting process of development involves more than 300 genes indexed to date.[6] Numerous studies on elucidating the early molecular events during development has helped in understanding the concepts of mesenchymal epithelial interaction, epithelial cell polarization, branching, morphogenesis as well as identifying human genes responsible for renal disease/cancer.[7]

In 1892, the French biologists Lobstein and Recamier introduced for the first time the concept of the embryonic origin of tumors. It was a few years later Prof. Bush pointed out that the cancer cell formation was the result of reactivation of repressed gene in the process of normal embryonic development.[8] In renal development, several genes such as neurogenic locus notch homolog protein (NOTCH), sonic Hedgehog (SHH), wingless-type (WNT), Wilms' tumor 1 (WT1), paired box2 (PAX2) and homeobox protein lim-1 (LIM1) have been identified in the past participating in molecular pathways/networks and further their re expression in human renal tumors. Thus, altered expression or regulation of certain genes important in the renal development may re express and contribute to carcinogenesis.[7] Further the evaluation of these molecules could pave the way in identification of prognostic markers, innovative therapies in the treatment of renal cancers.[7]

Renal cell carcinoma (RCC) accounts for approximately 3% of all human malignancies and remains a major health issue due to increase in incidence and mortality rates.[19] It is heterogeneous in nature owing to overlapping histomorphologic features among its variants such as chromophobe RCC, granular variant of clear cell RCC, collecting duct RCC.[10] Diagnosis and treatment of RCC still remains a challenging task to health care personnel's and biomarkers could be helpful in achieving these goals. Currently imaging biomarkers such as PET/CT with radio tracers, perfusion MRI (pMRI) and diffusion MRI, texture analysis, radiomics, serum biomarkers such as vascular endothelial growth factor, Interleukin 6 (IL-6), urine biomarkers neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule (KIM-1), tissue biomarkers PAX8 and PAX2 transcription factors, vimentin, CK7 cytokeratin, c-kit, cathepsin K are

being studied in order to understand the characteristics of RCC.[11] Therapy targeted towards RCC includes surgical resection, chemotherapy and immunotherapy. RCC is resistant to most chemotherapeutic drugs because of multidrug resistance mediated by p-glycoprotein present in all cells but infrequent reproducible responses to immunotherapeutic agents such as interferon alpha (INF-α) and interleukin 2 (IL-2) have been identified with modest benefits. Sorafenib and sunitinib are tyrosine kinase inhibitors that interfere with platelet derived growth factor receptor (PGDF-R) and vascular endothelial growth factor, and Temsirolimus is a rapamycin (mTOR) inhibitor. These drugs are used in the treatment of RCC.[12] However, drug induced resistance and fewer validated biomarkers drives researchers in identifying more effective therapy against this disease.[7]

AnxA1 and AnxA2, first two from the family of twelve annexins (Calcium dependent pholipid binding proteins) participate in multiple biological cellular processes, membrane organization and regulation of Ca^{2+} waves across membranes and within cells.[13] They have been explored as oncogenic signals in the molecular pathways of various types of tumors. Their deregulation in expression and activity is correlated with human cancers.[13]

AnxA1, act as intracellular sensors, identify incoming signals and provide each cell type a specific function.[14] It is a calcium sensitive and phospholipid binding protein which has the ability to sense changes in pH and interact specifically with lipid bilayer and protein molecules at the plasma membrane.[14] AnxA1 (Calpactin II), a 38 KD protein like other members of annexin family consists of calcium binding sites at its core domain and an unique NH2 terminal end with 40 residues that engages in specific molecular

interactions.[14-16] It has multiple potential sites that can undergo acetylation, lipidation, tyrosine, serine, and threonine phosphorylation. On exposure to Ca^{2+}, its N- terminal domain is exposed and through its interactions with several other molecules it participates in the regulation of biological processes such as cell proliferation, differentiation, cell death signaling, apoptosis, and phagocytosis.[17] Several studies show the activation of EGF and HGF receptor-tyrosine kinase is followed by the phosphorylation of AnxA1 on serine residues by protein kinase c and phosphorylation on thyrosine residues. This results in the activation of PI3 kinase and ERK MAP kinase glucocorticoid pathway which play important signaling functions in cell proliferation and migration.[18] Another function attributed to AnxA1 is its putative role in facilitating phagocytosis of apoptotic cells and secondary necrotic cells, and recruitment of phagocytes. Several studies support AnxA1 as an endogenous engulfment ligand that involves caspase dependent recruitment, release of intracellular calcium and colocalization with phosphatidyl serine on cell surface during apoptosis, followed by AnxA1 mediated recognition of apoptotic population and their engulfment.[19] Several studies document the interaction of AnxA1 with cytoskeletal proteins such as tubulin and actin.[18] In a Ca^{2+} dependent manner it's found to bind with F actin at the ruffles, cell to cell contacts, and cell surface of various cell types involved in the regulation of biological function such as cytoskeletal reorganization.[20] In addition, it is thought to stimulate VEGF- mediated angiogenesis. Although it has been studied in wide variety of normal tissues and abnormal tumor cells, its function in the developing embryonic tissues remain to be elucidated.

As far as its expression in cells and tissues are concerned, it is present abundantly in the smooth muscle, endothelial cells & specifically in the digestive and ductal

organs.[14,16] Several authors have detected low levels of AnxA1 in the tissues like brain, muscle and liver.[15] Moreover a strong AnxA1 immunoreactivity in mesangial cells, epithelial cells of Bowman's capsule, collecting duct cells of normal rabbit and rat kidneys has been reported indicating their role in specific physiological renal functions.[16,21] AnxA1 initiate Ca^{2+} dependent intracellular membrane repair and thus regulate cell survival and proliferation.

AnxA1 overexpression has been elucidated in esophageal adenocarcinoma, gastric adenocarcinoma, colorectal, pancreatic adenocarcinoma, hepatocellular carcinoma, clear cell carcinoma, hairy cell leukemia, lung adenocarcinoma and loss of AnxA1 expression in head and neck squamous carcinoma, esophageal squamous carcinoma, breast carcinoma and prostatic adenocarcinoma.[22] The role of AnxA1 protein is still unclear in tumor progression and carcinogenesis due to its inconsistent expression, up regulated in few tumors and down regulated in others.[17]

AnxA2 belongs to a family of calcium sensitive phospholipid binding proteins. It has been recognized to play roles in wide range of biological (cellular and molecular) processes such as angiogenesis, proliferation, apoptosis, cell migration, invasion, adhesion, exocytosis, endocytosis, membrane organization, ion channel conductance and to link F-actin cytoskeleton to the plasma membrane based on its localization.[23] It has been shown to bind to calcium and form complexes with other proteins. The interaction between annexin A2-S100A10 complex with calcium channel transient receptor potential channels TRPV5 and TRPV6 is essential for the active uptake of Ca^{2+} by kidney. It has also been recognized to interact with PI (4, 5) P2, in the regulation of membrane domains and cortical actin dynamics. Dysregulation and atypical expression of this 36-kDA

protein has been linked to wide spectrum of cancers, emphasizing its participation in tumor cell adhesion, proliferation, and invasion, metastasis and tumor neovascularization.[24]

Cancer development consists of cascade of events such as driver mutation (s), tumorigenesis followed by metastasis. However during these transitions, it comes across a physiological mechanism of apoptosis to eliminate damaged or abnormal cells.[22] A balance between cell survival, apoptosis and clearance of apoptotic cells is essential to maintain normal homeostasis in developing renal tissue. The fact that AnxA1 can mediate signal for cell proliferation or apoptosis prompted us to do a systematic understanding of this protein during various phases of renal development and renal cancer. Thus in the current study we report the expression profile of this apoptotic related protein during renal development at various gestational ages to adult kidney.

AnxA2 is a calcium binding protein with cell adhesive property and largely participates in transport across ion channels. These properties of AnxA2 are essential both in the development and functioning of normal adult kidney. Since the behavior and molecular basis of cancer largely mimics the development of embryo,[8] we tried to study the expression of metastasis associated protein AnxA1 and AnxA2 in the renal developmental, adult and cancer cells. This is primarily an attempt to elucidate a possible correlation of the several functional proposals made with respect to these proteins, their potential role during development and secondly, to speculate the role of the proteins in renal cancer from the perspective of development, anticipating their role as oncogenes. Thus provide an insight into kidney tumorigenesis. Although AnxA1 and AnxA2 have been studied in wide variety of tissues, there is limited data pertaining to their expression

in embryonic and adult renal tissues. Thus understanding renal carcinoma (RCC) from the perspective of developmental biology could pave way to newer therapeutic avenues and further strengthen support in making clinical decisions.

References:

1. Moore KL, Persaud TVN. The Developing Human: Clinically Oriented Embryology. 8th ed. Philadelphia: Saunders; 2008.

2. Lacson A, Bernstein J, Risdon RA, Gilbert-Barness E. Renal system. In: Gilbert-Barness E, editor. Potter's Pathology of the Fetus, Infant and Child.2nd ed. Philadelphia; 2007.p.1281–1344.

3. Alexander DP, Nixon DA. The foetal kidney. Br Med Bull 1996;17(2):112–117.

4. Tong GX, Yu WM, Beaubier NT, Weeden EM, Hamele-Bena D, Mansukhani MM, O' Toole KM. Expression of PAX8 in normal and neoplastic renal tissues: an immunohistochemical study. Mod Pathol 2009;22(9):1218–1227.

5. Jeon YJ, Kim J, Chae JIl. Comparative proteomic analysis of kidney development-related proteins in the pig. Vitr.Cell. Dev. Biol. Anim 2013;49(5):315–323.

6. Leroy X, Devisme L, Buisine MP, Copin MC, Aubert S, Gosselin B, Aubert JP, Porchet N. Expression of human mucin genes during normal and abnormal renal development. Am J Clin Pathol 2003;120(4):544–550.

7. Dormoy, V, Jacqmin D, Lang H, Massfelder T. From development to cancer: lessons from the kidney to uncover new therapeutic targets. Anticancer Res 2012;32(9):3609–3617.

8. Ma Y, Zhang P, Wang F, Yang J, Yang Z, Qin H. The relationship between early embryo development and tumourigenesis. J Cell Mol Med 2010;14(12):2697–2701.

9. El-shorbagy S, Alshenawy H. Diagnostic utility of vimentin, CD117, cytokeratin-7 and caveolin-1 in differentiation between clear cell renal cell carcinoma, chromophobe renal cell carcinoma and oncocytoma. J Microsc Ultrastruct 2016;5:90.

10. Ozcan A, Zhai J, Hamilton C, Shen SS, Ro JY, Knshnan B, Truong LD. PAX-2 in the diagnosis of primary renal tumors : Immunohistochemical comparison with renal cell carcinoma marker antigen and Kidney-Specific cadherin. Am J Clin Pathol 2009;13:393–404.

11. Farber NJ, Singer EA, Modi PK, Sadimin ET, Kim CJ, Hon JD. Renal cell carcinoma: the search for a reliable biomarker. Transl Cancer Res 2017;6(3):620–63.

12. Koul, Jung-Sik Huh, Kyle O. Rove, Luiza Crompton, Sweaty Koul, Randall B. Meacham et al. Molecular aspects of renal cell carcinoma: a review. Am J Cancer Res 2011;1(2):240-254.

13. Gerke V, Moss SE. Annexins: from structure to function. Physiol Rev. 2002;82(2):331-371.

14. Monastyrskaya K, Babiychuk EB, Draeger A. The annexins: spatial and temporal coordination of signaling events during cellular stress. Cell Mol Life Sci 2009;66(16):2623–2642.

15. Kang WY, Chen WT, Huang YC, Su YC, Chai CY.Overexpression of annexin 1 in the development and differentiation of urothelial carcinoma. Kaohsiung J Med Sci 2012;28(3):145–150.

16. Markoff A, Gerke V. Expression and functions of annexins in the kidney. Am J Physiol Renal Physiol 2005;289: F949-56.

17. Biaoxue R, Xiguang C, Shuanying Y. Annexin A1 in malignant tumors: Current opinions and controversies. Int J Biol Markers 2014;29:e8–e20.

18. Pin AL, Houle F, Fournier P, Guillonneau M, Paquet ÉR, Simard MJ, et al. Annexin-1-mediated endothelial cell migration and angiogenesis are regulated by vascular

endothelial growth factor (VEGF)-induced inhibition of miR-196a expression. J Biol Chem. 2012;287(36):30541–51.

19. Arur S, Uche UE, Rezaul K, Fong M, Scranton V, Cowan AE, et al. Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. Dev Cell. 2003;4(4):587–98.

20. Belvedere R, Bizzarro V, Popolo A, Dal Piaz F, Vasaturo M, Picardi P, et al. Role of intracellular and extracellular annexin A1 in migration and invasion of human pancreatic carcinoma cells. BMC Cancer. 2014;14(1):1–15.

21. Tribolo S, Maroux S, Massey-Harroche D. Kidney proximal tubule cells: epithelial cells without EGTA-extractable annexins? Biochem. Cell Biol 2000;78(4):495–502.

22. Fernald K, Kurokawa M. Evading apoptosis in cancer. Trends Cell Biol 2013;23:620–633.

23. Bharadwaj A, Bydoun M, Holloway R, Waisman D. Annexin A2 heterotetramer: Structure and function. Int J Mol Sci 2013; 14:6259–6305.

24. Xu XH, Pan W, Kang LH, Feng H, Song YQ. Association of Annexin A2 with cancer development (review). Oncol Rep 2015; 33:2121–2128.

Chapter 2

AIMS AND OBJECTIVES

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

2. Aims and Objectives

2.1: The following specific aims were proposed to accomplish our goal

a) To demonstrate the expression pattern of AnxA1 and AnxA2 in various renal structures of developing human fetal kidneys at different gestational ages.

Immunohistochemistry staining was performed on charged slides of renal tissues from aborted or dead human fetal kidneys of gestational age ranging from 14 to 39 weeks. Staining intensity of primary antibodies AnxA1 and AnxA2 was recorded in various renal structures.

b) Expression analysis of AnxA1 and AnxA2 in renal carcinoma and normal renal tissue

Immunohistochemistry staining was performed on charged slides of renal tissues obtained from surgical specimen or biopsy specimen (renal cell carcinoma) and cadaveric renal tissues. Staining intensity of primary antibodies AnxA1 and AnxA2 was recorded in various renal structures of normal cadaveric non neoplastic and neoplastic renal tissues and compared.

c) To compare the expression pattern of AnxA1 and AnxA2 in various renal structures of developing human fetal kidneys, adult kidneys with those observed in renal carcinomatous tissues.

2.2: Research Hypothesis

- AnxA1 and AnxA2 play important role in renal development, normal kidney functions and, elucidation of the expression pattern of AnxA1 and AnxA2 aids in understanding the underlying mechanism of kidney organogenesis and further progression to renal carcinoma.

The fact that AnxA1 and AnxA2 can mediate signals for the regulation of cell proliferation, apoptosis, epithelial mesenchymal interactions, their functions such as membrane organization and transport, properties such as cell to cell adhesion and calcium binding has prompted us to perform a systematic investigation of these phospholipid binding proteins by studying the distribution of their expression in embryonic, adult and renal carcinomatous tissues.

Therefore, briefly the purpose of the study is to lend support to the existing information pertaining to the expression status of AnxA1 and AnxA2 in the human fetal and adult kidneys and renal carcinomatous tissue and thereby provide new information (the importance of these proteins in renal development) in the field of kidney nephrogenesis research and further their role in renal carcinogenesis.

Chapter 3

REVIEW OF LITERATURE

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

3. Review of Literature

3.1: Introduction to Renal Development

The pre-embryonic, embryonic and fetal periods of human renal development is characterized by three successive primitive forms of excretory system, the pronephros, mesonephros and metanephros. The metanephros (primordial of permanent kidney) develops from the nephrogenic cord of intermediate mesoderm by reciprocal inductive interactions between the ureteric bud and metanephric blastema and there by inducing the formation of filtration units (nephrons) of adult kidney.[1,2] The metanephric blastemal cell condenses to form nephrogenic vesicles, S-shaped bodies. The invagination of capillaries formed in situ, into the nephrogenic vesicle helps in the formation of glomerulus, the elongation of the limbs of nephrogenic vesicle differentiate into proximal and distal tubules and loop of Henle. The ureteric bud progresses to form the collecting tubules, ureters and renal pelvis.[3] This process of tubulogenesis and branching morphogenesis is under the influence of many growth factors and signaling molecules, involving more than 300 genes.[4] The Wilm's tumor suppressor gene 1 stimulates the ureteric bud to induce differentiation and branching by regulating the production of glial - derived neutrotrophic factor (GDNF) and hepatocyte growth factor (HGF). Fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 7 (Bmp7) are known to block apoptosis and further aid in the proliferation of the metanephric mesenchyme. Paired-box gene 2 (pax2) and wingless-related (WNT4) up regulation also promotes the differentiation of mesenchyme into nephron epithelium.[5]

From the beginning of renal development around five weeks of gestation to cessation of new nephron formation at about 34 to 37 weeks of gestation, a reiterative inductive process establishes the nephron complement, generating distinct kidney components and driving kidney assembly.[6]

3.2: Nephrogenesis- Pronephros, Mesonephros and Metanephros: During gastrulation, the intermediate layer of the human embryo forms the intra embryonic mesoderm. This differentiates into paraxial mesoderm, intermediate mesoderm and lateral plate mesoderm.[7,8] Intermediate mesoderm connects paraxial mesoderm with lateral plate differentiates into urogenital structures as illustrated in figure 1. In the cervical and upper thoracic regions, it undergoes segmentation to form future nephrotomes where as more caudally it forms unsegmented mass of tissue called the nephrogenic cord.[8]

Embryologically urinary system and genital system are closely associated to each other. The urogenital system develops from the intermediate mesoderm from the dorsal body wall of the embryo. A longitudinal elevation of the mesoderm –urogenital ridge forms on each side of dorsal aorta. The part of the urogenital ridge giving rise to the urinary system is the nephrogenic cord.[9]

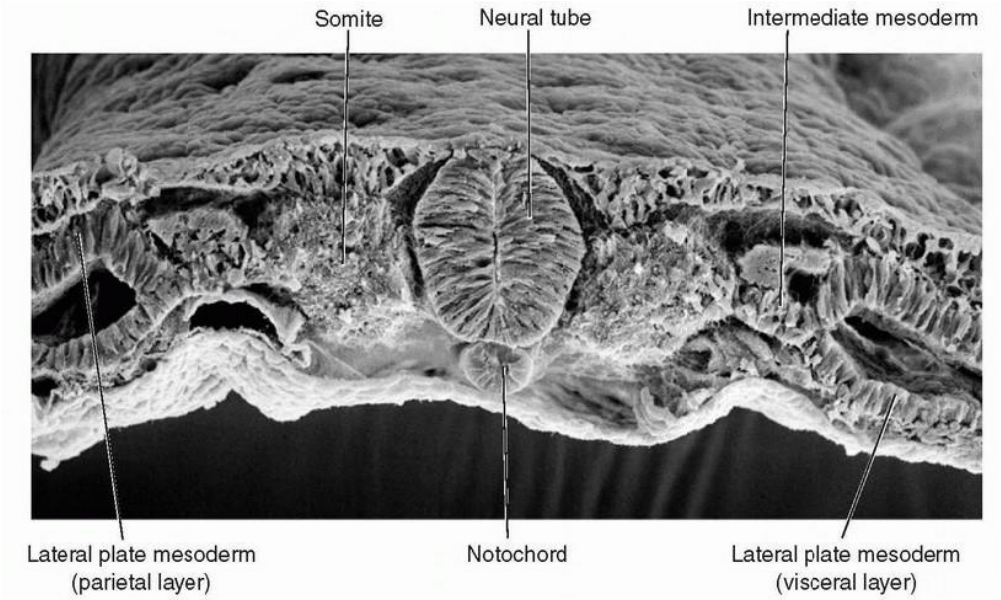

Figure 1: A representative view of notochord, neural tube and, the mesoderm that organizes into paraxial (somites), intermediate and lateral plate mesoderm.

Image courtesy: This image is from a tutorial developed by Drs. Kathleen K. Sulik and Peter R. Bream Jr with the assistance of Mr. Tim Poe and Ms. Kiran Bindra.

Three primitive forms (pronephros, mesonephros and metanephros) of definitive kidney are successively formed from the cranial, intermediate and caudal parts of the nephrogenic cord.[7] These spatially and temporally different kidney generations maintain extracellular fluid homeostasis and participate in the elimination of nitrogenous waste in few species.[10] The first formed embryonic pronephric kidneys are functional in fish and ambhibian larvae. In humans, they are represented by few transitory nonfunctional pronephric tubules associated with pronephric duct. This duct forms the collecting duct system of mesonephros and metanephros.[10-14] Since the pronephros serves no excretory function in the mammals, it quickly degenerates.[15] However gene expression analysis

show high expression of $Na^{2+/}K^{+}$-Atpase and ROMK channel involved in ion transport, speculating its role in extracellular fluid homeostasis.[16-20]

As the development continues, the condensation of the nephrogenic mesenchyme caudal to the pronephros results in the formation of mesonephros- the first kidney to serve excretory function. It gives rise to male reproductive ducts (wolffian duct) and the primordial to permanent kidney- the Metanephros.[15] Mice studies reveal that the mesonephric tubules develop via mesenchymal to epithelial transition (MET) within the mesonephric mesenchyme as comparable to a similar process of tubule formation of metanephric nephrons. Wnt signaling pathway is activated for induction of MET.[21] Other factors such as Wnt9b, FgF8, Wnt 4, Pax2, Lim1, Osrl, Salll, Wnt1 are expressed in both the former tubules.[22-30] Though there is a similarity in the factors expressed by both the generations, only the metanephric kidney becomes functional. The structure that is evidently absent in the second generation of tubules, is the branching in collecting duct of metanephros.[21] Some studies also debate the lack of juxtaglomerular apparatus and loop of Henle.

Though the pronephros, mesonephros are the transient organs of excretory system, they are very much essential in the formation of definitive kidney, the metanephros.[7] The pronephros appear as a mass of cells in the cranial portions of the nephrogenic cord and few rudimentary tubules caudally which eventually disappears, the mesonephros consists of similar type of cells that become vesicular and join the primary excretory duct. The pronephros and mesonephros contain tubular structures oriented cranio caudally in the nephrogenic cord resulting in the production of hypotonic urine. However in the

metanephros, the concentric arrangement of the tubules and the loop of Henle directed towards the pelvis enables it to produce concentrated hypertonic urine.[31]

3.2A: Metanephros- The Definitive Kidney:

The tubules of the metanephros do not join the primary excretory duct (mesonephric duct) however joins an evagination of the duct (ureteric bud that branches from the caudal end of the wolffian duct) (figure2)[32], which branches to several generations to form the collecting component of the excretory system.[33]

The metanephros develops largely from three sources:

A) Metanephric blastema

B) Ureteric bud

C) Angiogenic mesenchyme[31].

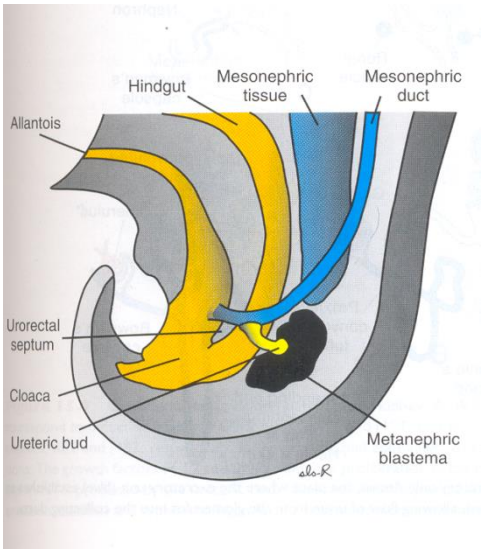

Figure 2: Representative view showing the invasion of ureteric bud into the metanephric blastema.[32]

An epithelial mesenchymal interaction between the branching ureteric bud and the metanephric blastema cells occurs in the metanephros. The ampulla of each branching bud induces formation of new nephrons. Besides each ampulla, a condensation of metanephric blastemal cells develop slit like cavities to form renal vesicle which elongates to form S shaped bodies (figure 3 & figure 4). While one end of it fuses with the ampulla on the other end the capillaries formed in situ invaginates to form the glomerulus. The limbs of the vesicle elongates further, to form the proximal, distal tubules and the loop of Henle.[3]

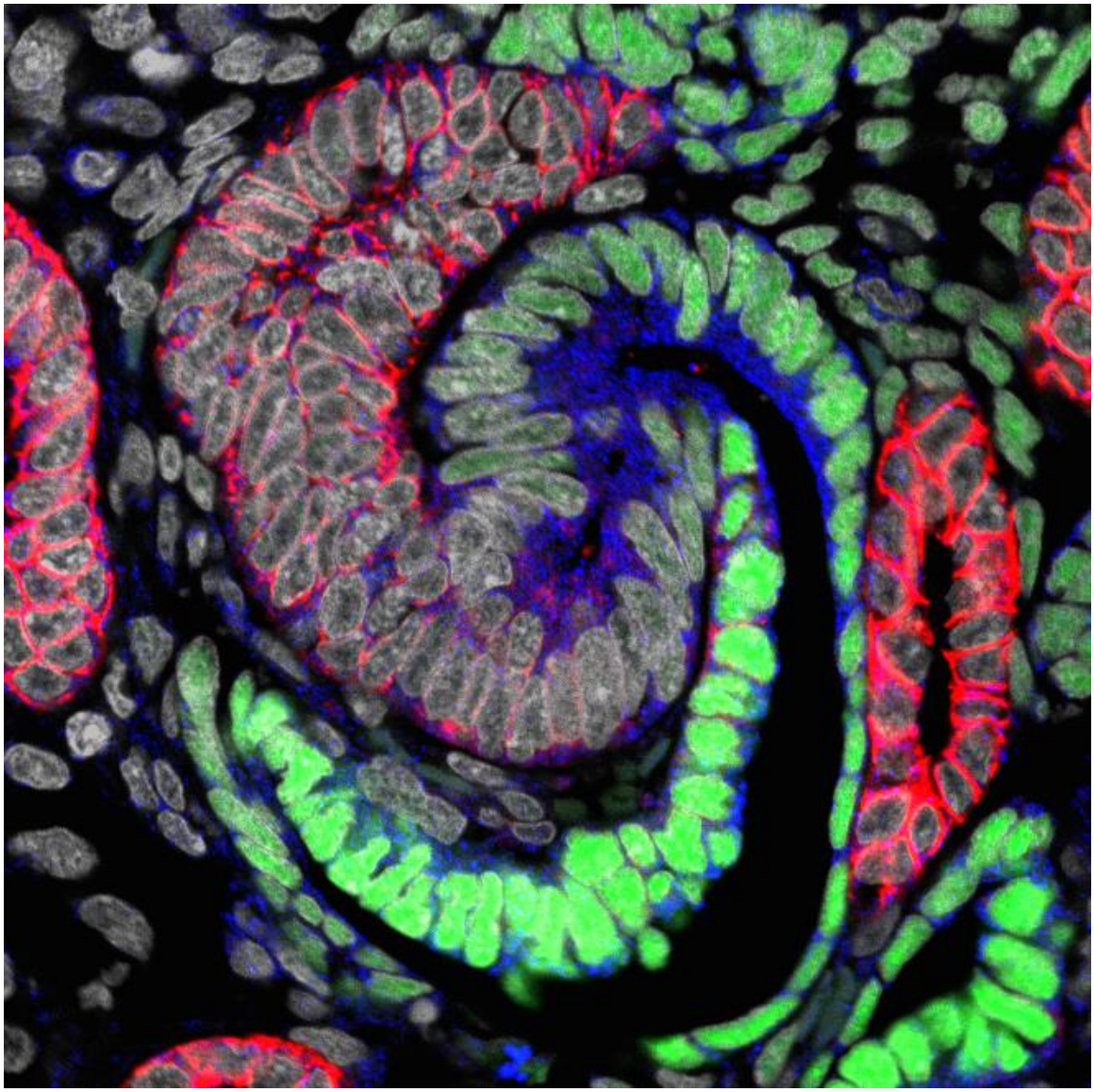

Figure 3: Early stage of nephron development showing 'S'- shaped bodies.

Image courtesy: Stacy Moroz and Tracy Tran/McMahon Lab

https://stemcell.keck.usc.edu/never-accept-a-kidney-donation-from-a-mouse/

Vascular development in the kidney is marked by two cellular processes- angiogenesis and vasculogenesis. During angiogenesis vasculature arises from progenitor cells within the metanephric mesenchyme while in vasculogenesis, there is migration of existing endothelial cells into developing mesenchyme.[33-35] Despite its importance, the

origin of the kidney vasculature is not completely understood. Studies suggests that with the invagination of renal vesicle, signals released by the cells of the vascular cleft recruit angioblasts or endothelial cells into the developing glomerulus. With the migration of these endothelial cells into the vascular cleft they undergo mitosis and form capillary loops within the developing glomerulus. The glomerular basement membrane is laid from extracellular matrix components produced by the endothelium and podocytes. As development continues mesangial cells occupy the glomerulus and they contribute to the stabilization of the glomerular capillary tuft.[36]

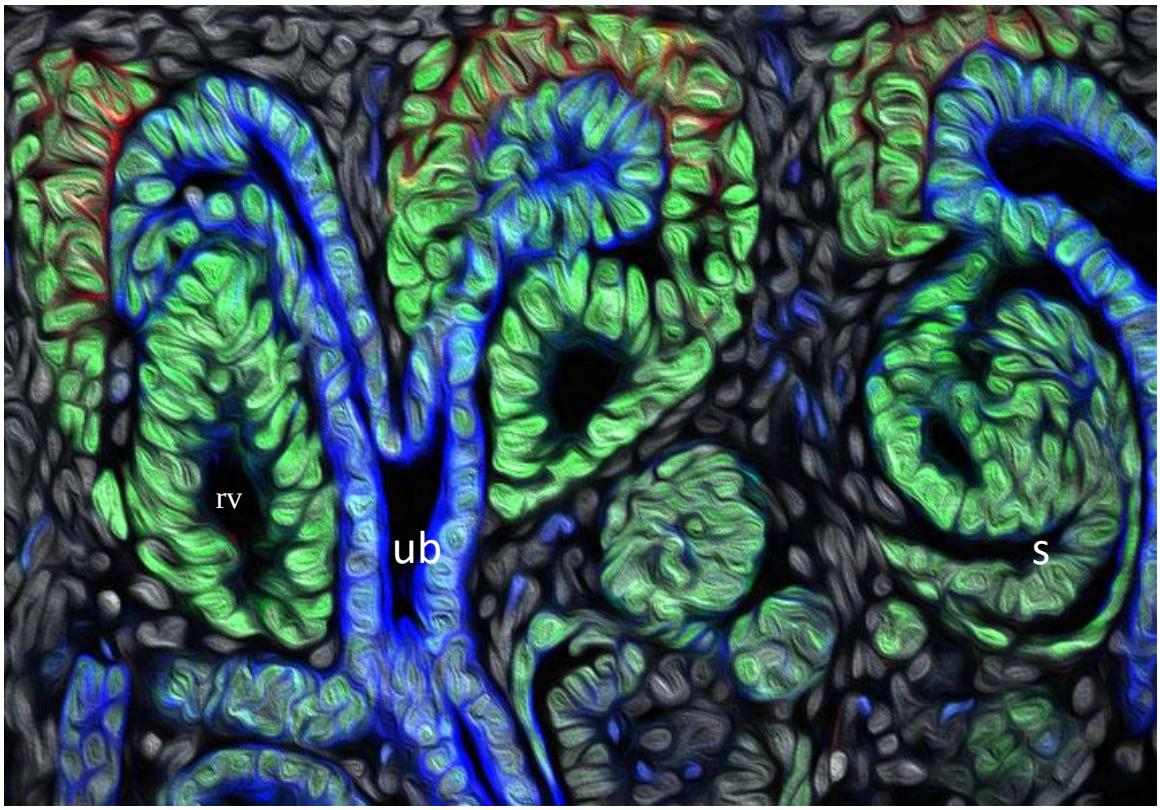

Figure 4: Developing nephron- the filtration unit. Cap mesenchymal cells overlying ureteric bud (ub) tips. Primitive forms of glomeruli- the renal vesicle (rv) and 'S' shaped bodies.
Image courtesy: Stacy Moroz and Tracy Tran/McMahon Lab
https://news.usc.edu/143998/perfectly-punctual-or-fashionably-late-it-takes-all-kinds-of-cells-to-build-a-kidney/

3.2B: Development of collecting duct system: The collecting duct, calyces, pelvis and ureter that form the collecting component of adult kidney is formed by sequential branching of ureteric bud that is completed by 20 to 22 weeks of gestation (figure 5a, 5b, 5c, 5d & 5e).[2,9] Extracellular matrix molecules such as chondroitin sulphate proteoglycan and chondroitin sulphate glygosaminoglycans are essential for the dichotomous branching of the ureteric bud. With the elongation of the collecting duct an adhesion molecule syndecan is detected in the condensing mesenchymal cells, the cells also express neural cell adhesion molecule, fibronectin, liver cell adhesion molecule.[31]

During human nephrogenesis, ureteric bud branches repeatedly to give rise to collecting ducts. During later stages, the first five generations of the ureteric bud undergo transfor mation into the pelvis and calyces by increased growth and dilatation of these tubules. Each ampulla of the branch is capable of inducing about 100 mesenchymal cells to proliferate, differentiate, undergo mesenchymal to epithelial transformation leading to the formation of the epithelial cells of the nephron.[36]

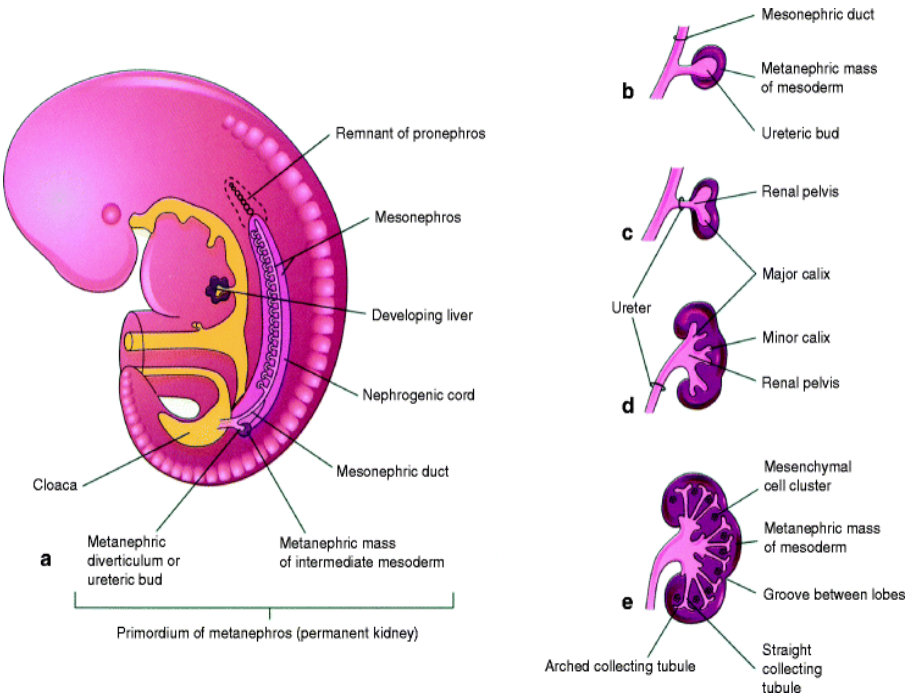

Figure 5a: Section of developing embryo showing three overlapping renal systems.
Figure 5b, 5c, 5d & 5e: Section depicting the formation of collecting component of kidney, branching of ureteric bud giving rise to minor calyces, major calyces, renal pelvis and ureter.[9]

3.3: Regulatory functions of genes in renal development: Experimentation on frog, chick embryos, and mice has enabled better understanding of complex architecture of renal organ system.[37] The development of the mammalian kidney has been studied at the genetic, biochemical, and cell biological level for several years. The interaction of several genes are important in specifying the renal epithelial cells of the nephron and is required in the maintenance of normal renal function.[38]

Association between **PAX/eye absent homolog (EYA)**, **sine oculis homeobox homolog (SIX)** transcription factors, **LIM1**, **odd-skipped related 1 (ODD1)** transcription factors is required during the early phases of renal patterning.[33,37,39-41] The proto-oncogene c-Ret

(RET) / **glial-derived neurotrophic factor (GDNF)** (receptor/ligand) pathway, controls bud outgrowth and branching morphogenesis.[33,42] The developing collecting duct is dependent on various molecular carriers of signals that arise from the surrounding mesenchymal cells. Glial Cell line derived Neutrotrophic factor, drives the arborization and can also induce the formation of supernumerary ureteric buds in culture. It is produced by the mesenchyme while its c-ret receptor is produced by the ureteric bud.[8, 43]

LIM1 described in Xenopus belonging to LIM-hd family of homeobox is expressed throughout in the urinary tract. It is thought to control the notch signaling pathway essential for tubule patterning and cell – cell communications.[37,44,39] Mesenchymal-to-epithelial transitions, development and maturation of podocytes is regulated by zinc finger protein **WT1**. Through its interactions with WNT proteins and **PAX2**, it plays a crucial role during kidney development.[45,46] It is a transcription factor released by the mesenchyme of metanephric blastema that renders it competent to interact with the ureteric bud. It also regulates the production of **GNDF** and **Hepatocyte Growth Factor**.[8] **Hepatocyte Growth Factor, Fibroblast growth Factor and BMP-7** produced by the mesenchyme is essential for the normal collecting duct development.[8,43] Studies show WNT proteins are involved in the induction of the kidney mesenchyme. **PAX2 and WNT4** upregulated in the metanephric mesenchyme by **WNT9B and WNT6**, promote condensation of mesenchyme and tubule formation respectively.[8,43] **Matrix metalloprotenases MMP-9** is required for the specific integrin and extracellular matrix interactions.[8,43] Other factors in various stages of kidney development documented are the phosphoinositide-3-kinase (PI3K)/protein kinase B alpha (AKT), nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB), mitogen-activated protein kinase

(MAPK), and Jun kinase pathways.[47-49] The SHH-glioma-associated oncogene homolog (GLI) signaling pathway has essential roles in cell differentiation and proliferation during embryogenesis.[50]

3.4: Cellular processes in kidney development:

The complex biological mechanisms, signaling pathways, transcription factors largely decide the fate of cells required for renal development. Cell proliferation, apoptosis, migration and differentiation, cell to cell communications and morphogenesis are the key events occurring during nephrogenesis.

Cell proliferation: Cell proliferation is essential in the formation of kidney. In vitro transmembrane culture studies utilizing undifferentiated mouse metanephric blastemal cells separated by an inductor tissue observed a burst of proliferative activity post the induction of metanephric blastemal cells. Studies show proliferation of cells particularly in the superficial germinal layer of renal cortex.[51]

Cell death (Apoptosis): In the context of renal development, a programmed cell death is an essential component in the establishment of tissue architecture, and helps to both remodel developing structures to their mature form and eliminate unessential cells. Thus, dysfunctions in apoptosis can manifest as developmental abnormalities. A classic example of apoptosis in the genitourinary tract, include the regression of the pronephros and the selective loss of portions of the mesonephros in females during kidney development. In the mammalian kidney, the number of nephrons (probably determined by the process apoptosis) generated at birth in any one individual is thought to be an important determinant of adult kidney health. The morphological changes in apoptosis

induced cells are thought to identify specific cells for removal in a controlled fashion, without the activation of inflammatory cells (in contrast to cell death attributed to necrosis).[52]

Morphogenesis: It is a process in which a developing system acquires a three dimensional architecture by the rearrangement of cells. Epithelial branching morphogenesis is critical to formation of various organs such as the vasculature, and kidneys in vertebrate embryos. For example the branching morphogenesis of a simple epithelial tube that grows by reiterative branches to form a complex epithelial tree structure.[53] Another example being the elongation of the tubules of loop of Henle. The unique spatial arrangement of these tubular components is essential for the regulation of urine concentration and other specialized kidney functions. The proper and timely assembly constituents of medulla is an important morphogenetic event leading to the formation of a functioning metanephric kidney.[2]

Migration and Differentiation:

Tissue organization in kidney is achieved during development by the combined cellular processes of cell differentiation and morphogenetic cell movements. In the kidney, each nephron is an epithelial tubule that is subdivided into distinct segments with specific transport functions. In several instances of morphogenesis, renal epithelial cells move as intact clusters, sheets, or tubes, in a process termed ‘‘collective cell migration’’.[54] Within the developing kidney the glomerular cell population may also be determined by cell migration.[7]

Cell cell/ Cell matrix adhesion: Cell adhesion is important for initiation of kidney development. Integrin's are heterodimeric transmembrane glycoproteins expressed during kidney development. They bind several components of the ECM (Extracellular matrix). The integrin α-8 subunit is expressed in mesenchymal cells surrounding the Wolffian duct and expression is upregulated as branching of ureter bud starts. Expression is not detected in the primitive forms of the glomerulus, comma- and S-shaped bodies. Study on rodents lacking the integrin α8 subunit have severe kidney abnormalities leading to death or born without ureters and kidney.[55] Two groups of adhesion molecules have been so far detected in the ontogeny of renal development, those that bring about cell to cell contact, e.g: neural cell adhesion molecule (NCAM), E-cadherin, a calcium dependent adhesive molecule, Nephrin, and those that enable cell matrix adhesion such as fibronectin, tenacin, laminins, galectin 3.[7]

It is apparent that the developing embryo largely simulates cancer (vice versa) in terms of biological behavior such as cell proliferation, invasion, migration, angiogenesis and, molecular pathways. Cancer is a disease that involves uncontrolled cell division, disruption of cell differentiation, invasion and, metastasis that recruits angiogenesis.

3.5: Renal neoplasias:

Renal cell carcinoma (RCC) is a common tumor of kidney and accounts for ~3% of all human malignancies worldwide. RCC is a heterogeneous disease, consists of different histological variants with a distinct clinical course, genetics, and response to treatment. Adult renal epithelial neoplasms are heterogeneous and consist of subtypes that have distinct gross, histological, ultrastructural, and immunohistochemical features.[56] Most of

the renal cell neoplasms arise from are thought to be from the renal tubules. Renal cell carcinoma (RCC) is divided into several histological subtypes such as clear cell (CRCC), papillary, chromophobe, collecting duct, and unclassified RCC. Clear cell, papillary, and chromophobe RCCs are the common types accounting about 70% to 80%, 14% to17%, and 4% to 8% of all RCCs, respectively. Collecting duct carcinoma is the rarest type amongst the various histological types of RCC (1%). 10% of renal tumors are benign such as oncocytoma, angiomyolipoma, and papillary adenoma.[57]

Most of these tumors are diagnosed incidentally as small renal masses. 30% of cases are benign while 20 to 30% can potentially progress and metastasize. It seems to occur during the sixth and seventh decades of life affecting both the gender. Radical nephrectomy or nephron sparing procedures are currently the treatment options. However systemic therapies used as adjuvants may not always bring about a positive outcome. There is a growing need for identifying molecular biomarkers of tumors which are aggressive in nature.[58]

3.5A: Etiology: Renal cell carcinoma has been associated with several exogenous risk factors such as smoking, obesity, hypertension and, genetic susceptibility as in individuals with first degree relative having RCC. It is also associated with other renal disorders that includes hemi hypertrophy, situs inversus totalis, teratoma, supernumerary kidney and von Hippel-Lindau disease.[7,59]

3.5B: Clinically less than 15% of individuals with RCC lack classic symptoms such as flank pain, renal mass and gross hematuria at the time of diagnosis. Other non specific symptoms include muscle tenderness, fever, fatigue, weight loss, anorexia in fewer cases while in others there is evidence of anemia (20-40%), hypercalcemia (13-20%), erythrocytosis (1-8%), elevated serum renin levels (37%), and gynaecomastia, musculinization in women suffering from RCC.[7,59]

3.5C: Grading and staging renal carcinoma: RCC is graded and widely accepted based on the grading system as proposed by Fuhrman et al in North America (Table 1).[60] Despite its shortcomings such as vague terminology and fewer number of cases studied, it is still accepted globally.[7] **TNM staging of RCC** is done to aid the clinician to assess the extent of spread, clinical behavior and outcome of RCC (Table 2).[61]

Table 1: Fuhrman nuclear grading

1.	Small round uniform nuclei (10µm), inconspicuous /absent nucleoli
2.	Irregular nuclei (15µm), nucleoli observed at high power (X400)
3.	Irregular nuclei (20 µm), prominent nucleoli observed at low power (X100)
4.	Similar to grade 3, bizarre, multilobed and clumped chromatin.

Nuclear grading of RCC as grade1, grade 2, grade 3, grade 4 based on the morphology of nucleus and nucleolus.[60]

Table 2: TNM staging of RCC

Stage I	T1	N0	MO
Stage II	T2	N0	M0
Stage III	T1 or T2 T3	N1 N0 or N1	M0 M0
Stage IV	T4 Any T	Any N Any N	M0 M1

TNM staging based on:
Primary Tumor (T), involvement of Lymph Nodes (N), and Metastasis (M)[61]

3.5D: Clear cell renal cell carcinoma: The commonest form of RCC is believed to be derived from the proximal tubular cells is characterized by cytogenetic abnormalities that involves loss of genetic material from chromosome 3p and mutations of VHL gene. VHL gene acting as a tumor suppressor gene on the 3p chromosome along with inactivation of other genes in its vicinity such as FHIT gene is anticipated as the cause of clear cell carcinoma. The VHL gene is found to be inactivated or hypermethylated, as observed in 96% of cases. CRCC are associated with several familial settings such as VHL disease (autosomal dominant) with an early onset of renal carcinoma in adolescence, mean age at diagnosis at 39 years following death of the untreated individuals. Other familial cause includes families segregating constitutional chromosomal 3 translocations. Histologically they appear as conventional RCC amidst a background of uniform spindle cells resembling smoothmuscle cells.[7,59]

Conventional CRCC histologically demonstrates neoplastic cells with clear cytoplasm, acinar, alveolar, cystic pattern. As the nuclear grades progress there is granular cytoplasmic changes.[62]

3.5E: Papillary renal cell carcinoma (PRCC): It occurs sporadically, inherited renal cancer developing in families with hereditary papillary renal carcinoma. It is caused due to mutations in the MET oncogene at 7q31.[7,59]

The male to female ratio of occurrence of PRCC is 2.4: 1. Radiologically they are evident mural calcifications, multifocal and necrotic. Grossly well circumscribed, cut sections appears light grey or golden yellow to red brown.[7,59] Histologically characterized by the tubular or papillary structures lined by tumor cells with or without pseudostratification.[62] They are classified into two distinct subtypes such as Type1 PRCC and Type2 PRCC (Table 3).[59]

Table 3: Papillary renal cell carcinoma

Sl.No	TYPE1 PRCC	TYPE2 PRCC
1.	Mean diameter 6cm	Mean diameter 9.5cm
2.	Low nuclear grade	Higher nuclear grade, nuclear pseudostratification
3.	Inconspicuous nucleoli	Prominent nucleoli
4.	Papillae are thin, short and delicate	Dense and fibrous papillae
5.	Glomeruloid bodies are many.	Less prevalent glomeruloid bodies
6.	Longer survival rates	Shorter survival rates

Differentiating Features of Type 1 and Type 2 PRCC

3.5F: Chromophobe renal cell carcinoma: Chromophobe RCC is believed to be derived from the intercalated cells of collecting duct either sporadic in occurrence or inherited as a part of Birt –Hogg- Dube syndrome that is characterized by skin disorder, cutaneous nodules particularly involving the head and neck.[7] Both the gender affected equally. Maximum number of cases witnessed in the Middle East for causes unknown. Grossly the tumors are 1.5 to 25cm in diameter. Cut surface is either light brown, rarely light pink or yellow white.[7] Histologically it is composed of pale cells giving a soap bubble appearance of cytoplasm, an eosinophilic variant has nested, alveolar appearance.[56] The chromophobe cells are usually admixed with granular eosinophilic cells, binucleated and presence of perinuclear halos.[7,59]

Similarity in the presenting features of oncocytoma and chromophobe renal cell carcinoma poses a challenge to pathologist in differentiating the two types. While several molecular biomarkers are nonspecific, molecular biomarkers such as S-100A1, proline rich region are yet to be extensively studied. CK7 is potential in distinguishing both.[7]

Two years postsurgical resection, the cancer survival rate is 25% in patients having chromophobe RCC with sarcomatoid differentiation and 96% without sarcomatoid differentiation.[7,59]

3.5G: Collecting duct carcinoma: This rare neoplasm arises from the principal cells of the collecting ducts of Bellini. Mean age of occurrence is 55years. Onset can be as early as 6 years. Male to female ratio of occurrence is 2:1. It was found patients with strong familial history or where in there were failure of renal allografts. Urine analysis show malignant cells in few cases. Usually metastasizing to bone. Tumor size ranges from 1 to

16cm. It has a wide origin either from cortex or medulla of kidney, extending into the perinephric fat, gerota fascia, renal veins and regional lymph nodes.[7] It has a papillary or cord like growth pattern with prominent desmoplastic stroma.[57] High grade nuclei, prominent nucleoli, "hobnail" appearance of tumor cells at the luminal structures are other typical features of Collecting duct RCC. Morbidity rates are way too high due to advanced tumor stage at the time of presentation.[7,59]

3.5H: Recent emerging new renal epithelial tumors: Tubulocystic renal cell carcinoma (TCRCC), a low grade tumor consists of tubules and cysts separated by fibrovascular septa. Sometimes it presents with papillary features making it indistinguishable from Type 2 PRCC. It is positive to markers such as vimentin, CD10, and CK7.[63]

Hereditary leiomyomatosis RCC syndrome associated renal cell carcinoma (HLRCC), aggressive tumor having similar features of Type 2 PRCC. S-(2 –succino)-cysteine (2SC) is a reliable marker.[63]

Acquired cystic disease associated renal cell carcinoma (ACDRCC) - is associated with acquired cystic kidney disease. Though has overlapping features like that of conventional RCCs', identified by the presence of intratumoral oxalate crystals. It is positive for CD10, vimentin and glutathione S transferase alpha.[63]

Clear cell (tubule) papillary renal cell carcinoma (CCPRCC) is less aggressive showing clear cells with inverted polarity with respect to the nuclear profile. It is positive to CK7, PAX2 and PAX8.[63]

Thyroid like follicular renal cell carcinoma (TLFRCC) resembling that of a thyroid follicular carcinoma having low grade tumor and low metastasis potential.

Anaplastic Lymphoma kinase translocation and renal cell carcinoma, **ALK translocation RCC,** a rare type of RCC affecting adults with sickle cell trait. Renal cell carcinoma with angio leiomyomatous stroma is a distinct neoplasm with characteristic morphological, immunohistochemical and molecular features, they do not exhibit chromosome 3p deletion as in CCRCC or associated with trisomy 7 and 17 as in case of papillary RCC.[63]

3.5G: Biomarkers in renal cancers:

The immunohistochemical markers of clear cell RCC include cytokeratin, CD10, epithelial membrane antigen, RCC marker, PAX8 and PAX2. The diffuse staining in the membranes of the tumor cells for CAIX is indicative of the inactivation of von Hippel-Landau (VHL) gene and further activation of hypoxia inducible factor pathway.[64] A study conducted by Mjones et al., showed the expression of erythropoietin and neuroendocrine markers in clear renal cell carcinoma while papillary renal cell carcinomas and chromophobe renal cell carcinomas where negative for the same markers.[65] PAX-2 has important role in nephrogenesis, it promotes cell proliferation and is inhibitor of apoptosis enabling cancer progression, this kidney specific transcription factor is expressed with high intensity in most of the renal tumors except sarcomatoid type of RCC.[66] Almost all cases of clear cell RCC and papillary RCC express a cell surface glycoprotein –CD10 and papillary RCC particularly expresses α- Methyl acyl coenzyme A Racemase. While chromophobe RCCs and oncocytomas are positive for cell cell adhesion molecules such as E-Cadherin and kidney specific cadherin, Parvalbumin, claudin 7, 8, and CD117, the clear cell and papillary RCCs are negative for the same immunohistochemical markers.[57]

3.6: Annexins: Annexins are a family of twenty members of calcium and phospholipid-binding proteins.[67] They are designated as *ANX* (human) or *Anx* (other species) and have synonyms such as lipocortins, calpactins, endonexins, synexin, anchorin, calcimedins, calelectrins, calcyclin-associated protein-50, calphobindins, placental or vascular anticoagulant proteins, p35, p36 and p68.[68] The members of the annexin family consists of 70-amino acid sequence that repeats four or eight times. These annexin repeats forms a compact core domain preceded by a unique amino-terminal region. The N terminal domain is capable of undergoing changes such as phosphorylation and acylation and also form binding sites for other proteins.[69] The NH2-terminal domains are unique in sequence, length, and structural fold and thus specific for a given member of the annexin family. The NH2-terminal domains can be extrapolated and its location at the concave side of the curved disc of the core enables the NH2-terminal domains to engage specific interactions. The 12 annexins identified in the human species show a tissue and cell specific localization.[70] The annexins are not only identified in the vertebrates but are also found in the metazoans, fungi, moulds and plants.[71]

Functionally they have been associated with membrane-membrane, membrane cytoskeletal binding, calcium regulated exocytosis, endocytosis and also proposed to function with respect to RNA binding and nucleotide binding activities.[72]

Annexins are thought to be involved in various mechanisms such as regulation and binding of extracellular matrix molecules and regulation of inflammatory processes, vesicular transport. Annexins (not all) are linked with phosphorylation in certain metabolic states of cells. Since they are expressed in various types of cells and their expression varies from cell to cell and also species variation their precise function is

currently not known. Annexin derives its name from the term to Annex which refers to linking or binding to various structural domains and further facilitate static and dynamic interactions based on their subcellular localization from nuclear, to cytosol, to membrane and extracellular localization. They were detected for the first time during untargeted approaches such as response to cellular stress. The probable functions of the annexins suggests that annexin research still needs to be expanded and explored for newer innovations targeting annexins for their role seems to be exciting in various cell types.[67]

3.6A: AnnexinA1 and AnnexinA2 in kidney and renal tumor development: AnxA1 (p35) is developmentally regulated in embryos and has restricted expression in adults. It is an important endogenous anti-inflammatory molecule and involved in variety of biological activities, including regulation of cell proliferation, cell death signaling and promoting efficient phagocytosis of apoptotic cells.[73] Immunohistochemistry studies of normal rat kidney showed that AnxA1 is enriched in epithelia of Bowman's capsule, the macula densa, and medullary/papillary collecting ducts, suggesting that is related to specialized renal functions.[74] Studies also reveal that AnxA1 can act as a second messenger in glucocorticoid –induced cellular responses, which may be important for kidney development and recovery from physiological stresses.[70]

AnxA1 has been reported to be overexpressed in conventional renal cell carcinomas. Several reports have demonstrated that cytoplasmic AnxA1can be induced to translocate into the nucleus by treatment with specific stimuli such as PMA (phorbol 12-myristate 13-acetate) suggesting the role of AnxA1 in cellular proliferation and tumor invasion.[75] Conventional Renal Cell Carcinomas are often composed of clear or eosinophilic cells to varying extent, and the amount of eosinophilic cells is thought to indicate the degree of

differentiation. A study by U. Zimmermann et al demonstrated a significant correlation between amount of eosinophilic cells, AnxA1 score and Fuhrman grade. Therefore expression of AnxA1 being most probably a feature of eosinophillic cells and thus characterized the grade of tumor differentiation. AnxA1 could serve as a useful prognostic marker for outcome of RCC. However, the biological function of AnxA1 in renal cancer cells remains unclear.[76]

3.6B: AnxA2 in kidney and renal tumors: AnxA2, calcium binding cytoskeletal protein is expressed in some of the tumor cells, endothelial cells, macrophages and mononuclear cells. It participates in phagocytosis, fibrinolysis and anticoagulation, angiogenesis and cell metastasis.[70] AnxA2 and related proteins are also known to regulate apoptosis and cell proliferation (figure 6).[77] AnxA2 has been implicated in wide range of physiological activities such as membrane organization, membrane transport events, required for the establishment or maintenance of epithelial polarity. AnxA2, a 36kDa protein is located in the chromosome 15q22.2 has been reported to play roles in exocytosis, endocytosis, and membrane trafficking. Knockdown of AnxA2 inhibits cell division and proliferation.[78]

In the kidneys, it participates in the membrane domain and cortical actin dynamics during establishment of cell to cell contacts in polarized monolayers of kidney epithelial cells.[75]

As for the role of AnxA2 in cancer, recent studies show that AnxA2 interacts with plasminogen and tissue plasminogen activator, which converts plasminogen to plasmin, thus mediating the lysis of fibrin polymers and extracellular matrix degradation and promotes cell invasion and migration.[70]

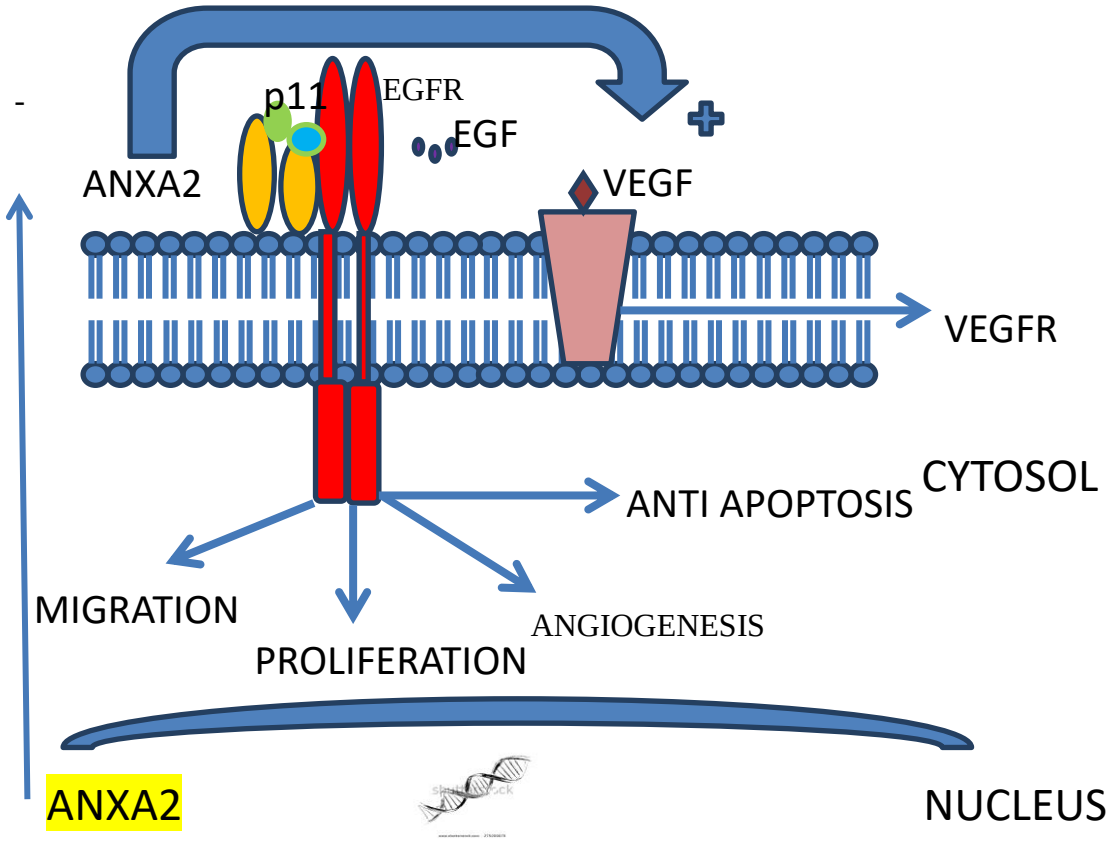

Figure 6: Regulation of epidermal growth factor receptor (EGFR) mediated downstream signaling functions of AnxA2.

Several studies relate AnxA2 to the regulation of membrane domain and cortical actin dynamics during the establishment of cell-cell contacts in polarized monolayers of kidney epithelial cells. Hansen et.al showed direct interaction of AnxA2 with Rac1 containing

complexes, which are involved in membrane dynamics of developing cell to cell contacts in epithelial cells.[75] A comparative proteomic analysis of pig kidney conducted by Young-Joo Jeon et.al, suggested the expression of AnxA2 and AnxA4 decreased during early development (E40), but slightly increased during late development (E93) than early developmental stage.[77]

The expression and the subcellular localization of Anx I, II, IV, & XIII in renal epithelial cells were investigated by Sandra Tribolo et.al, using immunological techniques. AnxA2 was detected in the collecting duct cells and was distributed in stippled pattern along the plasma membrane, where as in the proximal tubular cells this annexin was absent. However, when these cells were grown in primary culture, they were found to express Annexins I, II, IV, & V. As well as being located along the basolateral membrane, AnxI & II are also present on vesicles, which suggest that these Annexins may be involved in vesicular traffic under cell culture conditions.[79] A study conducted by Keith Alvares et.al, showed the binding of dentin phosphoprotein with Anx2 and 6 present in rat ureteric bud cell line and their colocalization restricted to the cell membrane of the ureteric bud branches of embryonic metanephric kidney. Hence postulating its role in calcium transport.[80]

A study conducted by Y Ohno et.al, on the expression of AnxA2 in primary renal cell carcinoma tissues showed upregulation of AnxA2 at both mRNA and protein levels in 14 of 18 primary RCC tissues in comparison to the corresponding normal renal tissues. Immunohistochemical analysis of 154 primary RCCs and 24 metastatic tumors was performed. The AnxA2 staining was observed on the cell membranes or in the cytoplasm. The AnxA2 staining was heterogeneous in the tumor sections, and the staining intensity

was relatively higher in the periphery of the tumor and around the vessels.[81] AnxA2 was upregulated in primary clear cell renal carcinoma cells compared with normal renal cortex. 47.4% of primary clear cell RCCs tissue and 87.5% of metastatic tumors were positive for AnxA2.[78] Immunohistochemistry analysis conducted by Shun-Fa Yang et al, showed that the AnxA2 expression level was generally elevated to various degrees in RCC tissues. In adjacent non-cancerous tissues AnxA2 was mainly expressed in glomeruli and slightly expressed in the cytoplasm of proximal tubules. AnxA2 expression level found in RCC was prominently expressed in cancer cell membranes.[75]

A study aimed at expression analysis of AnxA2 a natural ligand of S100A10 conducted by Tomohiro Domoto et.al, showed the immunolocalization of S100A10 and AnxA2 was basically the same, regardless of whether the kidney tissue was non-neoplastic or cancerous. In non-neoplastic kidney tissues, positive reactions were clearly found in the collecting duct system, the thin portion of Henle's loop and Bowmann's capsule. Moreover reaction was also observed in many of the distal convoluted tubules, a part of the thick portion of the Henle's loop and some glomerular podocytes and few of the proximal tubules showed reaction. The immunoreactivity in the tubular epithelia showed a membranous especially luminal staining pattern with or without faint, granular cytoplasmic staining. In RCC samples, immunopositivity was also seen on the plasma membrane with some faint cytoplasmic staining. In addition positive reactions were found in endothelial cells in non-neoplastic and cancerous tissues.[82]

Vivek Kumar et.al, demonstrated the presence of AnxA2 on apical surface of renal epithelial cells (MCCK cell line) which avidly binds calcium oxalate monohydrate crystals and thus could promote crystal retention and possibly kidney stone formation.[83]

Analysis of mRNA expression of intracellular calcium and phospholipid binding proteins in acute tubular necrosis induced mice demonstrated selective expression of AnxA2 in the renal cortex with marked elevation in embryonic day 3 and gradual decline on day 7 and further attenuation on day 14. On immunohistochemistry and western blot analysis the expression was increased and reached the peak level on day 7 and then gradually declined on day 14. Collectively these findings demonstrate that AnxA2 expression, initiated in response to tubular injury, persist in parallel throughout the recovery process of tubular cells in acute renal failure.[84]

Although previous studies on AnxA1 and AnxA2 expression have dealt with tumor pathology in an attempt to identify their possible role as a biomarker in the differential diagnosis of various carcinomas, little attention though has been paid on their possible role in normal and embryonic renal tissue. Taking into account of these reports, the calcium binding property and their involvement in various biological processes, it would be expected that AnxA1 and AnxA2 could appear during the developmental stages of nephrogenesis. Comparative proteomic analysis of the developing human kidney not only contributes to the better understanding of human nephrogenesis but can also be used as a complimentary approach in the study of biomolecular mechanisms of various renal diseases and carcinomas. Hence the present study aims at investigating the expression pattern of AnxA1 and AnxA2 in various renal structures of developing human fetal kidneys at different gestational ages and compares their expression pattern with those observed in adult renal tissues and renal carcinomatous tissues.

References:

1. Čukuranović Rade and Vlajković Slobodan. Age related anatomical and functional characteristics of human kidney. Med Biol 2005; 129(2):61-69.
2. Yosypiv IV and Song R. Development of the kidney medulla. Organogenesis 2012; 8(1):10-17.
3. Lacson A, Bernstein J, Risdon RA, Gilbert-Barness E. Renal system. In: Gilbert-Barness E (ed). Potter's Pathology of the Fetus, Infant and Child. 2nd ed. China:Mosby Elsevier; 2007.p.1281-344.
4. Leroy X, Devisme L, Buisine MP, et al. Expression of human mucin genes during normal and abnormal renal development. Am J Clin Pathol 2003; 120(4):544-550.
5. Sulemanji M, Vakili K. Neonatal renal physiology. Semin Pediatr Surg 2013; 22(4):195-198.
6. Lindström NO, McMahon JA, Guo J. Conserved and Divergent Features of Human and Mouse Kidney Organogenesis. J Am Soc Nephrol 2018; 29(3): 785–805.
7. Woolf AS, Jenkins D. Development of the kidney. In: Charles JJ , Olson JL, Schwartz MM, Silva FG, eds. Hepstinstal Pathology. 6th ed. Philadelphia: Lippincott Williams & Wilkins; 2007.p.71-95.
8. Sadler TW, Langman J. Langman's medical embryology. 13th ed. Philadelphia: Lippincott Williams & Wilkins; 2016.
9. Moore KL, Persaud TVN, editors. The developing human: clinically oriented embryology, 8th ed. Philadelphia: Saunders, 2008:244-260.
10. Haugan BM, Halberg KA, Jespersen A, Prehn LR, Møbjerg N. Functional characterization of the vertebrate primary ureter: structure and ion transport

mechanisms of the pronephric duct in axolotl larvae (Amphibia). *BMC Dev Biol*. 2010;10:56.

11. Brändli AW. Towards a molecular anatomy of the Xenopus pronephric kidney. *Int J Dev Biol*. 1999;43(5):381-395.
12. Drummond IA, Majumdar A, Hentschel H, et al. Early development of the zebrafish pronephros and analysis of mutations affecting pronephric function. *Development*. 1998;125(23):4655-4667.
13. Møbjerg N, Larsen EH, Jespersen A. Morphology of the kidney in larvae of Bufo viridis (Amphibia, Anura, Bufonidae). *J Morphol*. 2000;245(3):177-195.
14. Vize PD, Seufert DW, Carroll TJ, Wallingford JB. Model systems for the study of kidney development: use of the pronephros in the analysis of organ induction and patterning. *Dev Biol*. 1997;188(2):189-204.
15. Zhou W, Boucher RC, Bollig F, Englert C, Hildebrandt F. Characterization of mesonephric development and regeneration using transgenic zebrafish. *Am J Physiol Renal Physiol*. 2010;299(5):F1040-F1047.
16. Zhou X, Vize PD. Proximo-distal specialization of epithelial transport processes within the Xenopus pronephric kidney tubules. *Dev Biol*. 2004;271(2):322-338.
17. Tran U, Pickney LM, Ozpolat BD, Wessely O. Xenopus Bicaudal-C is required for the differentiation of the amphibian pronephros. *Dev Biol*. 2007;307(1):152-
18. Eid SR, Brändli AW. Xenopus Na,K-ATPase: primary sequence of the beta2 subunit and in situ localization of alpha1, beta1, and gamma expression during pronephric kidney development. *Differentiation*. 2001;68(2-3):115-125.

19. Kumano T, Konno N, Wakasugi T, Matsuda K, Yoshizawa H, Uchiyama M. Cellular localization of a putative Na(+)/H (+) exchanger 3 during ontogeny in the pronephros and mesonephros of the Japanese black salamander (Hynobius nigrescens Stejneger). *Cell Tissue Res*. 2008;331(3):675-685.
20. Uochi T, Takahashi S, Ninomiya H, Fukui A, Asashima M. The Na+,K+-ATPase alpha subunit requires gastrulation in the Xenopus embryo. *Dev Growth Differ*. 1997;39(5):571-580.
21. Georgas KM, Chiu HS, Lesieur E, Rumballe BA, Little MH. Expression of metanephric nephron-patterning genes in differentiating mesonephric tubules. *Dev Dyn*. 2011;240(6):1600-1612.
22. Carroll TJ, Park JS, Hayashi S, Majumdar A, McMahon AP. Wnt9b plays a central role in the regulation of mesenchymal to epithelial transitions underlying organogenesis of the mammalian urogenital system. *Dev Cell*. 2005;9(2):283-292.
23. Grieshammer U, Cebrián C, Ilagan R, Meyers E, Herzlinger D, Martin GR. FGF8 is required for cell survival at distinct stages of nephrogenesis and for regulation of gene expression in nascent nephrons. *Development*. 2005;132(17):3847-3857.
24. Stark K, Vainio S, Vassileva G, McMahon AP. Epithelial transformation of metanephric mesenchyme in the developing kidney regulated by Wnt-4. *Nature*. 1994;372(6507):679-683.
25. Torres M, Gómez-Pardo E, Dressler GR, Gruss P. Pax-2 controls multiple steps of urogenital development. *Development*. 1995;121(12):4057-4065.
26. Kobayashi A, Shawlot W, Kania A, Behringer RR. Requirement of Lim1 for female reproductive tract development. *Development*. 2004;131(3):539-549.

27. Wang Q, Lan Y, Cho ES, Maltby KM, Jiang R. Odd-skipped related 1 (Odd 1) is an essential regulator of heart and urogenital development. *Dev Biol*. 2005;288(2):582-594.
28. Nishinakamura R, Matsumoto Y, Nakao K, et al. Murine homolog of SALL1 is essential for ureteric bud invasion in kidney development. *Development*. 2001;128(16):3105-3115.
29. Xu PX, Zheng W, Huang L, Maire P, Laclef C, Silvius D. Six1 is required for the early organogenesis of mammalian kidney. *Development*. 2003;130(14):3085-3094.
30. Sainio K, Hellstedt P, Kreidberg JA, Saxén L, Sariola H. Differential regulation of two sets of mesonephric tubules by WT-1. *Development*. 1997;124(7):1293-1299
31. Standring S. Gray's anatomy: the anatomical basis of clinical practice, 41st ed. Philadelphia:Elsevier; 2016:1199-1208.
32. Sadler TW, Langman J. Langman's Medical Embryology. 11th ed. Philadelphia: Lippincott Williams & Wilkins; 2010:235-263.
33. Reidy KJ, Rosenblum ND. Cell and molecular biology of kidney development. *Semin Nephrol*. 2009;29(4):321-337.
34. Sariola H. Interspecies chimeras: an experimental approach for studies on embryonic angiogenesis. *Med Biol*. 1985;63(2):43-65.
35. Eremina V, Baelde HJ, Quaggin SE. Role of the VEGF--a signaling pathway in the glomerulus: evidence for crosstalk between components of the glomerular filtration barrier. *Nephron Physiol*. 2007;106(2):p32-p37.

36. Daković-Bjelaković M, Stefanović N, Vlajković S, Rade C, Antić S, Goran B et al. HUMAN KIDNEY DEVELOPMENT. Acta Facultatis Medicae Naissensis. 2004;21(3):163-170.
37. Dormoy, V, Jacqmin D, Lang H, Massfelder T. From development to cancer: lessons from the kidney to uncover new therapeutic targets. Anticancer Res2012;32(9):3609–3617.
38. Patel RS and Dressler RG. The Genetics and Epigenetics of Kidney Development. Semin Nephrol 2013 July; 33(4): 314–326.
39. Kobayashi A, Kwan KM, Carroll TJ, McMahon AP, Mendelsohn CL, Behringer RR. Distinct and sequential tissue-specific activities of the LIM-class homeobox gene Lim1 for tubular morphogenesis during kidney development. *Development*. 2005;132(12):2809-2823.
40. Drews C, Senkel S, Ryffel GU. The nephrogenic potential of the transcription factors osr1, osr2, hnf1b, lhx1 and pax8 assessed in Xenopus animal caps. *BMC Dev Biol*. 2011;11:5.
41. Cirio MC, Hui Z, Haldin CE, et al. Lhx1 is required for specification of the renal progenitor cell field. *PLoS One*. 2011;6(4):e18858.
42. Venegas-Ferrín M, Sudou N, Taira M, del Pino EM. Comparison of Lim1 expression in embryos of frogs with different modes of reproduction. *Int J Dev Biol*. 2010;54(1):195-202.
43. Davies JA, Perera AD, Walker CL. Mechanisms of epithelial development and neoplasias in the metanephric kidney. Int J Dev.Biol 1999; 43:473-478.

44. Holland PW, Booth HA, Bruford EA. Classification and nomenclature of all human homeobox genes. *BMC Biol*. 2007;5:47.

45. Kreidberg JA, Sariola H, Loring JM, Maeda M, Pelletier J, Housman D, Jaenisch R. WT-1 is required for early kidney development. Cell 1993; *74*:679-691.

46. Md Zin R, Murch A and Charles A. Pathology, genetics and cytogenetics of Wilms' tumour. Pathology 2011;*43*: 302-312.

47. Sourbier C, Danilin S, Lindner V, et al. Targeting the nuclear factor-kappaB rescue pathway has promising future in human renal cell carcinoma therapy. Cancer Research. 2007 Dec;67(24):11668-11676.

48. Sourbier C, Lindner V, Lang H, et al. The phosphoinositide 3-kinase/Akt pathway: a new target in human renal cell carcinoma therapy. *Cancer Res*. 2006;66(10):5130-5142.

49. Huang D, Ding Y, Luo WM, et al. Inhibition of MAPK kinase signaling pathways suppressed renal cell carcinoma growth and angiogenesis in vivo. Cancer Research. 2008 Jan;68(1):81-88.

50. Gill PS and Rosenblum ND. Control of murine kidney development by sonic hedgehog and its GLI effectors. Cell Cycle 2006;5:1426-1430.

51. T Nadasdy, Z Lajoie. Cell proliferation in the developing human kidney. Pediatric and developmental pathology 1998;1:49-55.

52. Ho J. The regulation of apoptosis in kidney development: implications for nephron number and pattern? Front Pediatr 2014;2:128.

53. Michos O. Kidney development: from ureteric bud formation to branchingmorphogenesis.*Curr Opin Genet Dev* 2009 October; 19(5): 484–490.

54. Vasilyev A, Liu Y, Mudumana S, Mangos S, Lam P-Y, et al. Collective cell migration drives morphogenesis of the kidney nephron. PLoS Biol2009; 7(1): e1000009.

55. Satu Kuure, Reetta Vuolteenaho, Seppo Vainio. Kidney morphogenesis: cellular and molecular regulation, Mechanisms of Development 2000; 92: 31-45.

56. El-Shorbagy S, Alshenawy H. Diagnostic utility of vimentin, CD117, cytokeratin-7 and caveolin-1 in differentiation between clear cell renal cell carcinoma, chromophobe renal cell carcinoma and oncocytoma. J Microsc Ultrastruct 2017;5(2):90-96.

57. Truong LD, Shen SS. Immunohistochemical diagnosis of renal neoplasms. Arch Pathol Lab Med 2011; 135(1):92-109.

58. Walter Henriques da Costa, Rafael Malagoli Rocha, Isabela Werneck da Cunha, Gustavo Cardoso Guima-raes, Stenio de Cassio Zequi. Immunohistochemical expression of CD44s in renal cell carcinoma lacks independent prognostic significance. Int Braz J Urol. 2012; 38 (4):456-65.

59. Maclennan GT, Cheng L. Neoplasms of the kidney. In: Bostwick DG, Cheng L. (eds.) Urologic Surgical Pathology. Philadelphia:Elsevier Saunders; 2014. p.76-155.

60. Fuhrman S, Lasky LC, Limas L. Prognostic significance of morphologic parameters in renal cell carcinoma. Am J Surg Pathol 1982;6:655-663.

61. Edge SB, Byrd DR, Compton CC, et al. American Joint Committee on Cancer staging manual. New York: Springer, 2010.
62. Kuroda N, Tanaka A, Ohe C, et al. Recent advances of immunohistochemistry for diagnosis of renal tumors. Pathol Int 2013; 63(8):381-390.
63. AyhanOzcan. Immunoprofiles of adult renal epithelial tumors: Immunohistochemistry is still essential for diagnosis of renal tumors (a comprehensive update. J Interdiscip Histopathol 2015;3(3):81-101.
64. Hillary Ross MD, Guido Martignoni MD, Pedram Argani MD. Renal Cell Carcinoma With Clear Cell and Papillary Features. Arch Pathol Lab Med 2012;136:391–399.
65. Mjønes PG, Nordrum IS, Qvigstad G, Sørdal Ø, Rian LL, Waldum HL. Expression of erythropoietin and neuroendocrine markers in clear cell renal cell carcinoma. APMIS 2017;125 (3):213-222.
66. Ozcan A, Zhai J, Hamilton C, et al. PAX-2 in the diagnosis of primary renal tumors : Immunohistochemical comparison with renal cell carcinoma marker antigen and Kidney-Specific cadherin. Am J Clin Pathol 2009; 131(3):393-404.
67. Mollenhauer J. Multi-author ReviewAnnexinsAnnexins: what are they good for? CMLS, Cell. mol. life sci 1997; 53:506–507.
68. Morgan RO and Ferna´ndez MP. Annexin gene structures and molecular evolutionary genetics. CMLS, Cell. mol. life sci 1997; 53: 508–515.
69. Rita Dreier, Kurt Werner SchmidVolker Gerke, Kristina Riehemann. Differential expression of annexins I, II and IV in human tissues: an immunohistochemical study.Histochem Cell Biol 1998; 110:137–148.

70. Markoff A, Gerke V. Expression and functions of annexins in the kidney. Am J Physiol Renal Physiol 2005; 289:F949-56.
71. Mussunoor S and Murray GI. Review ArticleThe role of annexins in tumour developmentand progression. *J Pathol* 2008; 216: 131–140.
72. Ursula Rescher and Volker Gerke. Annexins – unique membrane binding proteins with diverse functions. Journal of Cell Science 2004;117:2631-2639.
73. Ka SM, Tsai PY, Chao TK, Yang SM, Hung YJ, Chen JS, Shui HA, Chen A. Urine annexin A1 as an index for glomerular injury in patients. Dis Markers 2014:1-12.
74. James A. McKanna, Aporn Chuncharunee, Karen A. Munger, Julia A. Breyer, Stanley Cohen and Raymond C. Harris et al. Localization of p35 (annexinI, lipocortin I) in normal adult rat kidney and during recovery from ischemia. Journal of cellular Physiology 1992; 153(3):467-76.
75. Shun-Fa Yang, Han-LinHsu, Tai-Kuang Chao, Chia- Jung Hsiao, Yung-Feng Lin, Chao-Wen Cheng et al. Annexin A2 in renal cell carcinoma :Expression, function, and prognostic significance. Urologic Oncology: Seminars and Original Investigations 2015;33: 22.e11–22.e21.
76. U. Zimmermann, C. Woenckhaus, S. Teller, S. Venz, M. Langheinrich, C. Protzel, S. Maile, H Junker and J. Giebel et al. Histology and Histopathol 2007; 22: 527-34.
77. Young-Joo Jeon, Jumi Kim & Jung-Il Chae. Comparative proteomic analysis of kidney development-related proteins in the pig. In Vitro Cell. Dev. Biol—Animal 2013; 49:315–23.

78. Chi-YunWang and Chiou-Feng Lin. Annexin A2: Its Molecular Regulation and Cellular Expression in Cancer Development. Disease Markers 2014;2014:10 pages. Available from: http://dx.doi.org/10.1155/2014/308976.

79. Sandra Tribolo, Suzanne Maroux and Dominique Massey-Harroche. Biochemistry and Cell Biology2000;78: 495-502.

80. Keith Alvares, Paula H. Stern and Arthur Veis. Dentin Phosphoprotein Binds Annexin 2 and Is Involved in Calcium Transport in Rat Kidney Ureteric Bud Cells.The journal of biological chemistry 2013; 28: 13036–45.

81. Y Ohno, M Izumi, T Kawamura, T Nishimura, K Mukai and M Tachibana. Annexin II represents metastatic potential in clear-cell renal cell carcinoma. British Journal of Cancer 2009; 101:287 – 94.

82. Domoto T, Miyama Y, Suzuki H, Teratani T, Arai K, Sugiyama T,Takayama T, Mugiya S, Osono S, Nozawa R et al. Evaluation of S100A10, annexin II and B-FABP expression of markers for renal cell carcinoma. Cancer Sci 2007; 98(1):77-82.

83. Vivek Kumar, Gerrard Farell, Sergio Deganello and John C. Lieske. AnnexinII is present on Renal Epithelial Cells and Binds Calcium Oxalate Monohydrate Crystals. J Am Soc Nephrol 2003; 14:289-97.

84. Chao-Wen Cheng, Abdalla Rifai, Shuk-Man Ka, Hao Ai Shui, Yuh Feng Lin, Wei Hwa Lee and Ann Chen et al. Calcium-binding proteins annexin A2 and S100A6 are sensors of tubular injury and recovery in acute renal failure. Kidney International 2005;68:2694-2703.

Chapter 4

MATERIALS AND METHODS

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

4. Materials and Methods:

4.1: TYPE OF STUDY : PROSPECTIVE STUDY.

4.2: STUDY DESIGN : OBSERVATIONAL EXPLORATIVE STUDY

4.3. DURATION OF COLLECTION OF DATA:

Prospective study –From 1st June 2016 to 30th Nov 2019

4.4: PLACE OF CONDUCT OF RESEARCH:

Central research laboratory**,** SDM College of Medical Sciences and Hospital, Dharwad.

4.5: STUDY POPULATION: Paraffin embedded renal tissues collected from archives of the Department of Pathology comprised the following:

- Forty two fetal renal tissue samples of gestational age ranging from 14 weeks to 39 weeks, thirty seven renal cancer tissue (biopsy) samples.
- Thirty seven normal adult renal tissues were collected by cadavers donated to the department of Anatomy fixed in 10% formalin and were subjected to tissue processing.

4.6: SAMPLE SIZE : With 95% confidence level, anticipated prevalence of renal cell carcinoma as 3.8% (WHO-GLOBOCAN 2012: Estimated Cancer Incidence, Mortality and Prevalence Worldwide) and desired precision as $\pm$5%, the minimum sample size is 37. The total sample size is 111. Thirty seven (37) samples each of fetal renal tissue, adult renal tissue and renal carcinomatous tissue.

The formula used to calculate the sample size

$$n=\frac{Z^2 P (1-P)}{d^2}$$

n=sample size

Z=Z statistic for a level of confidence (1.96)

p=expected prevalence

p=0.038

d=precision

=0.05

A minimum number of 111 cases coming under the inclusion criteria during the study period were included in the study.

4.7 SELECTION CRITERIA:

4.7A: INCLUSION CRITERIA: Renal tissues from aborted and dead human fetuses of gestational age ranging from 14-39 weeks. Fetal kidneys, which appeared macroscopically normal with no congenital malformations.

Biopsy specimens obtained from patients of all ages with renal carcinoma received by the Department of Pathology, SDM College of Medical Sciences and Hospital.

4.7B: EXCLUSION CRITERIA: Patient refusal, inadequate and inconclusive tissue material, renal tissues from autolysed, calcified, haemorrhagic kidneys, hypoplastic kidneys, renal tissues from the kidneys of anomalous fetus, malignant lesions having received chemotherapy or radiotherapy.

4.8. PLAN OF STATISTICAL ANALYSIS :

Chi-square (χ 2) test was employed to determine the significance of differences between groups for categorical data by Graphpad Prism7 software. The statistical significance was set at 5% level of significance ($p \leq 0.05$, $\alpha < 0.05$). The group data was compared using one way ANOVA.

Ethical clearance was obtained by the Institutional Ethical Committee of BLDE (Deemed to be University) (IEC No-182/2016-17, dated-13-10-2016) and SDM College of Medical Sciences & Hospital (IEC No-0747; 2016, dated 20-6-2016). For prospective cases paraffin embedded tissue blocks of biopsy specimens of renal carcinomatous tissue and autopsied fetal kidneys were availed from the department of pathology, adult kidneys were obtained from cadavers donated to department of Anatomy. However with due consent from the patient /attenders and parent-mother of the deceased fetus. The consent was taken for the use of tissues for the purpose of research after explaining in detail in a language (English / Kannada) understood by them.

4.9. DATA COLLECTION PROCEDURE:

The present work envisaged the expression of two closely related calcium sensitive phospholipid binding proteins AnxA1 and AnxA2 in fetal, adult and renal tumor tissues. The localization of these proteins were demonstrated and assessed by immunohistochemistry.

Parameters estimated: Immune Markers: AnxA1 and AnxA2.

Paraffin embedded renal tissues collected from archives of the Department of Pathology comprised the following: Forty two fetal renal tissue samples of gestational age ranging from 14 weeks to 39 weeks, thirty seven renal cancer tissue samples. Thirty seven normal adult renal tissues were collected by cadavers donated to the department of Anatomy and were subjected to tissue processing. The gestational age of the fetus as calculated by obstetrical methods were obtained from clinical records. The ultra sound scanning and anomaly scanning reports from maternal clinical records were reviewed to exclude kidney associated congenital anomalies.[1] The metanephric stage of kidney development was studied which included stage 2 (14-22weeks, n=15), stage 3 (22-36weeks, n=13) and stage 4 (36 weeks onwards that continues to adult life, n=14).[2] In the first stage the ureteric bud branches for six to eight generations inducing the formation of new nephrons.[2] Fetal kidneys at this stage were not included in the current study due to difficulty in obtaining the renal tissue from the aborted fetuses.[2] All the fetal and adult renal tissues underwent histopathological assessment to exclude any microscopic abnormalities and affirm the histological diagnosis of tumors. The morphology of glomerular cells was better assessed by subjecting the renal tissues to PAS staining. Thirty seven renal tumor tissues included clear cell renal cell carcinoma (CCRCC), papillary renal cell carcinoma, chromophobe renal cell carcinoma, collecting duct carcinoma and oncocytoma. Tumors were graded according to Fuhrman et al.[3]

5µ thick sections were obtained from the paraffin embedded tissue blocks and stained with haematoxylin and eosin to study the a) histogenesis of fetal kidney in various phases of fetal development. b) normal histological architecture of mature kidney c) types of

renal cell carcinomas and confirm the original diagnosis, before performing immunohistochemistry.

4.9A: Immunohistochemistry:

3µ thick sections were obtained from formalin fixed and paraffin embedded renal tissues. Immunohistochemical staining was performed using avidin biotin (vector labs) immune peroxidase method.[4] The tissue sections were de-waxed at 65^0C for 2 hrs in incubator followed by immersing the slides in xylene for 10 min, xylene was replaced 4 times to complete the dewaxing. The sections were rehydrated by incubating them in descending grades of ethanol (100%, 95%, 80% and 70%) and washing the sections in double distilled water for 5min each. This was followed by treating them with 0.01 M of citrate buffer (prewarmed for 5min) for 45 min at 100^0 C for unmasking the antigen. The endogenous peroxidase activity was blocked by incubating the renal tissue with 0.3% hydrogen peroxide (1:100 dilutions in methanol). Nonspecific binding sites were blocked by incubating the sections with normal horse serum (vector laboratories). The sections were incubated overnight with primary antibody against AnxA1 (DIL 1:100, Santa Cruz Biotechnology Inc; SantaCruz, CA Catalogue No. 12740). As a negative control, anti-mouse IgG whole molecule (Sigma– Aldrich) was used at 1:1000 dilution. This was followed by sequentially incubating the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse raddish peroxidase complex (ABC; VECTOR).[5] The antigen of interest was detected by use of a 3, 3^1-diamino benzidine (DAB) chromogen and by counterstaining with haematoxylin (table 4). The immunohistochemical detection of AnxA2 was performed as described previously.[6] Primary antibody against AnxA2 (1:100 dilutions) was purchased from Santa Cruz

Biotechnology Inc; (SantaCruz, CA catalogue no- Annexin II (H-50): sc- 9061). AnxA2 primary antibody was replaced by anti-rabbit IgG whole molecule (Sigma– Aldrich, USA, catalogue no- A0545) at 1:1000 dilution as negative control while triple negative breast cancer cell slides were used as positive controls vowing to the previous studies in our laboratory confirming the expression of AnxA2 in breast cancer cells. The tissues were evaluated under light microscope with Lieca image centre. IHC-stained samples were evaluated by two pathologists and all samples were blinded. The localization of AnxA1 and AnxA2 protein was counted in 10 random fields across the cortex and medulla of renal tissues. Tissue sections stained with Anti AnxA1 antibody and Anti AnxA2 were graded based on a) patterns of their expression as M-membranous, C–cytoplasmic, N–nuclear, Mx- Mixed (cytoplasmic and membranous).[7] b) Percentage of immunoreactive cells (0=no staining, 1-10% as 1, 11-50% as 2, 51-70% as 3, 71-100% as 4),[8] c) intensity of staining 0= no color, (1+) weak brown, (2+) moderate brown, (3+) dense brown, strong expression.[8] The intensity and percentage scores were multiplied. Score of more than three was considered significant.

Table 4: Immunohistochemistry was performed by following protocol

Deparaffinise the tissue sections at 65^0 C for 2 hours in incubator. This is followed by immersing the slides in xylene for 10 min. Xylene is replaced four times to complete dewaxing. This is followed by rehydration.
(Rehydration)
Rehydrate the the sections by incubating the slides in descending grades of alcohol (100%, 95%, 80%, 70% of ethanol) 5min each. Wash the sections in double distilled water for 5min, followed by antigen retrieval.
(Antigen Retrieval)
Treat the sections with 0.01 M of citrate buffer (prewarmed for 5min) for 45 min at 100^0 C for unmasking the antigen before blocking.
(Blocking)
The endogenous peroxidase activity is blocked by incubating the renal tissue with 0.3% hydrogen peroxide (1:100 dilutions in methanol). Nonspecific binding sites are blocked by incubating the sections with normal horse serum (vector laboratories). Followed by incubation with primary antibody.
(Treatment with primary antibody)
The sections are incubated overnight with primary antibody against AnxA1 (DIL 1:100, Santa Cruz Biotechnology Inc; SantaCruz, CA Catalogue no. 12740). Further treated with secondary antibody.
(Treatment with secondary antibody)
Sequentially incubate the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA) and avidin biotin horse raddish peroxidase complex (ABC; VECTOR).
(Detection)
The antigen of interest is detected by use of a 3, 3^1-diamino benzidine (DAB) chromogen and by counterstaining with haematoxylin
(Observation)
Sections observed and evaluated using light microscope (leica image centre)

4.10: Statistical analysis:

Chi-square (χ 2) test was employed to determine the significance of differences between groups for categorical data by Graphpad Prism7 software. The statistical significance was set at 5% level of significance ($p \leq 0.05$, $\alpha < 0.05$). The expression of AnxA1 in the cells of mature glomeruli at various gestational ages of fetus and mature adult kidney were compared. The expression of AnxA2 in the collecting duct and collecting tubular cells of fetal kidney at various gestational ages of fetus and mature adult kidney were compared. AnxA1 and AnxA2 expression was tested on non-neoplastic tissue and clear cell renal cell carcinoma. Data were calculated as average percentage of AnxA1 and AnxA2 expressing cells in the proximal tubular cells of non-neoplastic adult tissues against tumor cells of clear cell renal cell carcinoma at different tumor grades. The expression of AnxA2 in the proximal tubular cells of fetal kidney was compared to the expression status in that of adult and tumor cells of clear cell renal cell carcinoma using One-way ANOVA.

References:

1. Chikkannaiah P, Roy M, Kangle R, Patil PV. Glomerulogenesis: can it predict the gestational age? a study of 176 fetuses. Indian J Pathol Microbiol. 2012;55:303–307.
2. Lacson A, Bernstein J, Risdon RA, Gilbert-Barness E. Renal system. In: Gilbert-Barness E, editor. Potter's pathology of the fetus, infant and child. 2nd ed. Philadelphia: Elsevier Mosby, 2007:1281–1344.
3. Eble NJ, Grignon JD, Young HR. Tumors of the urinary tract. In: Fletcher MDC, editor. Diagnostic histopathology of tumors. 4th ed. Philadelphia: Elsevier Saunders, 2013:559-601.
4. Cheng CW, Rifai A, Ka SM, Shui HA, Lin YF, Lee WH, Chen A. Calcium-binding proteins annexin A2 and S100A6 are sensors of tubular injury and recovery in acute renal failure. Kidney Int. 2005;68:2694–2703.
5. Hudkins KL, Giachelli CM, Cui Y, Couser WG, Johnson RJ, Alpers CE. Osteopontin expression in fetal and mature human kidney. J Am Soc Nephrol. 1999;10:444–457.
6. Shetty P, Patil VS, Mohan R, et al. Annexin A2 and its downstream IL-6 and HB-EGF as secretory biomarkers in the differential diagnosis of Her-2 negative breast cancer. Ann Clin Biochem. 2017; 54(4):463-471.
7. Lacunza E, Ferretti V, Barbeito C, Segal-Eiras A, Croce MV. Immunohistochemical evidence of Muc1 expression during rat embryonic development. Eur J Histochem. 2010;54:e49.
8. Kang WY, Chen WT, Huang YC, Su YC, Chai CY. Overexpression of annexin 1 in the development and differentiation of urothelial carcinoma. Kaohsiung J Med Sci. 2012;28:145–150.

Chapter 5

RESULTS

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

5. Results and observation:

The evaluation of AnxA1 immunostaining in the cortical and medullary compartments of the developing kidney was based on the histomorphologic features of renal tissues stained with haematoxylin and eosin.

5.1. Validation of anti AnxA1 antibody for current immunohistochemistry study: To verify the antigen specificity of anti AnxA1 monoclonal antibody (DIL 1:100, Santa Cruz Biotechnology Inc.; Santa Cruz, CA Catalogue No. 12740), we used two different prostate cancer cell lines. Hormone dependent prostate cancer AnxA1 null LnCaP cell line and AnxA1 abundant metastatic prostate cancer PC-3 cell lines. We confirmed the expression using western blot analysis (fig 7a) and immunohistochemistry of these cell lines that showed +3 expressions in PC-3 cells and null in LnCaP cells (fig 7b).

5.2. Mesangial cells demonstrate increased expression of AnxA1 at 14 to 22 weeks of gestational age: The second stage of nephrogenesis is a period of nephron arcade formation and cessation of ureteric bud branching.[1] H & E sections of fetal kidney revealed densely stained undifferentiated mesenchyme cells just beneath the renal capsule and growing ureteric bud lined with cuboidal cells, capped with nephrogenic cells.[2] These cells continued to add newer developing glomeruli, such that the older mature glomeruli were located deeper in the cortex and newer immature forms were located under the capsule (Fig 7c).[3] In between the developing glomeruli within the connective tissue, the developing tubules with eosinophilic cytoplasm were observed. The medulla showed groups of tubules indicating the formation of collecting tubules. Few primitive blood vessels lined with squamous epithelium were appreciated.[2] (Fig 7d).

As shown in figure 7, overall expression of AnxA1 in the fetal kidney was observed more in cytoplasm with little membranous staining and nuclear in fewer cell types. Immature forms of glomeruli such as the renal vesicle (rv), 'S' shaped 'C' capillary loop structures in the nephrogenic zone demonstrated weak expression of AnxA1 in renal tissues from 14 weeks to 22 weeks of gestation. There was no immunostaining in the ureteric bud and cap mesenchymal cells overlying them (Fig 7e). AnxA1 was negative in immature and maturing tubular structures of cortex and medulla (Fig 7f). A striking strong AnxA1 immunoreactivity was observed in the cells of maturing glomeruli in the deeper parts of the cortex. These cells were the mesangial cells, podocytes and endothelial cells as delineated by PAS stain (Fig 7g). Although hematoxylin and eosin stained renal sections enabled the recognition of the glomerular cells such as mesangial, podocytes, parietal epithelial cells and endothelial cells, we used PAS stain that permitted precise resolution of relationship between cells and matrix as it accentuates the matrix and basement membrane constituents.[4] The intra glomerular mesangial cells were found to be embedded within the mesangium, the podocytes were found protruding into the capsular space of Bowman's capsule, investing the outer surface of mesangium and the endothelial cells with oval nuclei lined the inner surface of glomerular capillaries.[4,5] (Fig 7g & 7h). Strong expression of AnxA1 was observed in the mesangial cells and podocytes during early phase from 14-19weeks of gestation. Though there was faint AnxA1 expression in the parietal cells of Bowman's capsule of most of the glomeruli, few glomeruli also demonstrated moderate expression of AnxA1 in the parietal cells. However the staining intensity declined from 20 weeks onwards. Of the 15 cases studied under this age group, 10 cases showed strong positivity (+3) for AnxA1 in the mesangial cells and podocytes

(P<0.0001) (table 5 & table 6), only 4 cases showed mild to moderate AnxA1 reactivity (+3) in the glomerular endothelial and parietal epithelial cells, respectively (fig 8) (table 7 & table 8).

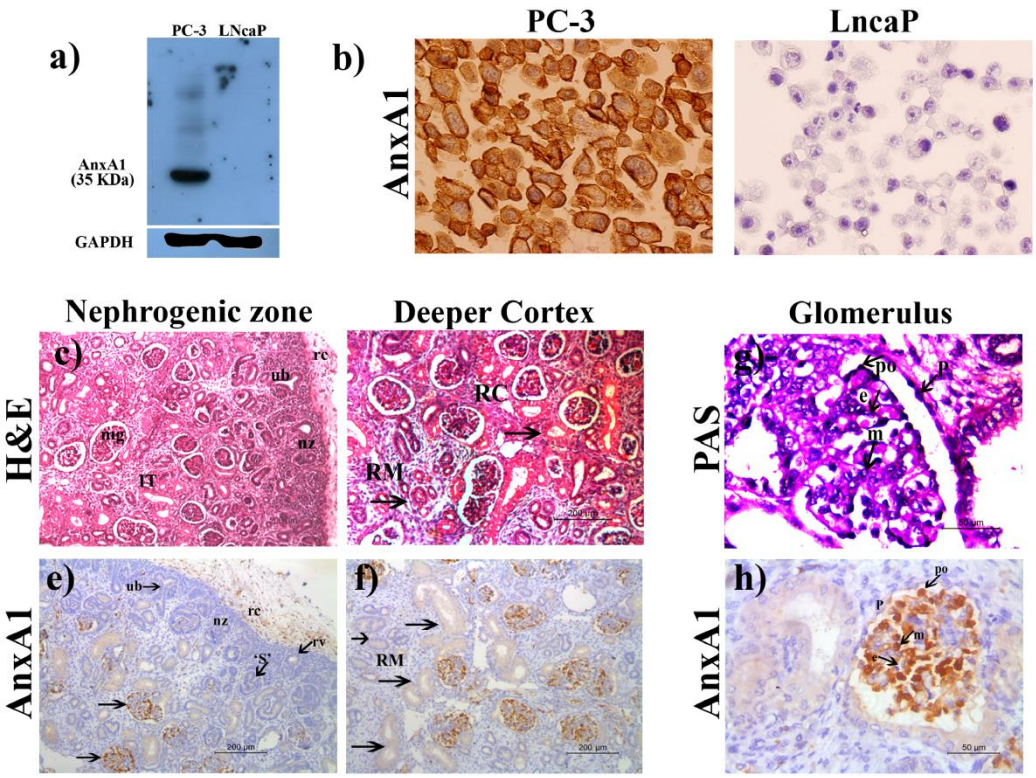

Fig 7: *AnxA1 expression pattern in developing human kidney at 14 week gestational age.* Antigen specificity of anti AnxA1 monoclonal antibody was verified using two different prostate cancer cell lines. Hormone dependent prostate cancer AnxA1 null LnCaP cell line and AnxA1 abundant metastatic prostate cancer PC-3 cell lines. The correlation of altered expression was demonstrated by western blot analysis (fig7a) and immunohistochemistry of these cell lines that showed +3 expressions in PC-3 cells and null in LnCaP cells (fig7b). c) H&E staining showing differentiating outer cortex and inner medulla and general structural features. Cortex showing nephrogenic zone (nz) beneath the renal capsule (rc). Ureteric bud (ub) capped by blastemal cells. Mature glomeruli (mg) in the deeper part of cortex. Immature tubules (IT) between the glomerulus. d) H&E of deeper cortex with maturing glomeruli and immature tubules in renal cortex (RC) and differentiating renal medulla (RM) e) Embryonic kidney showing strong AnxA1 immunoreactivity in the glomerular cells (arrows), with lack of staining in the nephrogenic zone, immature forms of glomeruli renal vesicle (rv), S-shaped bodies

'S', and ureteric bud. f) Absence of staining in the immature tubules of renal cortex and medulla (RM) (arrows). g) Glomerular cellular details are better appreciated using Periodic acid-Schiff staining (PAS), demonstrating various glomerular cells podocyte (p), mesangial cell (m), endothelial cell (e) and parietal cell (P). h) Mature glomerulus showing strong AnxA1 expression in the mesangial cells (m), podocytes (p) and endothelial cell (e). Magnification 10X, Bar=200µm & 40X, Bar=50µm.

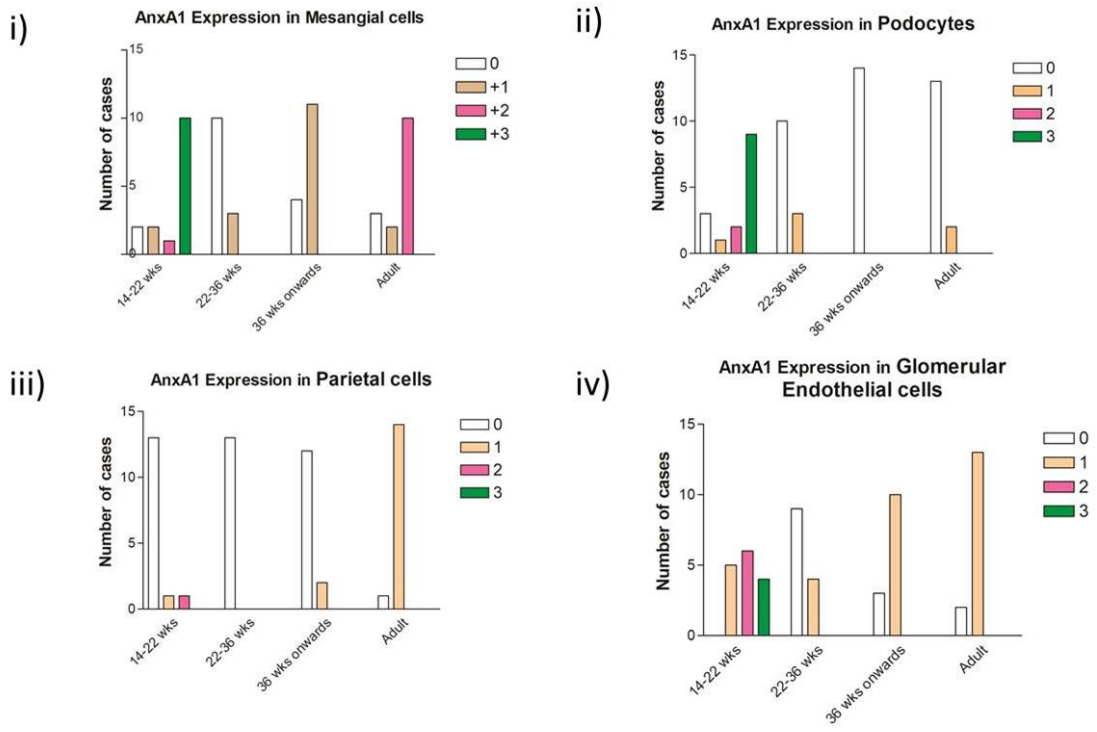

Fig 8: *Semiquantitative expression analysis of AnxA1 by IHC.* Data shown are representative of number of cases immunoreactive for AnxA1 in glomerular cells at various gestational periods of renal development and in adult kidney. (i) mesangial cells, P<0.0001; (ii) podocytes, P<0.0001; (iii) parietal cells, P = NS; (iv) glomerular endothelial cells, P<0.0001.

5.3. Decline in the expression of AnxA1 in the glomerular cells: During 22 to 36 weeks of nephrogenesis, the cortex appeared more mature with increase in number of mature glomeruli and fewer immature forms. PCTs' and DCTs' could be easily identified. PCTs' were lined by cuboidal cells with acidophilic cytoplasm and an apical brush border. They were larger in cross sections unlike fewer, smaller DCTs' lined by cuboidal cells with less abundant paler cytoplasm. Ureteric bud, a derivative of the Wolffian duct invaded the mesenchymal cells. The cells of the ureteric bud appeared cuboidal with centrally located nuclei. There was no further branching of ureteric bud. In the deeper medulla, thin and thick loops of Henle were lined by simple squamous and simple cuboidal epithelium respectively and appeared regularly round in cross section.[5] The collecting tubules derived from the ureteric bud resembled the thick loop of Henle but were less regular in shape. Collecting duct was clearly identified by larger diameter and tall columnar cell lining (Fig 9a & 9b).[5] The expression of AnxA1 increased in the glomerular cells during early development (stage 2) but decreased during later stage (stage 3). The podocytes, parietal, and endothelial cells were negative for AnxA1 in most of the cases. Few cases in this group demonstrated mild immunoreactivity (+1) in mesangial cells and podocytes (fig 8). There was no immunostaining for AnxA1 in either mesonephric duct derived ureteric buds or metanephric blastema derived early structures such as vesicle, S-shaped or C-shaped body (Fig 9c & 9e). No significant immunostaining was detected in maturing tubules, stromal or vascular structures of fetal renal compartments at this growth phase (Fig 9c, 9d & 9f).

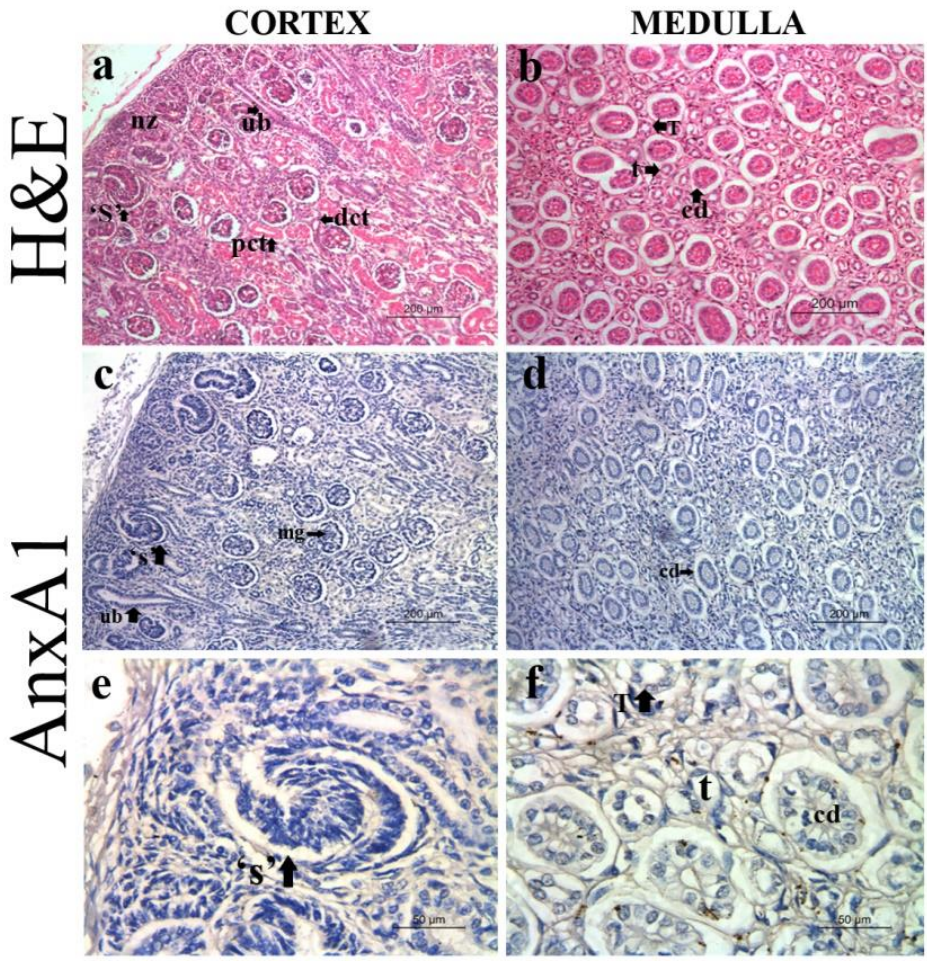

Fig 9: *AnxA1 immunohistochemical staining of developing human kidney at 23 weeks of gestation.* a) H&E staining of fetal renal cortex showing nephrogenic zone, ureteric bud, immature glomeruli (S-shaped), mature glomeruli (mg) and clearly discernable PCTs' with intense eosinophilic cytoplasm and centrally located nuclei and DCTs', less numerous and with scanty cytoplasm. b) H&E staining of fetal renal medulla, displays the developing collecting ducts (cd), Thick loop of Henle (T), thin loop of Henle (t), and blood capillaries. Cortex (Fig 9c & 9e) and medulla (Fig 9d & 9f) showing absence of AnxA1 staining in all renal tubular structures. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.4. Lack of AnxA1 expression at 36 weeks of gestation: During the fourth stage of kidney development, no new nephrons are formed. The volume density of glomeruli increases. The loops of Henle continue to increase in length and convoluted tubules become longer and tortuous.[1] The thickness of the cortex and medulla; renal vascularity increases with increase in gestational age where as the size of the nephrogenic zone decreases with increase in fetal age as shown in figure 10.[2] Decrease in the expression of AnxA1 was observed in the mesangial cells and endothelial cells of renal corpuscle. The podocytes and parietal cells of Bowman's capsule, cortical and medullary tubules, demonstrated absence of AnxA1 staining (Fig 10c & 10e). Renal interstitium and blood vessels exhibited low expression of AnxA1 (Fig 10c, 10e, 10d & 10f).

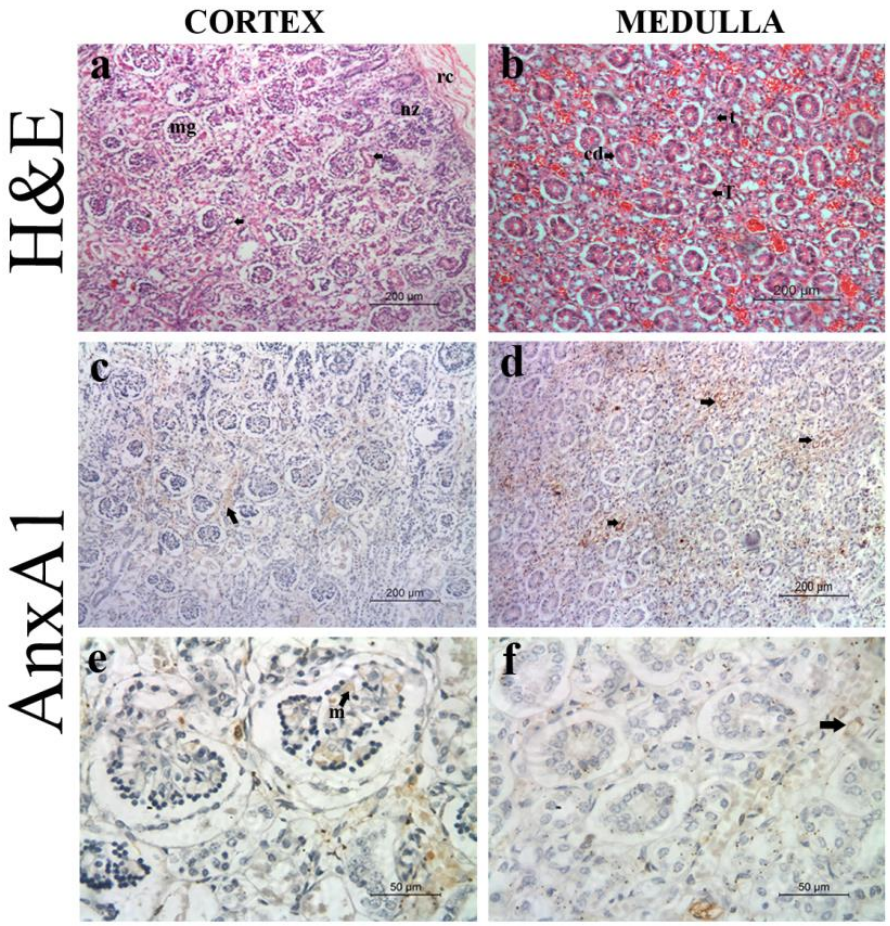

Fig 10: *AnxA1 immunostaining of developing human kidney cortex and medulla at 36 week of gestational age.* a) H&E section of fetal renal cortex showing decrease in the thickness of nephrogenic zone with numerous maturing tubules (arrows) and glomeruli (mg). b) H&E section of fetal renal medulla showing abundant tubules representing collecting ducts, thick and thin loop of Henle, and increased vascularity. Fig c, d & f) Mild expression of AnxA1 can be appreciated in the cortical and medullary interstitium and blood vessels (arrows). e) low expression of AnxA1 staining in the mesangial cells (m) of mature glomeruli and f) Absence of staining in the medullary tubules. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.5. Moderate expression of AnxA1 in mesangial cells of adult kidney: The adult kidney shows a well demarcated outer cortex and inner medulla. It is composed of numerous uriniferous tubules bound by connective tissue stroma. The cortex is mainly occupied by renal corpuscle, PCT and DCT.[6,7] The renal corpuscle consists of glomerular plexus of capillaries invaginating into Bowman's capsule. The vascular mesentery consists of mesangial cells. The capsule consists of outer parietal layer lined with flattened epithelium and an inner visceral layer is lined by large polyhedral cells. Both the layers are separated by capsular space. The PCT are numerous, with smaller lumen when compared with fewer, shorter DCT devoid of microvilli and faint eosinophilic stain (Fig11a). The medulla contains straight portions of tubules, segments of LOH and collecting duct. The thin segments of LOH are lined by simple squamous epithelium and resemble the capillaries and are distinguished from the latter by the absence of blood cells in the lumina and thicker epithelial lining, as shown in fig11b.[7] Our immunohistochemical data revealed AnxA1 staining in the adult kidney was consistent in the mesangial cells of the renal corpuscle and few blood capillaries in the medulla as demonstrated in fig 11c, 11d & 11e. Adult renal tissues demonstrated moderate immunostaining for AnxA1 in the mesangial cells (fig 11e). In addition the endothelial lined capillary loop, parietal epithelial cells of renal corpuscle also demonstrated a moderate expression of AnxA1 in few cases. There was absence of staining in the cortical tubules. No staining was seen in the medullary collecting ducts and segments of LOH (Fig 11c & 11f). A summary of the observed immunostaining in various renal structures is presented in table 9. Note the semiquantitative expression analysis of AnxA1 showing

representative number of cases immunoreactive to AnxA1 in glomerular cells of fetal and adult kidney (Fig 12, 13, 14, 15).

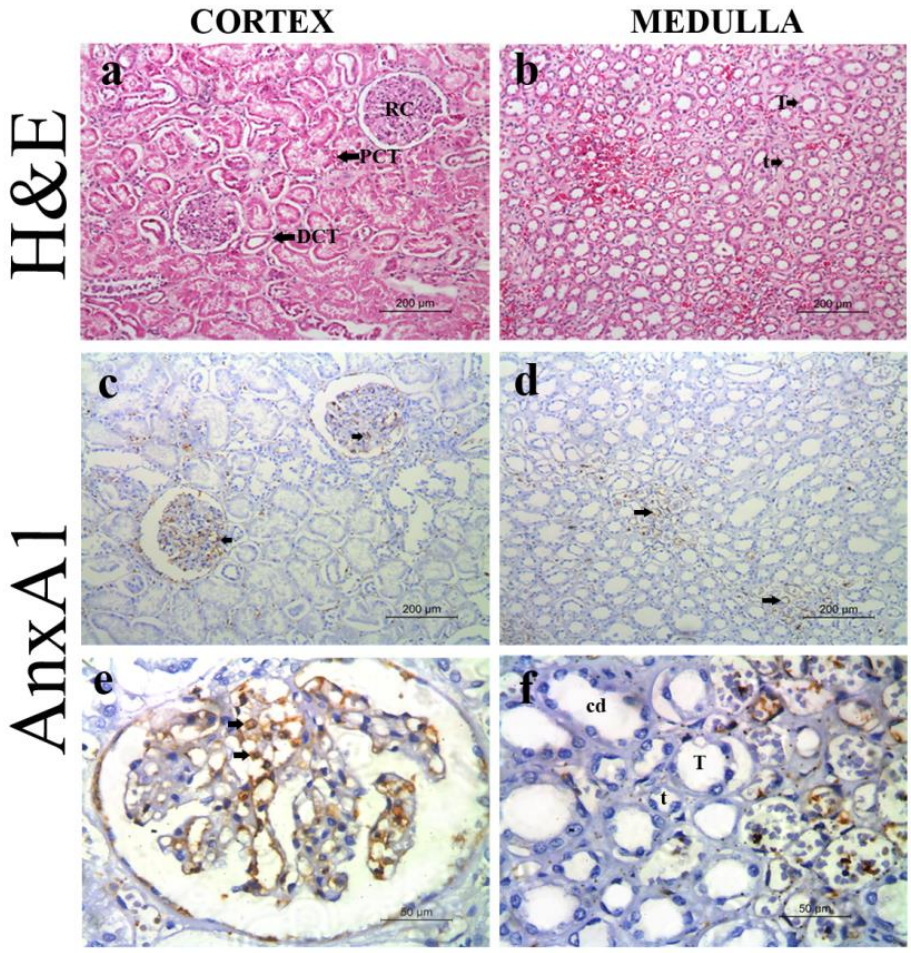

Fig 11: *AnxA1 immunostaining of mature adult kidney.* a) H&E section of kidney cortex occupied by numerous mature PCT, few pale staining DCT lined with cuboidal cells and renal corpuscles (RC). b) H&E section of kidney medulla demonstrating mature tubules. Fig 11c & 11e) Immunohistochemical expression of AnxA1 in adult renal cortex shows positive moderate expression in the mesangial and capillary loop of renal corpuscle. Fig 11d & 11f) AnxA1 staining in the medulla is restricted to blood capillaries (arrows) and negative in the tubules. Magnification 10X, Bar=200µm &40X, Bar=50µm.

STATISTICAL ANALYSIS OF EXPRESSION STATUS OF ANXA1 IN GLOMERULAR CELLS OF FETAL AND ADULT KIDNEY

Table 5: AnxA1 Expression in Mesangial cells

AnxA1 Expression in Mesangial cells (n=no of cases)					P Value
Group	0	+1	+2	+3	P<0.0001 (α<0.05)
14-22 weeks (n=15)	2.0	2.0	1.0	10.0	
22-36 weeks (n=13)	10.0	3.0	0.0	0.0	
36 weeks onwards(n=14)	4.0	11.0	0.0	0.0	
Adult (n=15)	3.0	2.0	10.0	0.0	

Statistical analysis of expression status of AnxA1 in Mesangial cells of fetal kidney at different gestational periods and adult kidney by Chi-square test (P<0.0001).

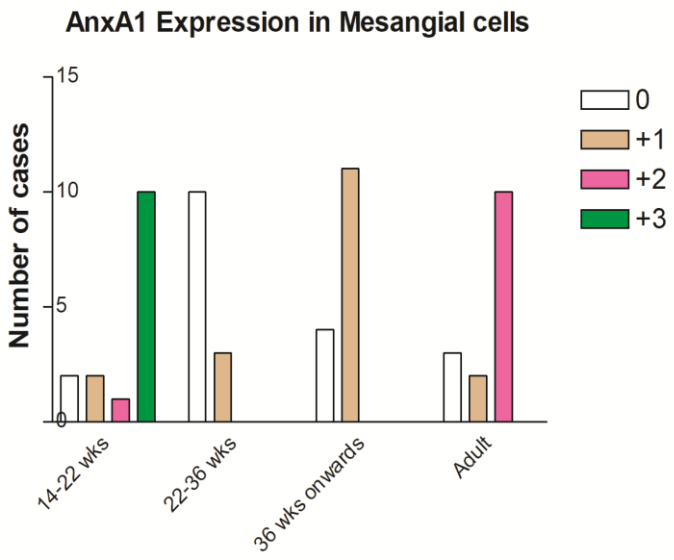

Fig 12: Semi quantitative expression analysis of AnxA1 by *IHC*. Data showing representative number of cases immunoreactive for AnxA1 in mesangial cells at various gestational periods of renal development and in adult kidney.

Table 6: AnxA1 Expression in Podocytes

AnxA1 Expression in Podocytes (n=no of cases)					P Value
Group	0	+1	+2	+3	P<0.0001 (α<0.05)
14-22 weeks (n=15)	3	1	1	10	
22-36 weeks (n=13)	10	3	0	0	
36 weeks onwards(n=14)	14	0	0	0	
Adult (n=15)	13	2	0	0	

Statistical analysis of expression status of AnxA1 in Podocytes of fetal kidney at different gestational periods and adult kidney by Chi-square test (P<0.0001).

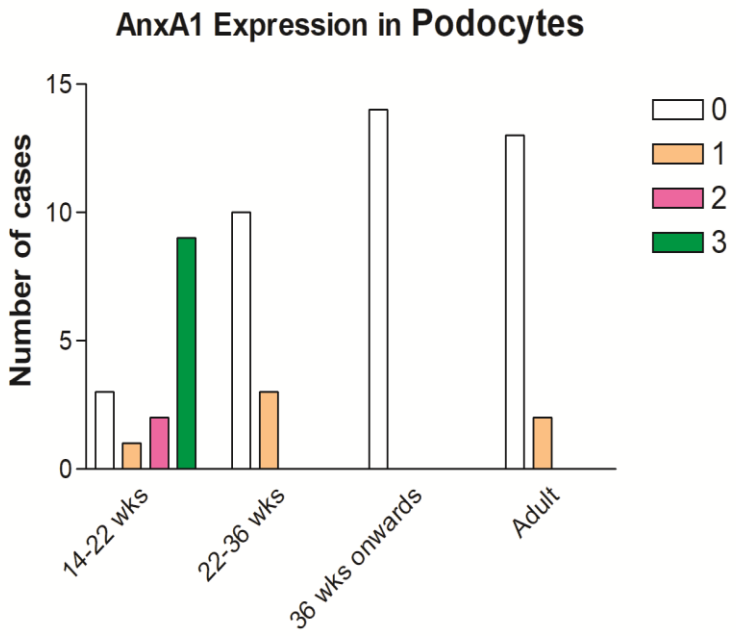

Fig 13: Semi quantitative expression analysis of AnxA1 by *IHC*. Data showing representative number of cases immunoreactive for AnxA1 in podocytes at various gestational periods of renal development and in adult kidney.

Table 7: AnxA1 Expression in Endothelial cells

AnxA1 Expression in Glomerular Endothelial Cells (n=no of cases)					P Value
Group	0	+1	+2	+3	
14-22 weeks (n=15)	06	5	4	0	
22-36weeks (n=13)	9	4	0	0	P<0.0001 (α<0.05)
36weeks onwards(n=14)	3	10	0	0	
Adult (n=15)	2	09	04	0	

Statistical analysis of expression status of AnxA1 in Endothelial cells of fetal kidney at different gestational periods and adult kidney by Chi-square test (P<0.0001).

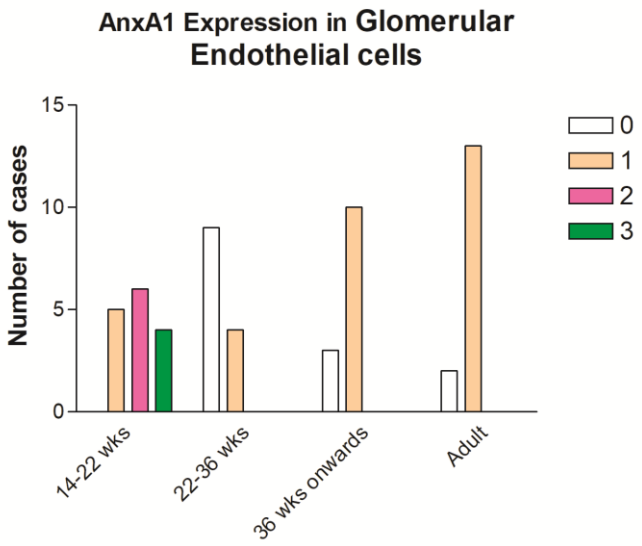

Fig 14: Semi quantitative expression analysis of AnxA1 by *IHC*. Data showing representative number of cases immunoreactive for AnxA1 in Glomerular Endothelial Cells at various gestational periods of renal development and in adult kidney.

Table 8: AnxA1 Expression in Parietal cells

AnxA1 Expression in Parietal cells (n=no of cases)					P Value
Group	**0**	**+1**	**+2**	**+3**	
14-22 weeks (n=15)	**09**	**1**	**4**	**0**	
22-36 weeks (n=13)	**13**	**0**	**0**	**0**	**NS**
36 weeks onwards(n=14)	**12**	**2**	**0**	**0**	
Adult (n=15)	**1**	**14**	**0**	**0**	

Statistical analysis of expression status of AnxA1 in Parietal cells of fetal kidney at different gestational periods and adult kidney by Chi-square test (P= NS).

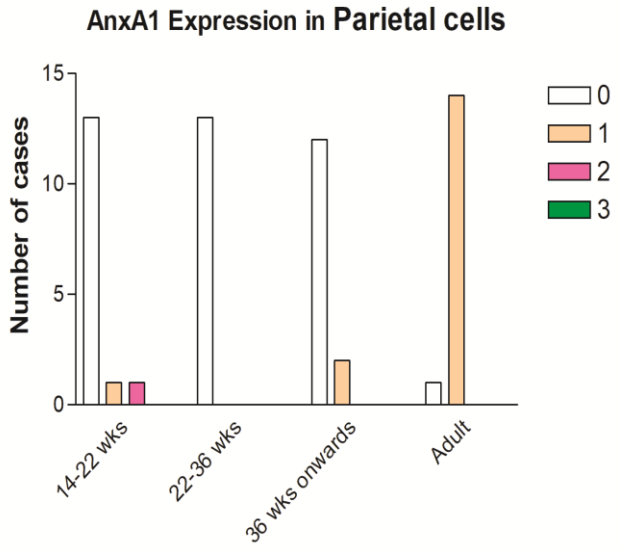

Fig 15: Semi quantitative expression analysis of AnxA1 by *IHC*. Data showing representative number of cases immunoreactive for AnxA1 in Parietal Cells at various gestational periods of renal development and in adult kidney.

Table 9:

EXPRESSION OF ANNEXINA1 IN NORMAL HUMAN FETAL AND ADULT KIDNEY

Renal structures n= no of cases		**1**4 n=4	**1**6 n=2	**1**7 n=1	**1**8 n=3	**1**9 n=1	20 n=1	21 n=1	22 n=2	22-36 weeks n=13	**36weeks onwards** n=14	**Mature Adult** n=15
Immature forms of glomeruli		+	+	0	+	+	0	+	0	0	mv	mv
Ureteric bud		0	0	0	0	0	0	0	0	0	mv	mv
Immature tubules (IT)		+	+	+	mv	mv	mv	mv	mv	mv	mv	mv
Mature glomeruli	Mesangial cells	+++	+++	++	+++	+++	+	+	+	+	+	++
	Podocytes	+++	+++	++	+++	++	0	+	0	0	0	0
	Parietal cells	0	0	0	0	++	0	+	0	0	0	+
	endothelial cells	+++	++	++	++	+	+	+	+	0	0	+
PCT		mv	mv	mv	0	0	0	0	0	0	0	0
DCT		mv	mv	mv	0	0	0	0	0	0	0	0
Thick LOH		mv	mv	mv	0	0	0	0	0	0	0	0
Thin LOH		mv	mv	mv	0	0	0	0	0	0	0	0
CD		mv	mv	mv	0	0	0	0	0	0	0	0

PCT, Proximal convoluted tubule; DCT, Distal convoluted tubule, LOH, Loop of Henle; CD, Collecting duct; mv,missing value; Immunohistochemical staining for AnxA1,0=no staining, 1-10% as 1+, 11-50% as 2+, 51-70% as 3+, 71-100% as 4+). A summary of expression status of AnxA1 in various structures of human fetal kidney and adult kidney, showing increased expression of AnxA1 in the glomerular mesangial and podocytes in earlier weeks of gestational period in comparison to decrease in its expression in latter weeks of gestation in fetal kidney to adult kidney.

5.6.1. Upregulation of AnxA1 in kidney cancer: Immunohistochemical staining of normal parts of adult kidneys showed absence of AnxA1 staining of proximal tubular cells as shown in fig 16c & 16d. Our study comprised predominantly of clear cell type renal cell carcinoma (CCRCC) and was graded based on Fuhrman nuclear grades. The tumor cells either presented itself with clear cytoplasm or admixed with eosinophillic or granular cytoplasm.[8] Evaluating the expression of AnxA1 in the tumor cells of CCRCC , grade 1 tumors showed mild positivity for AnxA1 (fig 16g & 16h), the staining intensity increased in grade 2 and grade 3 clear cell renal cell carcinomas (+2) with tumor progression (fig 16k, 16l, 16o & 16p). AnxA1 expression was tested on non-neoplastic tissue and CCRCC. Data were calculated as average percentage of AnxA1 expressing cells in the proximal tubular cells of non-neoplastic adult tissues against tumor cells of CCRCC at different tumor grades. The percentage of tumor cells staining positive for AnxA1 increased with increasing tumor grade (fig 16B) ($p < 0.0001$).

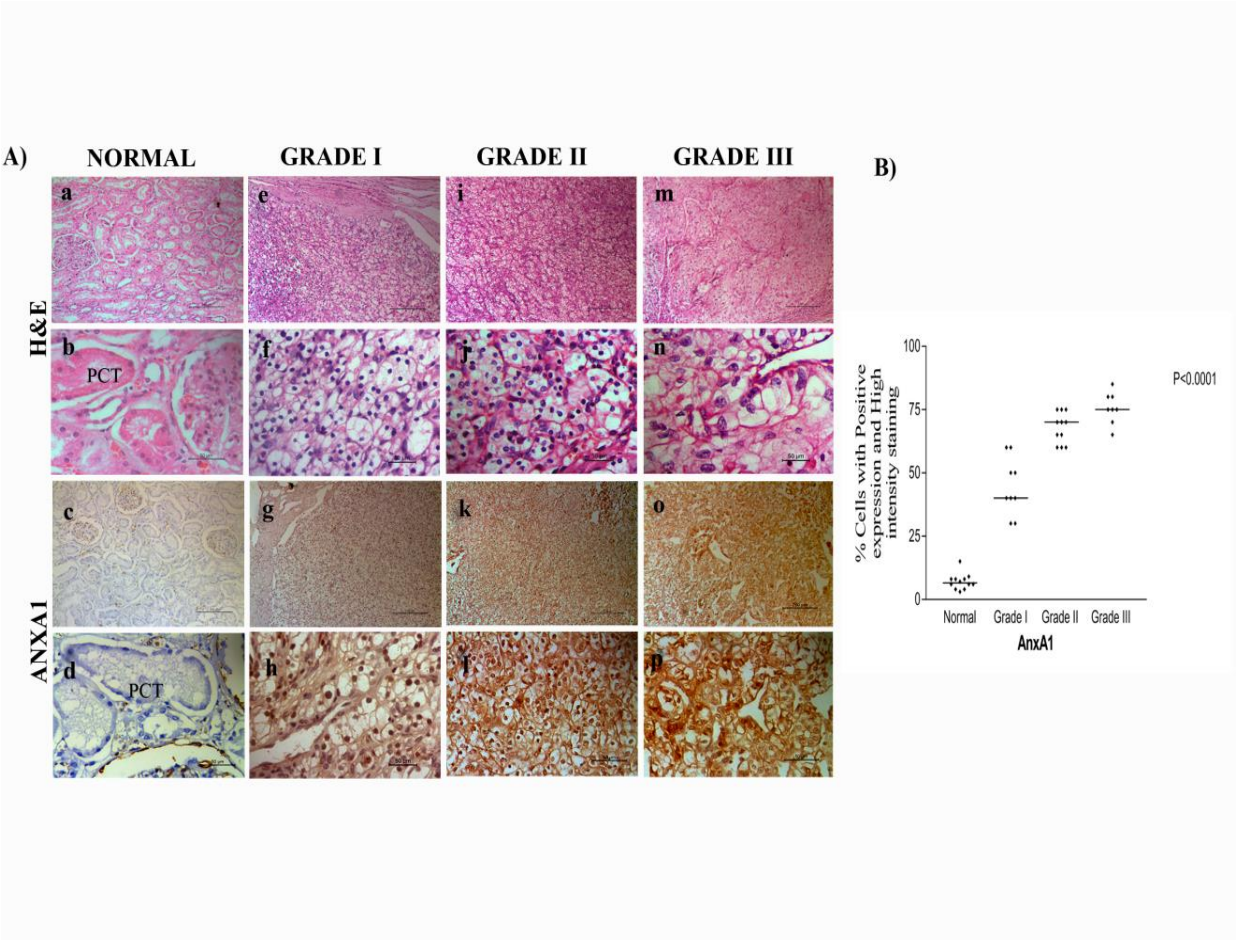

Fig 16A: *AnxA1 immunostaining of clear cell renal cell carcinoma (CCRCC) tissues:* H&E section of normal adult kidney cortex (fig 16a & 16b). Immunohistochemical expression of AnxA1 in adult renal cortex showing absence of AnxA1 staining in proximal tubular cells (fig 16c & 16d). H&E section of Fuhrman histological grade I clear cell renal cell carcinoma showing clear cells with small round uniform nuclei and absence of nucleoli (fig 16e &1 6f). Immunohistochemical expression of AnxA1 grade I CCRCC showing mild membranous staining in tumor cells (16g & 16h). H&E section of Fuhrman histological grade II clear cell renal cell carcinoma showing clear cells with relatively larger irregular nuclei and presence of nucleoli (16i & 16j). Immunohistochemical expression of AnxA1 grade II CCRCC showing moderate staining in tumor cells (16k & 16l). H&E section of Fuhrman histological grade III clear cell renal cell carcinoma showing clear cells with larger irregular nuclei and presence of prominent nucleoli (16m & 16n). Immunohistochemical expression of AnxA1 grade III CCRCC showing moderate staining in tumor cells (16o & 16p). Magnification 10X, Bar=200µm & 40X, Bar=50µm. Fig 16B: *AnxA1 vs. Tumor grade:* Comparison of AnxA1 expression in adult proximal tubular cells against tumor cells in CCRCC relative to tumor grade, P<0.0001.

5.6.2. AnxA1 expression in other types of renal cell carcinoma: The growth pattern of papillary renal cell carcinoma was either papillary or tubule-papillary type.[8] Fig 17a & 17b shows type 2 tumor cells having eosinophilic cytoplasm, high grade nuclei with pseudo-stratification.[9] In all the cases of papillary renal cell carcinoma studied, tumor cells were immunopositive for AnxA1 as demonstrated in fig 17c & 17d. Hematoxylin and eosin staining of collecting duct carcinoma was characterized by glandular or papillary growth pattern (fig 17e & 17f).[8] Tumor cells of collecting duct carcinoma showed moderate cytoplasmic and nuclear AnxA1 immunostaining (+2) as depicted in fig 17g & 17h. Chromophobe renal cell carcinoma consisted of large cells with reticulated cytoplasm and small cells with granular cytoplasm in H&E tumor sections (fig 17i & 17j).[8] Chromophobe renal cell carcinoma sections revealed strong immunostaining for AnxA1 (+3) as shown in fig 17k & 17l. Oncocytomas largely mimic chromophobe renal cell carcinoma histologically, however these tumor cells consisted of deeply eosinophilic cytoplasm, centrally located nuclei (fig 17m & 17n).[9] All cases of oncocytoma studied, tumor cells demonstrated moderate immunopositive staining for AnxA1 (+2) as depicted in fig 17o & 17p.

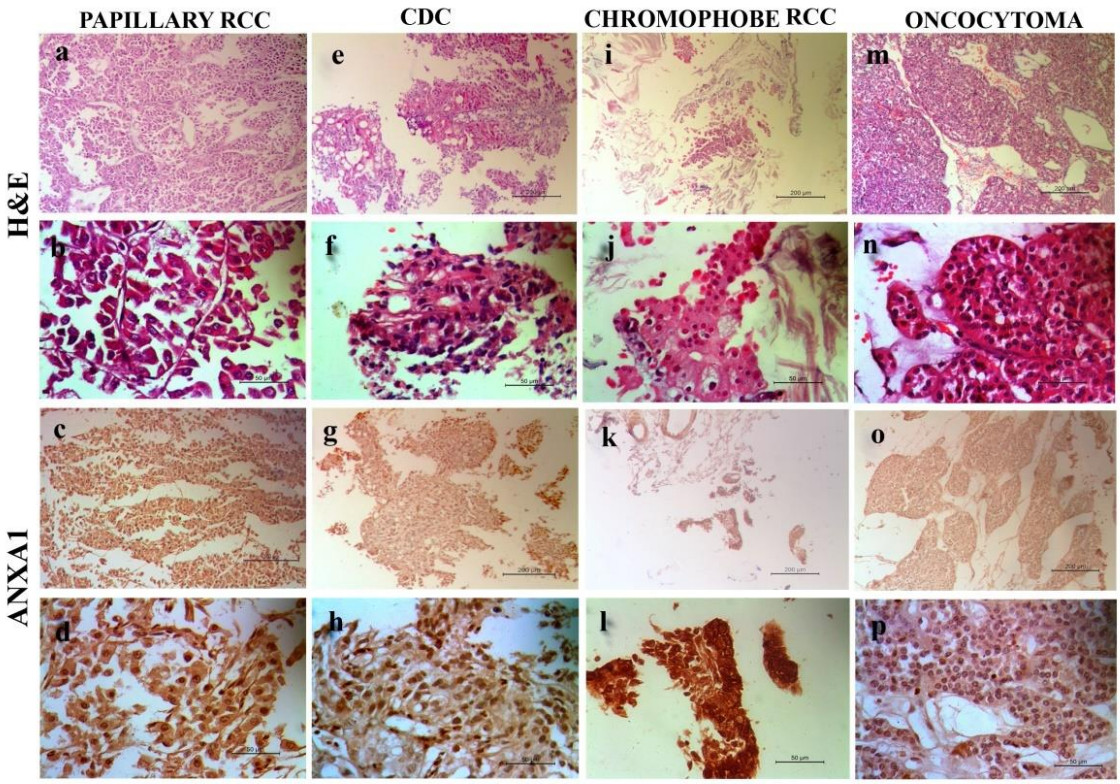

Fig 17: *AnxA1 immunostaining in other types of renal cell carcinomas:* H& E sections of papillary renal cell carcinoma, chromophobe renal cell carcinoma, collecting duct renal cell carcinoma (CDC) and oncocytoma (Fig 17a to 17h). Immunopositive AnxA1 staining was detected in papillary renal cell carcinoma, chromophobe renal cell carcinoma, collecting duct renal cell carcinoma and oncocytoma (Fig 17i to 17p).

5.7: AnxA2 in ureteric buds and collecting ducts of fetal kidneys: The nephrogenic zone (nz) was comprised of primitive forms of nephron such as vesicle 'V', S-shaped bodies 'S', capillary loop C and also the ureteric bud (ub) lined by cuboidal cells forming the collecting component of mammalian kidney (Fig 18a).[3] The cortex demonstrated maturing glomerulus (mg), developing proximal tubule (pt) and distal tubular cells (dt) and a differentiating medulla showing ureteric bud derived developing collecting duct cells (Fig 18b). AnxA2 staining of renal cortex of fetal kidneys from 14 to 22 weeks of gestation (nine out of 15 cases) revealed moderate cytoplasmic positivity in the developing proximal tubules (pt) ($p<0.0001$) (Fig 18c). At all-time points, a moderate to strong immune-expression for AnxA2 was observed in the membranes and cytoplasm of ureteric bud (as recognized up to 32 weeks of gestation), developing collecting tubules (ct) and collecting ducts (cd), endothelial cells lining blood vessels of all fetal renal tissues (Fig18c & Fig18d). As shown in Fig 18e & 18f, the expression was stronger in the membrane facing the lumens of ureteric buds and collecting ducts. AnxA2 imunolocalization was rarely seen in the immature forms of glomeruli derived from metanephricblastema.

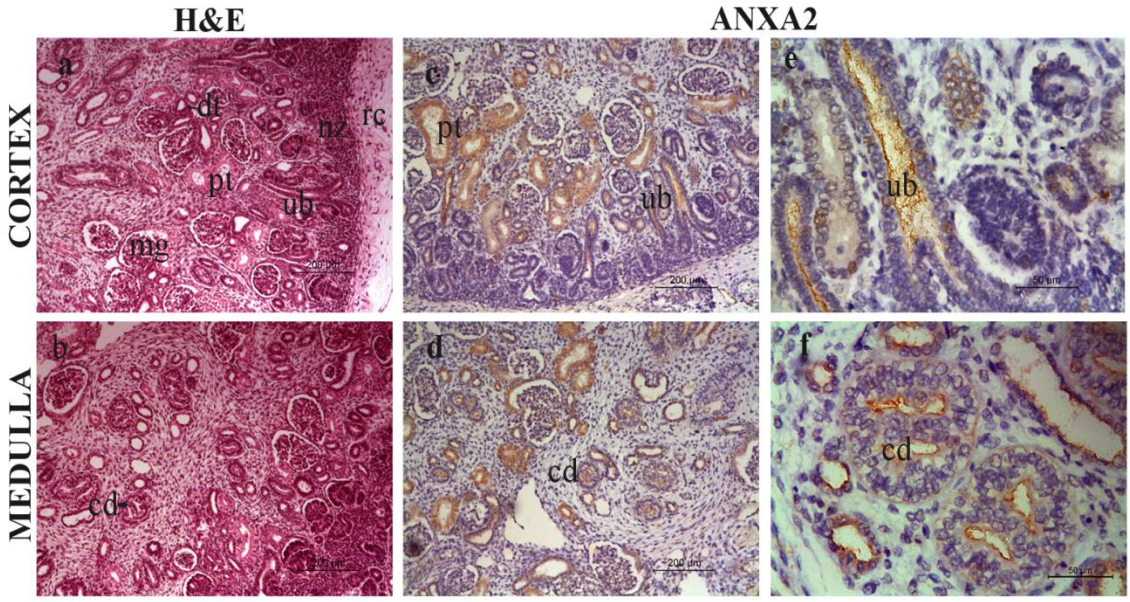

Fig 18: *AnxA2 expression pattern in developing human kidney at 14 week gestational age.*

a) H & E staining showing a renal capsule (rv), nephrogenic zone (nz) and differentiating outer cortex. Condensations of metanephricblastemal cells with slit like cavity (rv) besides the dilated tips of ureteric bud (ub) are seen in the nephrogenic zone. Developing proximal tubular (pt) distal tubular cells (dt) and mature glomeruli (mg) in the deeper part of cortex. b) H & E of differentiating renal medulla (RM) showing developing collecting duct cells lined by tall columnar epithelium. c) Embryonic kidney showing moderate AnxA2 immunoreactivity in the ureteric bud (membranous), cytoplasmic immunoreactivity in proximal tubular cells (pt). d) AnxA2 immunoreactivity seen in developing collecting duct cells. e) Moderate (+2) staining for AnxA2 observed in the membranes of the cells lining the ureteric bud. f) Collecting duct cells showing strong

AnxA2 expression in the membranes facing the lumen. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.8: Decline in the expression of AnxA2 in proximal tubular cells: A single section of fetal kidney at third stage of development show the nephrogenic zone (nz) with vesicle (rv), S-shaped bodies 'S', ureteric bud, developing maturing glomerulus (mg) (at various stages of maturation), proximal tubules pt, distal tubules dt, loop of Henle (LOH) (thick limb of loop of Henle (T) and thin loop of Henle (t), and collecting duct cells, cd (fig 19a & 19b). The ureteric bud and the collecting duct cells continued to express AnxA2 as shown in fig 19c, 19d, 19e & 19f, however a decline in the expression of AnxA2 in cells of pt was observed in fetal kidneys in third and fourth stages of nephron development (fig 19c) (table 10). The primitive forms of immature glomerulus such as the renal vesicle (rv) , 'S' shaped bodies in the nephrogenic zone showed either mild immune staining or absence of AnxA2 as shown in fig 19c.

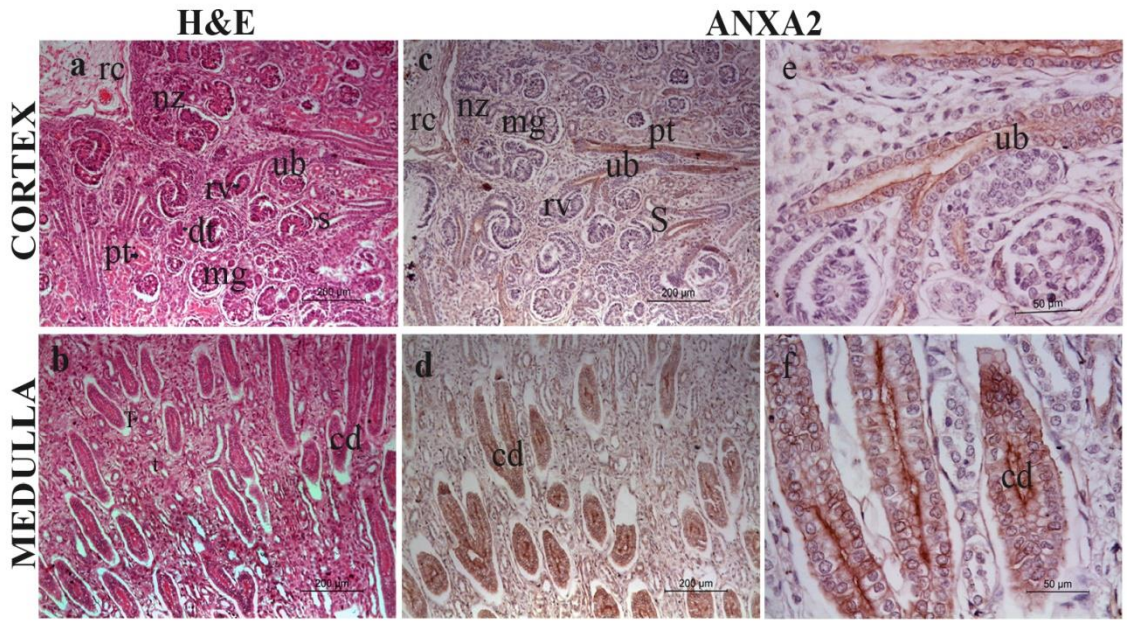

Fig 19: *AnxA2 immunohistochemical staining of developing human kidney at 24 weeks of gestation.* a) H & E staining of embryonic kidney showing outer renal cortex with renal capsule (rc) nephrogenic zone (nz), ureteric bud (ub), renal vesicle (rv), S-shaped bodies, mature glomeruli (mg), proximal tubular (pt) and distal tubular cells (dt). b) H&E staining of inner renal medulla, demonstrating the developing collecting ducts (cd), Thick loop of Henle (T), thin loop of Henle (t), and blood capillaries. Fig c) & e) Mild staining (+1) for AnxA2 in the proximal tubular cells (pt) and moderate (+2) staining in the ureteric bud cells (ub). Fig d & f) Collecting duct cells in the medulla showing moderate to strong staining of AnxA2. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.9: Unswerving expression of AnxA2 in the collecting tubular and duct cells: At the fourth stage of nephron development, the cortex appeared more mature with increase in volume of glomeruli and increase in tortuosity of proximal and distal tubular cells (fig 20a) and a well differentiated medulla as shown in fig 20b.[1] Immunohistochemical staining of these sections of fetal kidney showed a consistent expression of AnxA2 in the collecting tubular and duct cells (fig 20c, 20d, 20e & 20f) (p=0.0001) (table 10). The cells of renal corpuscles, distal tubules (dt), loop of henle (LOH) showed weak to moderate expression of AnxA2 in fewer cases.

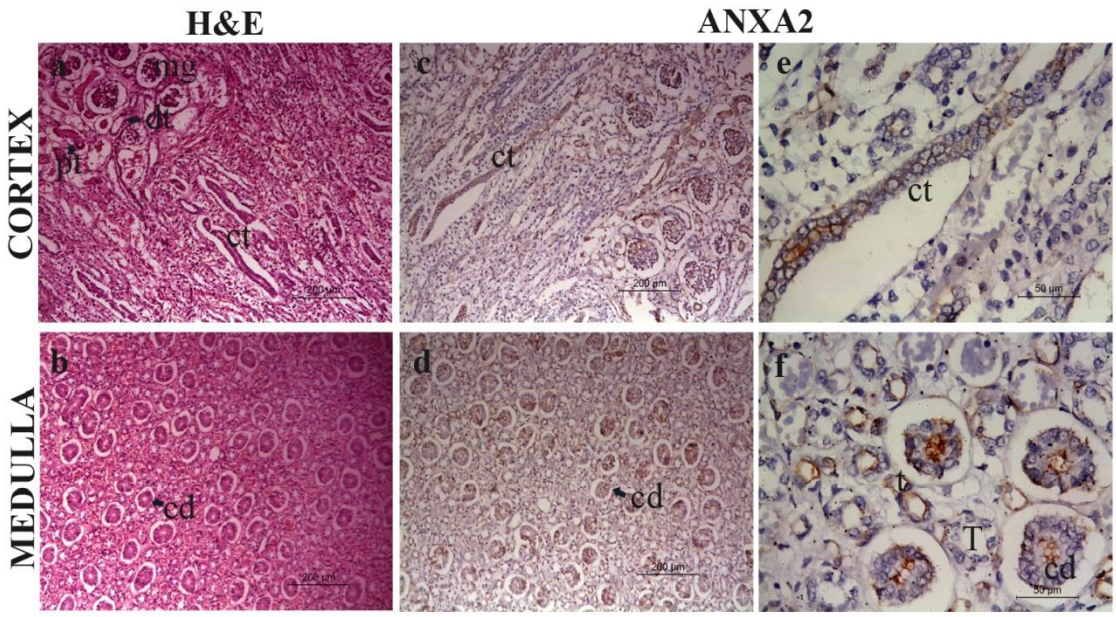

Fig 20: *AnxA2 immunostaining of developing human kidney at 36 week of gestational age.* a) H & E section of fetal renal cortex showing numerous maturing tubules, pt, dt cells and glomeruli (mg). b) H & E section of well differentiated fetal renal medulla showing numerous tubules such as collecting ducts, thick and thin loop of Henle. Fig c & e) Moderate expression of AnxA2 can be appreciated in the cortical collecting tubules. Fig d & f) Moderate AnxA2 staining in the membranes of collecting duct cells and thin limb of loop of Henle (t) Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.10: Lack of AnxA2 expression in proximal tubules of adult kidneys: The renal cortex is identified by the presence of renal corpuscles (RC), proximal (PT) and distal convoluted tubules (DT) (fig 21a). The proximal convoluted tubules usually found as a continuation of renal corpuscle is a coiled tube lined by cuboidal brush bordered epithelium and intensely stained cytoplasm whereas the distal tubule is a continuation of ascending limb of loop of Henle found amongst the proximal convoluted tubules clearly differentiated from the latter by the absence of brush border and a defined lumen as depicted in fig 22a. Medulla is distinguished by the presence of collecting tubules (lined by cuboidal epithelium, less regular lumen), collecting ducts (lined by columnar epithelium), the thick (T) and thin loop of Henle (t) (lined by cuboidal and squamous epithelium respectively) and blood vessels (Fig 21b).[5] All of the cases treated with anti AnxA2 antibody, showed either no staining or very little staining of epithelial cells of PT (+1) (fig 21c & 21e). The cells of DT, LOH exhibited AnxA2 positivity (+2) in fewer cases. Out of the 37 normal adult kidneys immunostained, expression of AnxA2 remained constant in the membranes and cytoplasm of collecting ducts and endothelial cells of arterioles and blood capillaries (+2) (fig 21d & 21f). AnxA2 was detected in the mesangial cells, podocytes and parietal cells of renal corpuscle (+2) in fewer normal renal tissues.

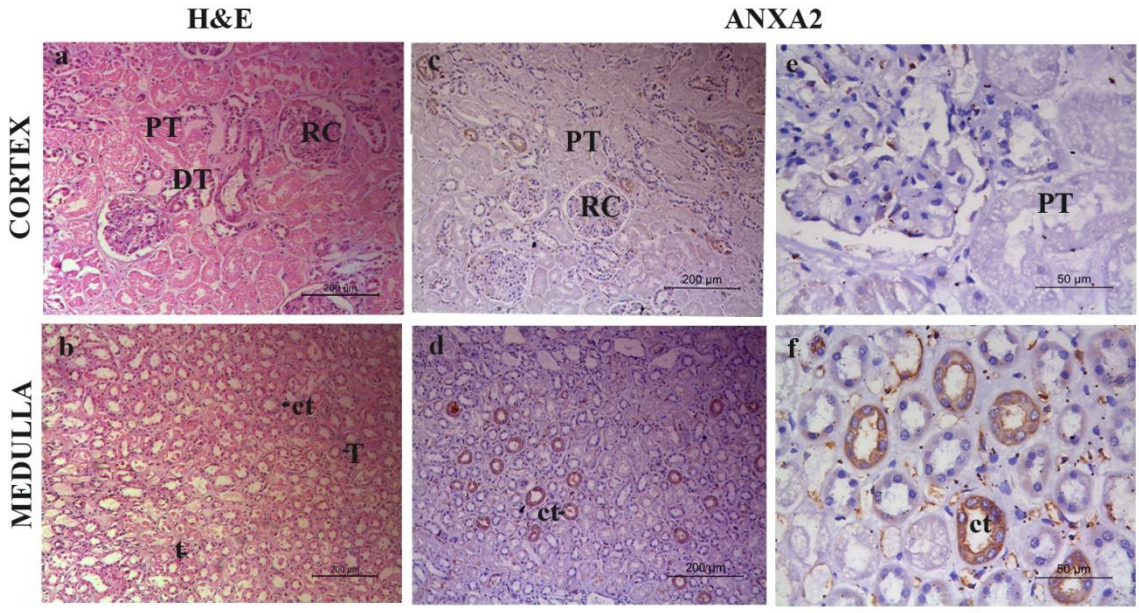

Fig 21: *AnxA2 immunostaining of mature adult kidney.* a) H & E section of adult kidney cortex occupied by many proximal tubular cells (PT) lined by cuboidal cells, strongly eosinophilic cytoplasm and presence of microvilli; few pale staining distal tubular cells (DT) lined with cuboidal cells and absence of microvilli admist the renal corpuscles (RC). b) H & E section of adult kidney medulla showing mature tubules such as the collecting tubule (ct), thin limb of loop of Henle (t) and thick limb of LOH (T). Fig 21c & 21e) Immunohistochemical expression of AnxA2 in adult renal cortex shows absence of staining in the PT cells and mild to moderate staining in fewer DT cells and blood vessels. Fig 21d & 21f) AnxA2 moderate staining in the medulla is restricted to collecting tubular cells (ct). Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.11:AnxA2 expression increases with the progression of Clear cell renal carcinoma (CCRCC): Immunohistochemical staining of normal parts of adult kidneys showed either absence or mild staining of proximal tubular cells for AnxA2 as shown in fig 22b & 22c. Our study comprised predominantly clear cell type of renal cell carcinoma. The growth patterns of clear cell RCC varied from typical solid, acinar to tubular, cystic and pseudo papillary type. The tumor cells either presented itself with clear cytoplasm or admixed with eosinophillic or granular cytoplasm (fig 22d, 22g & 22j).[8] Evaluating the expression of AnxA2 in the tumor cells of CCRCC showed primarily membranous, nuclear and cytoplasmic reactivity. While grade 1 tumors showed mild positivity for AnxA2 (fig 22e & 22f), the staining intensity increased in grade 2 and grade 3 clear cell renal cell carcinomas (+2) with tumor progression (fig 22h, 22i, 22k & 22l).

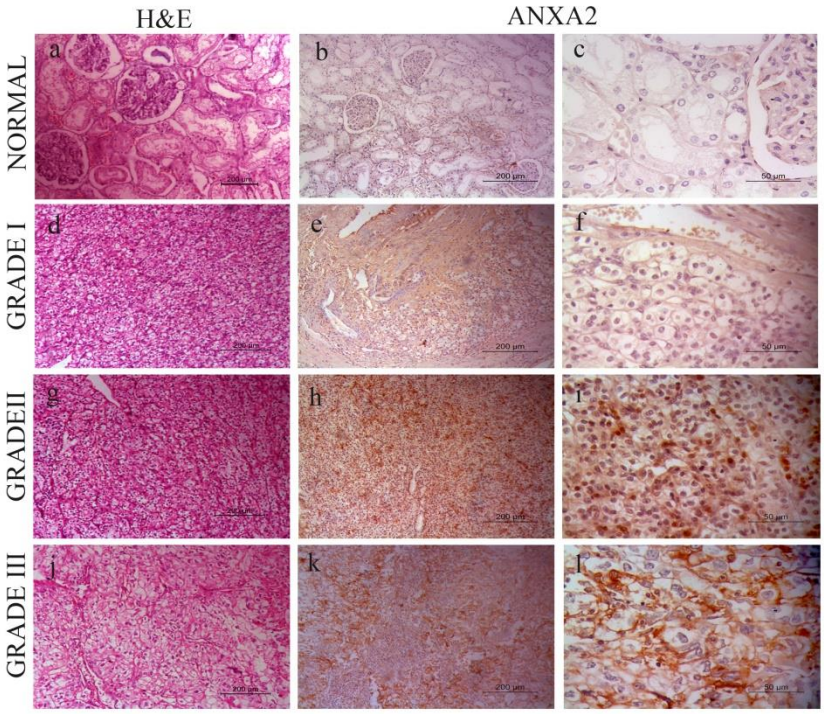

Fig 22: *AnxA2 immunostaining of CCRCC*. a) H & E section of normal adult renal cortex showing RC, PT and DT cells. Fig b & c) AnxA2 staining of normal adult renal cortex demonstrating lack of staining in the PT cells. d) H & E section of grade I CCRCC. Fig e & f) Mild staining for AnxA2 in the tumor cells and blood vessels of grade 1 CCRCC. g) H&E section of grade II CCRCC. Fig h & i) Moderate to strong expression of AnxA2 can be appreciated in the tumor cells of grade II CCRCC. j) H & E section of grade III CCRCC. Fig k & l) Moderate to strong expression of AnxA2 demonstrated in the tumor cells of grade III CCRCC. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

5.12: AnxA2 expression in variants of RCC:

Adult normal kidney collecting tubule demonstrated moderate staining of AnxA2 and null expression in the proximal tubules (fig 23b & 23c).The respective expression was compared with the tumor cells of papillary renal cell carcinoma. The growth pattern of Papillary renal cell carcinoma is either papillary or tubulopapillary type.[8] It is histologically differentiated as type 1, tumor cells presents with basophilic cytoplasm, low grade nuclei and absence of nuclear pseudo stratification. Type 2 tumor cells having eosinophillic cytoplasm, high grade nuclei with pseudo stratification as shown in fig 6d.[9] In all the cases of papillary renal cell carcinoma studied, tumor cells were immunonegative for AnxA2 as demonstrated in fig 23e & 23f.

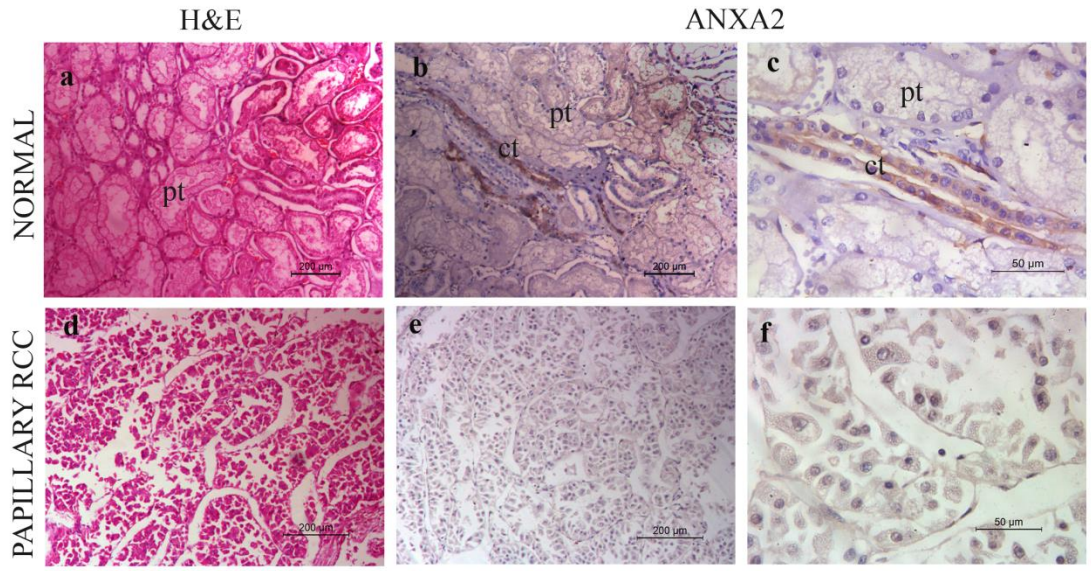

Fig 23: *AnxA2 immunostaining of Papillary RCC.* a) H&E section of normal adult renal cortex showing numerous PT cells. Fig b & c) AnxA2 staining of normal adult renal cortex demonstrating lack of staining in the PT cells and moderate staining in the collecting tubular cells. d) H & E section of papillary renal cell carcinoma. Fig e & f) Absence of AnxA2 staining in the tumor cells of papillary renal cell carcinoma. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

The adult kidney collecting duct cells showed moderate cytoplasmic and membranous staining for AnxA2 as demonstrated in fig 24a, 24b & 24c. Hematoxylin and eosin staining of collecting duct carcinoma was characterized by glandular or papillary growth pattern (fig 24d).[8] Tumor cells of collecting duct carcinoma showed strong AnxA2 immunostaining (+3) as depicted in fig 24e & 24f. Chromophobe RCC consisted of solid sheets of cells separated by hyalinized blood vessels, in addition consisted of large cells with reticulated cytoplasm and small cells with granular cytoplasm. The tumor cells exhibited nuclear wrinkling and distinct cell membranes in H & E tumor sections (fig 24g).[8] Chromophobe RCC revealed moderate to intense immunostaining for AnxA2 (+3) as shown in fig 24h & 24i. Oncocytomas largely mimic Chromophobe RCC histologically, however these tumor cells consisted of deeply eosinophilic cytoplasm, centrally located nuclei with smooth margin (fig 24j).[9] All cases of oncocytoma studied, tumor cells demonstrated mild immunopositive staining for AnxA2 (+2) as depicted in fig 24l.

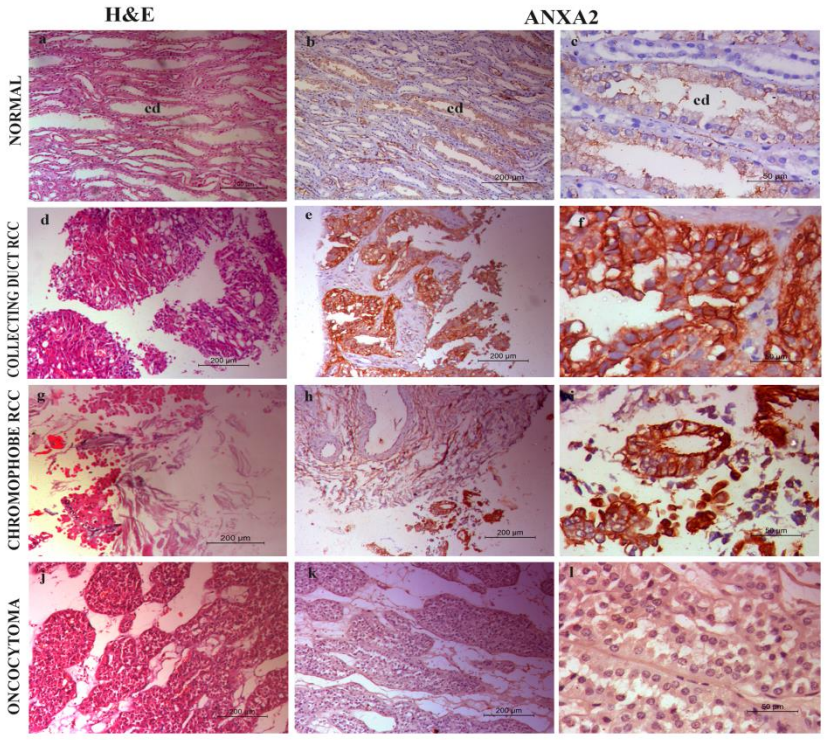

Fig 24: *AnxA2 immunostaining in variants of RCC.* a) H & E section of normal adult renal medulla showing mature tubules with collecting ducts (cd). Fig b) & c) AnxA2 staining of normal adult renal medulla demonstrating moderate membranous and cytoplasmic staining in the collecting duct cells. d) H & E section of collecting duct RCC. Fig e & f) Strong staining for AnxA2 in the tumor cells of collecting duct carcinoma. g) H & E section of Chromophobe RCC. Fig h & i) Strong expression of AnxA2 can be appreciated in the tumor cells of Chromophobe RCC. j) H & E section of Oncocytoma. Fig k & l) Mild expression of AnxA2 demonstrated in the tumor cells of Oncocytoma. Magnification 10X, Bar=200µm & 40X, Bar=50µm.

We retrospectively compared the expression of AnxA2 in the proximal tubules of fetal kidney at different stages of development with the staining of increasing grades of CCRCC tumor cells (Figures 25A, 25a to 25f). The expression of the protein in the proximal tubular cells of fetal kidneys at earlier gestational weeks was obvious and the further reappearance of AnxA2 in the tumor cells supports the precedential theory. Though both CCRCC and papillary RCC have similar embryonic origin (proximal tubules cells), AnxA2 gene mutation could be speculated only for CCRCC, in papillary RCC an inactivation of AnxA2 may be most probably implicated.

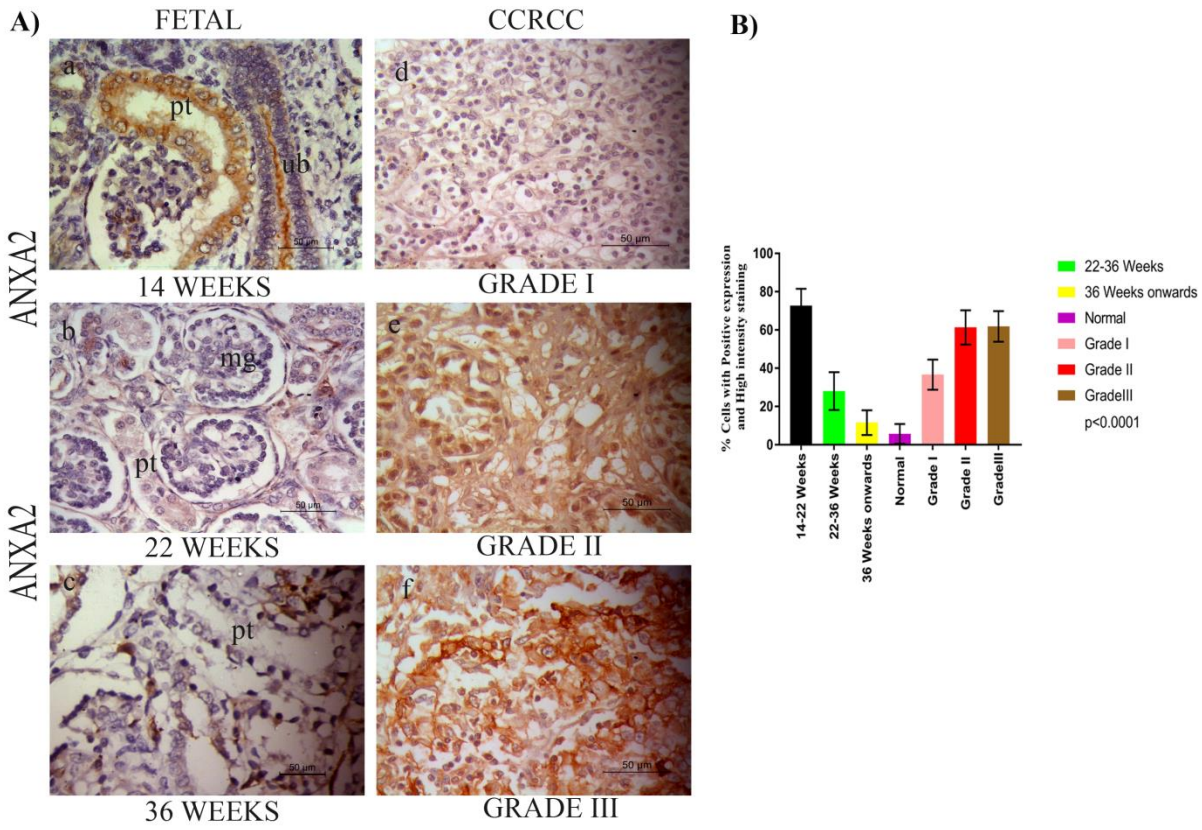

Fig 25A: Comparison of *AnxA2 immunostaining in proximal tubular cells of fetal kidney with tumor cells of CCRCC.* a) Moderate to strong staining for ANXA2 in the cytoplasm of proximal tubular cells of fetal kidney at 14 weeks of gestational age. b) AnxA2 staining of proximal tubular cells demonstrating mild cytoplasmic staining in fetal kidney at 24 weeks of gestational age. c) Lack of AnxA2 staining in the proximal tubular cells of fetal kidney at 36 weeks of gestation. Fig d, e & f) Mild to strong staining of tumor cells of CCRCC with progression of cancer from low grade to higher grade of poorly differentiated CCRCC. Magnification 40X, Bar=50µm. B) Data were calculated as average percentage of AnxA2 expressing cells in the proximal tubular cells of fetal, non-neoplastic adult tissues against tumor cells of CCRCC at different tumor grades. The percentage of tumor cells staining positive for AnxA2 increased with increasing tumor grade ($p < 0.0001$).

Table 10: AnxA2 Expression in Proximal tubular cells, collecting duct cells of fetal and adult kidney and, AnxA2 Expression in cRCC tumor cells

AnxA2 Expression in PT and cRCC tumor cells (n=no of cases)					P Value
Group	0	+1	+2	+3	
14-22 gestational weeks (n=15)	0	06	9	0	<0.0001
22-36 gestational weeks (n=13)	06	05	02	0	
36 gestational weeks onwards(n=14)	04	09	01	0	
Adult (n=37)	22	15	0	0	
Crcc (n=28)	02	08	15	03	
AnxA2 Expression in CD cells (n=no of cases)					**P Value**
Group	0	+1	+2	+3	
14-22 weeks (n=15)	0	4	11	0	0.0001
22-36 weeks (n=13)	0	1	7	5	
36 weeks onwards(n=14)	0	0	7	7	
Adult (n=37)	3	11	23	0	

Statistical analysis of expression status of AnxA2 in proximal tubular cells of fetal kidney at different gestational periods, adult kidney vs tumor cells of cRCC by One-

way Anova (P<0.0001). Statistical analysis of expression status of AnxA2 in collecting ducts of fetal and adult kidney by Chi-square test (P=0.0001).

References:

1. Lacson A, Bernstein J, Risdon RA, Gilbert-Barness E. Renal system. In: Gilbert-Barness E, editor. Potter's pathology of the fetus, infant and child. 2nd ed. Philadelphia: Elsevier Mosby, 2007:1281–1344.
2. Tank KC, Saiyad SS, Pandya AM, Akbari VJ, Dangar KP. A study of histogenesis of human fetal kidney. Int J Biol Med Res 2012;3:1315–1321.
3. Bertelli E, Regoli M, Fonzi L, Occhini R, Mannucci S, Ermini L, Toti P. Nestin expression in adult and developing human kidney. J Histochem Cytochem 2007;55:411–421.
4. Bonsib FM. Renal anatomy and histology. In: Jennette JC, editor. Heptinstall's pathology of the kidney. 7th ed. Philadelphia: Wolters Kluwer Health, 2015: 1-65.
5. Young B, Lowe JS, Stevens A, Heath JW. Wheater's functional histology: a text and color atlas, 5th ed. Philadelphia: Churchill Livingstone Elsevier Ltd, 2009:302-327.
6. Junqueira LC, Jose C, editors. Basic histology: text and atlas, 11th ed. New York: McGraw- Hill, 2005:373-391.
7. Eroschenko VP. Urinary system. In: Eroschenko VP, editor. Di Fiore's atlas of histology with functional correlations. Philadelphia: Lippincott Williams & Wilkins, 2005:309-327.
8. Truong LD, Shen SS. Immunohistochemical diagnosis of renal neoplasms. Arch Pathol Lab Med 2011;135:92-109.
9. Kuroda N, Tanaka A, Ohe C, Nagashima Y. Recent advances of immunohistochemistry for diagnosis of renal tumors. Pathol Int 2013;63:381–390.

Chapter 6

DISCUSSION

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

6. DISCUSSION

The fetal kidney during the early developmental period largely contributes in the regulation of amniotic fluid level and fetal blood pressure. Though pronephros disintegrates, the mesonephros functions for a brief period of time and produces urine by the 5th week of gestation. However it degenerates by 11-12th week of gestation. Few of the caudal mesonephric tubules develop into various components, primarily of male genital system.[1] The first human metanephric glomerulus is formed at 9th week and nephrogenic zone is seen until 36th week of gestation. The process of nephrogenesis is completed at 34th to 36th week of gestation.[1] The complex developmental and functional architecture of human kidney is regulated by molecular factors such as Glial cell derived neurotrophic factor (GNDF) and Wilms' tumor suppressor gene 1 (WT1), which stimulates the branching of ureteric bud to form the collecting component of kidney. Fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 7 (Bmp7) blocks apoptosis and stimulate proliferation of metanephric mesenchyme, Paired box gene 2 (pax2) and Wingless related gene (WNT4) participates in the differentiation of mesenchyme to nephron epithelium.[1]

The present study demonstrates the expression of AnxA1 in podocytes, mesangial, parietal epithelial cells and endothelial cells of developing mature glomeruli during early phase of nephrogenesis. The expression of AnxA1 in the mesangial cells persisted in adulthood. These results suggested a probable correlation of physiological roles of AnxA1 and certain functions of glomerular cells.

Mesangial cells occupy central position in the renal glomerulus, mostly found in the stalk of glomerular tuft. They may get originated from mesenchymal cells that

develop other cells of nephron or develop from extra renal components such as hematopoietic lineages.[2,3] Several vasoactive agents such as Angiotensin II and Vasopressin are thought to influence the glomerular mesangial function by activation of Phospholipase C for PIP_2 (Phosphotidyl-inositol 4, 5- bisphosphate) which in turn results in the formation of diacylgycerol (DAG) and inositol triphosphate (IP_3).[2]

AnxA1 act as sensors for lipid second messengers such as PIP_2 and DAG and further increase in Ca^{2+} levels promotes membrane binding of AnxA1.[4] On activation of Protein Kinase C (PKC) by its major activator DAG, PKC participates in various signaling pathways by phosphorylating target proteins. In the mesangial cells PKC has a role in hormone induced prostaglandin formation and acts as a negative feedback regulator of inositol lipid signaling cascade, having a major role in regulating glomerular filtration rate.[5] Upon elevation of Ca^{2+}, both PKC and AnxA1 are known to translocate from cytosol to plasma membrane to interact with each other.[4] *In-vivo* and *in-vitro* studies on rat mesangial cells demonstrated that AnxA1 phosphorylation catalyzed by PKC required the presence of Ca^{2+}, phospholipids and PKC activators such as angiotensin II and vasopressin and the phosphorylation occurred at serine and tyrosine residues of NH_2 terminal end of AnxA1.[6] This interaction brings about wide variety of cellular responses. Increase in intracellular Ca^{2+} also activates Phospholipase A_2 and arachidonic acid release from membrane phospholipids and thus local prostaglandin formation in mesangial cells contributing to its various functions.[2] AnxA1 together with cytosolic Phospholipase A_2 ($cPLA_2$) is translocated to the plasma membrane. Although cleaved AnxA1 maintains its interaction with $cPLA_2$, it fails to inhibit the phosphorylation of $cPLA_2$, thus leading to the

release of arachidonic acid.[7] The association between AnxA1 and PKC, AnxA1 and PLA_2 are therefore critical determinants in the regulation of the glomerular functions by the mesangial cells.

Although GFR is established during intra uterine life, it is barely significant, as the fetal kidneys do not primarily function in regulating water and fluid electrolytes. A gradual increase in blood flow during nephrogenesis suggests that the kidneys are able to autoregulate early in life but with relatively lower efficiency.[1]

In the present study, we found a mild positivity for AnxA1 in the mesangial cells at later stages of gestation. We predict that AnxA1 may aid in auto regulation, however at a lower base line.

Shuk man ka et al demonstrated that full length AnxA1 and its cleaved form are released by mesangial cells.[8] However the mode of secretion of AnxA1 protein remains an unsolved mystery. A new mode of secretion of AnxA1 through the release of microparticles or micro vesicles from its various cell sources has been hypothesized. These exosomes are shed from activated cells by a flippase-scramblase mechanism, where there is a reversing of its lipid bilayer exposing phosphotidyl serine to the exterior.[9] A recent study, demonstrated the involvement of AnxA1 in calcium dependent production and clustering of ceramides on the plasma membrane of cells. Ceramide is a key lipid mediator for apoptosis.[4] Under the influence of calcium, AnxA1 is known to bind to negatively charged phospholipids such as phosphatidylserine.[10] Evidence suggests that ceramides along with phosphatidyl serine promote binding of AnxA1 to apoptotic cells and mediate their clearance followed by AnxA1 induced apoptosis of surrounding cells.[4] In humans, apoptosis occurs both in developing and adult kidney but the rate of apoptosis

is particularly intense in early phases of nephrogenesis.[11,12] Glomerular mesangial cells behave as semi-professional phagocytes. They deploy various mechanisms for the phagocytosis and clearance of apoptotic bodies in order to eliminate unwanted cells, so as to fine tune the number of renal cells and also prevent a local inflammatory insult due to secondary necrosis of un-cleared apoptotic cells.[13] The strong expression of AnxA1 as observed in our study, in the mesangial cells of fetal kidneys of early gestational weeks could be correlated to this function of mesangial cells and the expression of AnxA1 could be speculated much earlier.

Angiogenesis (formation of new capillaries from existing preformed capillaries) is essential for enhancing the vascular network in normal processes. VEGF is the major promoter that increases the interaction between AnxA1 and actin which is essential for cytoskeleton remodeling into lamellipodia and thus migration and proliferation of capillary endothelial cells. Thus AnxA1 is an important regulator in VEGF mediated angiogenesis.[14] Immunopositive AnxA1 expression as demonstrated in endothelial lined capillary loops of fetal and adult glomeruli in the current study may presumably be attributed to this function of AnxA1.

Podocytes and parietal epithelial cells have been implicated in maintaining molecular sieve like structure of glomerulus and its functions. They aid in the maintenance of filtration barrier and transport respectively. If the podocyte structure is compromised, it leads to development of proteinuria and progressive glomerulosclerosis.[15] A proteomic study on cultured podocytes showed abundant actin cytoskeletal proteins, annexins and vascular endothelial growth factor and they also expressed glucocorticoid receptors.[16] Although AnxA1 promoter contains glucocorticoid

response elements, it's thought to act as a second messenger in glucocorticoid induced cellular responses which is required for both kidney development and recovery from physiological stress. Intracellular AnxA1 is also found to be associated with the endosomes at 1-26 residues of NH_2 terminal domain indicating its involvement in endocytotic processes.[17] The present investigation shows that AnxA1 is expressed in human fetal podocytes, and fewer parietal epithelial cells of maturing glomerulus which suggest a significant role in the biology of renal corpuscle. Perhaps the expression in both the cell types could be due to the fact that both cells have a common mesenchymal origin and are the result of divergent differentiation during embryogenesis.[18] Further a study on Adriamycin induced glomerulopathy in mice model demonstrated high levels of urinary AnxA1and its signals were appreciated in the renal podocytes. Their data suggested that the urinary AnxA1were derived from the apoptotic renal tissues.[8]

Renal cell carcinoma accounts for 3% of adult malignancies and 90% of which arise from the renal tubules. The histological subtypes of renal cell carcinoma such as the clear cell and papillary renal cell carcinoma are thought to be derived from proximal tubular cells and chromophobe and collecting duct from the distal portions of nephron and renal pelvis respectively.[19] Zimmermann et al demonstrated the up regulation of AnxA1 expression in the conventional renal cell carcinoma and correlated to Fuhrman grade, clinical outcome and metastatic potential.[20] A recent study by Yamanoi et al showed the expression of AnxA1 in the membranes of renal cell carcinoma cells received from 27 patient specimens with evidences suggesting its positive correlation with respect to patients' malignant outcome.[21] Our findings are in accordance with their study with strong expression for AnxA1 in the membranes of clear renal cell carcinoma cells which

increased with the progression of tumor. The absence of immunostaining in the proximal tubular cells of normal adult kidney indicates malignant transformation of these cells. Our findings also support the observation that low AnxA1 expression in normal tissues increase during tumor transformation.[22]

Current study reports the increased expression status in other histological subtypes such as chromophobe, papillary, collecting duct renal cell carcinoma and, oncocytoma. Zimmermann et al emphasized the expression of AnxA1 as a feature of eosinophilic cells in conventional renal cell carcinoma. The latter indicating the degree of tumor differentiation.[20] Renal neoplasms with granular or eosinophilic cells also includes oncocytoma, eosinophilic variant of chromophobe RCC, papillary RCC type 2, and collecting duct carcinoma speculating AnxA1 expression in these neoplasms. Contrary to normal cells the tumor cells are under constant oncogenic stress, genomic instability and cellular hypoxia. Under the influence of these stimuli it activates the intrinsic pathway of apoptosis triggering the release of pro-apoptotic proteins which could be disabled by the tumor cells by destabilizing the latter.[23] AnxA1 behaves differently in different tumor types. For example, in cancers such as breast, bladder, gastric and leukemia it could act both like a tumor suppressor and as an oncogene.[24] The subcellular localization of AnxA1 (cytosol, nuclear, membrane) is suggested to play important role in tumorigenesis through its interaction with various cognate partners. Several studies have indicated the role of AnxA1/FPR (formyl peptide receptors) in cancer progression. A study on keratinocyte proliferation in squamous cancer cells showed that AnxA1 interaction with $cPLA_2$ required the formation of S100A11/AnxA1 complex and on proteolytic cleavage AnxA1 lost its capacity to bind to S100A11 thus $cPLA_2$ maintained its active state enabling

keratinocyte proliferation. In few cancers, the disruption of the S100A11/AnxA1 complex and activation of EGFR downstream signaling pathways enabling enhanced migration has been established.[7] AnxA1 is a protein that inhibit inflammation while stimulate VEGF mediated angiogenesis.

To date, several studies have dealt with immunohistochemical markers expressed by renal cells of nephron lineage with little attention to markers of non-nephron lineage such as those originating from cortical, medullary interstitium or mesangium.[25] The expression of AnxA1 in the human kidney appears to be linked to several physiological functions of glomerulus. It restores intracellular homeostasis by participating in specific pathways and receptor interactions and thus making it an accessible target for therapy in altered physiology.[4,6] This biomarker may constitute the key to uncover early molecular events during nephrogenesis and may be used as a strategy to deal with further renal pathogenesis.

70% of renal cell carcinomas are clear cell carcinomas and are thought to be derived from proximal convoluted cells.[26] Since the expression of AnxA1 is observed to be absent in the fetal and adult proximal tubular cells, their expression in the renal tumor cells shows malignant transformation of the normal cells indicating an aberrant expression pattern unlikely to be due to reactivation of a repressed gene in the process of normal embryonic development. Further its interaction with formyl peptide receptors on the cell surface of tumor cells makes it a potential target to be assessed in therapy as the formyl peptide receptors can be activated or silenced by specific ligands.[7] In addition to this, AnxA1 is a known anti-inflammatory upstream regulatory molecule for all the downstream devastation of cytokines like TNF α, IL-6, IL-17 etc. Correlating the level of

this critical anti-inflammatory molecule during nephrogenesis, normal adult functional kidney and further during various clinical conditions will be ideal to know as in most of these diseased renal conditions, inflammation is the leading complication.

Cancer cell orchestrates early embryonic cells. Both kinds of cells undergo deprogramming to become immortal and invasive.[27] Currently, developmental biologists are focused on the genes involved in the biological process of cell differentiation, as tumorigenesis is mainly thought to be due to the disruption of normal cell differentiation process controlled by various genes; mutations of such genes have a major role in tumor initiation and progression.[28]

AnxA2 (36KDA, LIPOCORTIN II) is a multifunctional calcium regulated phospholipid binding protein present abundantly in endothelial, smooth muscle, trophoblast and few tumor cells.[29] AnxA2 functions as either a monomer or heterotetramer. The annexin A2–S100A10 heterotetramer interacts with cytoskeletal, membrane and extracellular matrix regulating a wide variety of biological processes that includes tissue remodeling, degradation of extracellular matrix, angiogenesis, actin cytoskeletal dynamics, endocytosis, exocytosis, cell-cell adhesion and cell polarity thereby mediating regulatory effects on cell behavior.[30,31] Kidney development is closely related to cell differentiation, proliferation and apoptosis and in addition, reorganization of cytoskeleton of metanephric mesenchyme by the formation of cell-to-cell contacts and extracellular matrix interactions is leading to the completion of developmental program.[11]

To understand the role of AnxA2 in human renal development and cancer, it is necessary to establish its localization in various developing renal structures. Our study on the ontogeny of AnxA2 during renal development reveals that this molecule is expressed in

the ureteric bud and its derivatives such as the collecting tubules and collecting ducts at all-time points (as early as 12th week). The expression often appeared within the cells or on the plasma membrane. In addition, moderate cytosolic expression was detected in the early developing proximal tubules. Similar expression pattern was observed in one of the fetal kidney (not included in the study group) at 12 week of gestation, speculating a much earlier AnxA2 expression. Our study confirmed that AnxA2 expression in the proximal tubules, declined (+1) with increasing gestational age. Earlier works on the expression of AnxA2 in animal models have emphasized its role in calcium homeostasis. Calcium (Ca^{2+}) pulses, waves and gradients are involved in coordination of cell movements, axis specification during early vertebrate development. Recent recordings of calcium wave in metanephric blastemal cells of explanted rat embryonic kidney suggested the requirement of calcium for kidney development. Gilbert et al, showed a restricted expression of AnxA2 in the ureteric bud and collecting ducts of embryonic mouse kidney, indicating its participation in Ca^{2+} homeostasis via membrane traffic or regulation of ion channel activities in the developing permanent kidneys.[32] A study conducted to examine the relationship between dentin phosphoprotein and AnxA2 showed their membranous and cytosolic co localization and physiological calcium dependent binding in rat kidney ureteric bud cells, this likely indicating the participation of the molecule in calcium transport.[33] Another member of annexin family, annexin IV is also acknowledged to play important role in pronephros morphogenesis.[32] The cytosolic and membranous expression of AnxA2 is often involved in the regulation of actin cytoskeleton dynamics, endocytosis and exocytosis, cell-cell adhesion, cell polarity and endosome formation.[30]

AnxA2 has specific roles in the kidney. In the collecting duct it mediates cAMP-induced trafficking of aquaporin2 while in the thick ascending limb of Henle (TAL), it participates in the recruitment and activation of Na^+-K^+-2Cl (NKCC2) co-transporter. NKCC2, responsible for urine concentration and systemic salt homeostasis interacts with AnxA2 in phosphorylation dependent manner. The latter promotes its apical translocation in response to vasopressin signaling.[34]

Several studies provide evidence of co localization of AnxA2- S100A10 complex with TRPV5 and TRPV 6 calcium channel in renal tubules, suggesting its role in apical ion transport in MDCK cells (an epithelial line with features of collecting ducts) and active calcium reabsorption.[17,35] Further, an association of the heterotetramer complex with AHNAK protein in the formation of cell-to-cell contacts and cell polarity in MDCK cells was observed.[17] Domoto et al, demonstrated the expression of both antigens (AnxA2 and S100A10) in the renal medulla of normal human kidney, primarily in the loop of Henle, distal convoluted tubules and collecting duct cells.[35] Current study also demonstrated a similar expression pattern, AnxA2 being largely expressed in the endothelial cells, collecting tubules and collecting duct cells of the normal adult kidney and occasional in the loop of Henle and distal tubular cells, this suggesting a possible role of the protein in calcium uptake from these nephron' components. The lack of AnxA2 expression in the proximal tubules of adult human kidney suggests that in these cells probably AnxA2 fails to play a role in structural organization of the membrane, housekeeping functions or regulation of the membrane intracellular traffic.[36]

AnxA2 is expressed in a wide spectrum of cancer that includes: cervical, breast, hepatic, non-small cell lung carcinoma and multiple myeloma.[37] Renal cell carcinomas are the

most common amongst the genitourinary cancers and AnxA2 has been found in a subset of renal neoplasms such as CCRCC, papillary and chromophobe RCC.[37] AnxA2 monomer exists in the cell cytoplasm and nucleus and AnxA2 heterotetramer is present on the cell membrane. Nuclear AnxA2 is thought to promote DNA replication and chromosomal instability explaining the rapid tumor growth and resistance to chemotherapy.[29] When it is located on the extracellular surface, AnxA2, a natural ligand of S100A10, also promotes the formation of plasmin and active forms of matrix metalloproteases, resulting in extracellular matrix degradation, with further membrane mediate cytoskeletal remodeling, thus permitting tumor invasion and metastases.[30,31,35,37]

Our findings corroborate earlier reports regarding the strong expression of AnxA2 in cancer cell membranes of CCRCC.[37] While the tumor cells of chromophobe, collecting ducts carcinoma and oncocytoma were AnxA2 immunopositive, the tumor cells of papillary RCC were immunonegative. CCRCC and papillary RCC are thought to be derived from the proximal tubular epithelium while chromophobe, collecting ducts carcinoma and oncocytomas are derived from the ureteric bud.[38] This probably explains the variability in the expression of AnxA2 in different types of renal tumors. Accordingly, we observed the presence of AnxA2 expression in the normal renal epithelia of the distal tubules, loop of Henle, collecting duct system, endothelial cells, but not in the proximal tubules (thought to be the origin of the most CCRCC), this possibly indicating a malignant transformation of the normal cells.

Our study points towards AnxA2 expression in embryonic and neoplastic kidney that could be of clinical relevance in designing new standard markers against CCRCC and maladies attaining ureteric bud and embryonic collecting ducts. The membranous and

cytosolic expression of AnxA2 in the fetal and adult renal tubules may presumably play an essential regulatory role in the maintenance of cell- cell contacts, membrane cytoskeletal dynamics, calcium homeostasis and other cell signaling events. If AnxA2 is not often expressed in the proximal tubules of normal adult kidney, fetal kidneys show moderate expression in the proximal tubules and a strong expression in the CCRCC suggesting a deregulation of the gene during tumorigenesis. AnxA2 is also expressed in the ureteric bud and its derivatives such as collecting tubules, collecting ducts and chromophobe, collecting duct RCC.

The expression analysis of AnxA1 and AnxA2 in the nascent, adult and neoplastic human kidney could be of interest to the developmental and cancer biologists in designating of molecular networks that could bridge the gap between the involvement of these molecules in the developing kidney and renal tumorigenesis.

References:

1. Sulemanji M, Vakili K. Neonatal renal physiology. Semin Pediatr Surg 2013;22:195–198.
2. SchlöndorffD. The glomerular mesangial cell: an expanding role for a specialized pericyte. FASEB J 1987;1:272–281.
3. Quaggin SE, Kreidberg JA. Development of the renal glomerulus: good neighbors and good fences. Development 2008;135:609-620.
4. Monastyrskaya K, Babiychuk EB, Draeger A. The annexins: spatial and temporal coordination of signaling events during cellular stress. Cell Mol Life Sci 2009;66:2623–2642.
5. Pfeilschifter J. Regulatory functions of protein kinase c in glomerular mesangial cells. Klin Wochenschr 1990; 68:1134–1137.
6. D'Acunto CW, Gbelcova H, Festa M, Ruml T. The complex understanding of Annexin A1 phosphorylation. Cell Signal 2014;26:173–178.
7. Boudhraa Z, Viallard B, Bouchon C, D'Incan M, Degoul F. Annexin A1 localization and its relevance to cancer. Clin Sci 2016;130:205–220.
8. Ka SM, Tsai PY, Chao TK, Yang SM, Hung YJ, Chen JS, Shui HA, Chen A. Urine annexin A1 as an index for glomerular injury in patients. Dis Markers 2014:1-12.
9. Perretti M, Dalli J. Exploiting the Annexin A1 pathway for the development of novel anti-inflammatory therapeutics. Br J Pharmacol 2009;158: 936–946.

10. Weyd H, Abeler-Dörner L, Linke B, Mahr A, Jahndel V, Pfrang S, Schnölzer M, Falk CS, Krammer PH. Annexin A1 on the surface of early apoptotic cells suppresses CD8+ T cell immunity. PLoS One 2013;8:e62449.
11. Jeon YJ, Kim J, ChaeJIl. Comparative proteomic analysis of kidney development-related proteins in the pig. Vitr Cell Dev Biol Anim 2013;49:315–323.
12. Sanz AB, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A. Mechanisms of renal apoptosis in health and disease. J Am Soc Nephrol 2008;19:1634–1642.
13. Cortes-Hernandez J, Fossati-Jimack L, Carugati A, Potter PK, Walport MJ, Cook HT, et al. Murine glomerular mesangial cell uptake of apoptotic cells is inefficient and involves serum-mediated but complement-independent mechanisms. Clin Exp Immunol. 2002;130(3):459–66.
14. Pin AL, Houle F, Fournier P, Guillonneau M, Paquet ÉR, Simard MJ, et al. Annexin-1-mediated endothelial cell migration and angiogenesis are regulated by vascular endothelial growth factor (VEGF)-induced inhibition of miR-196a expression. J Biol Chem. 2012;287(36):30541–51.
15. Bertelli E, Regoli M, Fonzi L, Occhini R, Mannucci S, Ermini L, Toti P. Nestin expression in adult and developing human kidney. J Histochem Cytochem 2007;55:411–421.
16. Schnenberger E, Ehrich JH, Haller H, Schiffer M. The podocyte as a direct target of immunosuppressive agents. Nephrol Dial Transplant. 2011;26(1):18–24.

17. Markoff A, Gerke V. Expression and functions of annexins in the kidney. Am J Physiol Renal Physiol 2005; 289:F949-56.
18. Miesen L, Steenbergen E, Smeets B. Parietal cells—new perspectives in glomerular disease. Cell Tissue Res. 2017;369(1):237–44.
19. Truong LD, Shen SS. Immunohistochemical diagnosis of renal neoplasms. Arch Pathol Lab Med 2011;135:92-109.
20. Zimmermann U, Woenckhaus C, Teller S, Venz S, Langheinrich M, Protzel C, et al. Expression of annexin AI in conventional renal cell carcinoma (CRCC) correlates with tumour stage, Fuhrman grade, amount of eosinophilic cells and clinical outcome U. Histol Histopathol. 2007;22:527–34.
21. Yamanoi M, Yamanoi K, Fujii C, Fukuda MN, Nakayama J. Annexin A1 expression is correlated with malignant potential of renal cell carcinoma. Int J Urol. 2019;26(2):284–90.
22. Biaoxue R, Xiguang C, Shuanying Y. Annexin A1 in malignant tumors: current opinions and controversies. Int J Biol Markers 2014;29:e8–e20.
23. Fernald K, Kurokawa M. Evading apoptosis in cancer. Trends Cell Biol 2013;23:620–633.
24. Sheikh, M.H, SolitoE. Annexin A1: Uncovering the many talents of an old protein. Int J Mol Sci 2018;19:1–20.
25. Gerosa C, Fanni D, Nemolato S, Di Felice E, Iacovidou N, Eyken PV, Faa G, Cataldi L, Fanos V. Shift of galectin-3 expression in the human kidney during development. J Pediatr Neonat Individual Med 2013;2:1–6.

26. Thedieck C, Kuczyk M, Klingel K, Steiert I, Müller CA, Klein G. Expression of Ksp-cadherin during kidney development and in renal cell carcinoma. Br J Cancer 2005;92: 2010–2017.
27. Monk M, Holding C. Human embryonic genes re-expressed in cancer cells. Oncogene, 2001, 20(56):8085-8091.
28. Ma Y, Zhang P, Wang F, et al. The relationship between early embryo development and tumourigenesis. J Cell Mol Med, 2010, 14(12):2697-2701.
29. Cheng CW, Rifai A, Shuk-Man KA, et al. Calcium-binding proteins annexin A2 and S100A6 are sensors of tubular injury and recovery in acute renal failure. Kidney Int, 2005, 68(6):2694-2703.
30. Xu XH, Pan W, Kang LH, et al. Association of Annexin A2 with cancer development (review). Oncol Rep, 2015, 33(5):2121-2128.
31. Hitchcock JK, Katz AA, Schäfer G. Dynamic reciprocity: The role of annexin A2 in tissue integrity. J Cell Commun Signal, 2014, 8(2):125-133.
32. Gilbert T, Leclerc C, Moreau M. Control of kidney development by calcium ions. Biochimie, 2011, 93(12):2126-2131.
33. Alvares K, Stern PH, Veis A. Dentin phosphoprotein binds annexin 2 and is involved in calcium transport in rat kidney ureteric bud cells. J Biol Chem, 2013, 288(18):13036-13045.
34. Dathe C, Daigeler AL, Seifert W, Jankowski V, Mrowka R, Kalis R, et al. Annexin A2 mediates apical trafficking of renal Na+-K +-2Cl- Cotransporter. J Biol Chem. 2014;289(14):9983–97.
35. Domoto T, Miyama Y, Suzuki H, et al. Evaluation of S100A10, annexin II and

B-FABP expression as markers for renal cell carcinoma. Cancer Sci, 2006, 98(1):77-82.

36. Tribolo S, Maroux S, Massey-Harroche D. Kidney proximal tubule cells: Epithelial cells without EGTA-extractable annexins? Biochem Cell Biol, 2000, 78:495-502.
37. Yang SF, Hsu HL, Chao TK, Hsiao CJ, Lin YF, Cheng CW. Annexin A2 in renal cell carcinoma: expression, function, and prognostic significance. Urologic Oncology Seminars and Original Investigations, 2015, 33(1):11-21.
38. Terpe HJ, Störkel S, Zimmer U, et al. Expression of CD44 isoforms in renal cell tumors: Positive correlation to tumor differentiation. Am J Pathol, 1996, 148(2):453-463.

Chapter 7

SUMMARY AND CONCLUSION

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

7. SUMMARY AND CONCLUSION:

- AnxA1 and AnxA2 were expressed in certain cell types (AnxA1-mesangium, podocytes, tumor cells) and (AnxA2- epithelial cells, ureteric bud cells, tumor cells) in accordance with the functional properties postulated.

- The expression of AnxA1 in the human kidney appears to be linked to several physiological functions of glomerulus. It restores intracellular homeostasis by participating in specific pathways and receptor interactions and thus making it an accessible target for therapy in altered physiology.[1,2]
- This biomarker may constitute the key to uncover early molecular events during nephrogenesis and may be used as a strategy to deal with further renal pathogenesis.
- Further its interaction with formyl peptide receptors on the cell surface of tumor cells makes it a potential target to be assessed in therapy as the formyl peptide receptors can be activated or silenced by specific ligands.[2]
- The membranous and cytosolic expression of AnxA2 in the fetal and adult renal tubules may presumably play an essential regulatory role in maintenance of cell-cell contacts, membrane cytoskeletal dynamics, calcium homeostasis and other cell signaling events.
- AnxA2 is also expressed in the ureteric bud and its derivatives such as collecting tubules, collecting ducts and, chromophobe and collecting duct RCC and can be used to treat any maladies with respect to the ureteric bud and its derivatives.

- 70% of renal cell carcinomas are clear cell carcinomas and are thought to be derived from proximal convoluted cells. Since the expression of AnxA1 is observed to be absent in the fetal and adult proximal tubular cells, their expression in the renal tumor cells is indicative of aberrant expression pattern unlikely to be due to reactivation of a repressed gene in the process of normal embryonic development. Moreover, AnxA2 is not often expressed in the proximal tubules of normal adult kidney; however fetal kidneys show moderate expression in the proximaltubules (thought to be the origin of renal cell carcinoma) and a strong expression in the clear cell carcinoma suggesting a deregulation of the gene during tumorigenesis.
- The expression analysis of AnxA1 & AnxA2 in the nascent to adult human kidney could be of interest to the developmental and cancer biologists in delineating molecular networks bridging the gap between involvement AnxA1 & AnxA2 in developing kidney and renal tumorigenesis.
- The purpose of our study points towards AnxA1 & AnxA2 expression in embryonic and neoplastic kidney could be of clinical relevance in designing newer therapeutic molecules against renal cell cancer.

References:

1. Monastyrskaya K, Babiychuk EB, Draeger A. The annexins: spatial and temporal coordination of signaling events during cellular stress. Cell Mol Life Sci 2009; 66:2623–2642.
2. D'Acunto CW, Gbelcova H, Festa M, Ruml T. The complex understanding of Annexin A1 phosphorylation. Cell Signal 2014;26:173–178.
3. Boudhraa Z, Viallard B, Bouchon C, D'Incan M, Degoul F. Annexin A1 localization and its relevance to cancer. Clin Sci 2016;130:205–220.

LIMITATIONS

- In the current study we could analyze the expression status and correlate the functions of AnxA1 and AnxA2 in the fetal, adult and renal tumor cells. It would be ideal and interesting if we could delineate the molecular mechanism and how these proteins regulate their respective functions in different status.

FUTURE DIRECTIONS:

- A direct link between the expression of AnxA1 and AnxA2 in specific cell type and biological function, their mode of subcellular localization (membrane, cytosolic to nuclear) and their association with other developmental and tumor related proteins still needs to be elucidated.

ANNEXURES

Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma

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Affiliated of Rajiv Gandhi University of Health Sciences, Bangalore & Recognized by Medical Council of India, New Delhi
GOI Notification No. U.12012/95/2001-ME (P-II) and MCI Notification No.MCI-34(41)/MED.2009/5527 dated 01.05.2009
Contact - Tel.No : +91836 2477574, 2477553, Tele fax: +91836 2461651 email: sdmcmshc@gmail.com Website: sdmmedicalcollege.org

Ref: SDMIEC: 0747: 2016 Date : 20-06-2016

To,
Dr. Roshni S.,
Tutor,
Department of Anatomy,
SDM College of Medical Sciences & Hospital
Sattur, Dharwad.

Dear Dr. Roshni S.,

SUB: - Institutional Ethics Committee permission.

I am happy to inform you that **permission** is granted to you to carry out your study titled **"Comprehensive Immunophenotypic Expression Analysis of Phospholipid Binding Proteins in Renal Organogenesis and in Kidney Carcinoma".**

Thanking you,

Yours sincerely

Dr. M.A. Kamdod
Member Secretary - SDMIEC

Dr. H. Mallikarjun Swamy
Chairman – SDMIEC

B.L.D.E. UNIVERSITY

(Declared vide notification No. F.9-37/2007-U.3 (A) Dated. 29-2-2008 of the MHRD, Government of India under Section 3 of the UGC Act,1956)

The Constituent College

SHRI. B. M. PATIL MEDICAL COLLEGE, HOSPITAL AND RESEARCH CENTRE,VIJAYAPURA

IEC Ref No- 182/2016-17

13 October, 2016

INSTITUTIONAL ETHICAL CLEARANCE CERTIFICATE

The Ethical Committee of this University met on 30th June 2016 at 11 AM to scrutinize the Synopsis / Research projects of Postgraduate student / Undergraduate student / Faculty members of this University / college from ethical clearance point of view. After scrutiny the following original / corrected & revised version synopsis of the Thesis / Research project has been accorded Ethical Clearance.

Title: *"Comprehensive immunophenotypic expression analysis of phospholipids binding proteins in renal organogenesis and in kidney carcinoma ".*

Name of Ph.D./ P. G. / U. G. Student / Faculty member: Dr.Roshni Sadashiv

Name of Guide: Dr. B.N.Bannur, Prof & HoD, Dept.of Anatomy.

Dr. Sharada Metgud
Chairperson, I.E.C
BLDE University,
VIJAYAPURA– 586 103

Dr.G.V.Kulkarni
Secretary, I.E.C
BLDE University,
VIJAYAPURA – 586 103.

Member Secretary,
Institutional Ethical Committee,
BLDE University, BIJAPUR.

Following documents were placed before Ethical Committee for Scrutinization:

- Copy of Synopsis / Research project
- Copy of informed consent form
- Any other relevant document's

INFORMED CONSENT:

"COMPREHENSIVE IMMUNOPHENOTYPIC EXPRESSION ANALYSIS OF PHOSPHOLIPID BINDING PROTEINS IN RENAL ORGANOGENESIS AND IN KIDNEY CARCINOMA"

I parent (mother) of deceased foetus unreservedly, in my full senses, give my complete and informed consent for microscopic study of its renal tissue. I, hereby confirm that I have been informed (in a language understood by me) that a study is being conducted on "Comprehensive immunophenotypic expression analysis of phospholipid binding proteins in renal organogenesis and in kidneycarcinoma" The study has been explained to me in detail. I understand that the information regarding me and the deceased , collected during the course of this study will remain confidential. I understand that the records maintained will be used only for research purpose.

Date:

Parent's signature	Witness signature	Name of doctor
(Name)	(Name)	(Name)

INFORMED CONSENT:

"COMPREHENSIVE IMMUNOPHENOTYPIC EXPRESSION ANALYSIS OF PHOSPHOLIPID BINDING PROTEINS IN RENAL ORGANOGENESIS AND IN KIDNEY CARCINOMA"

I unreservedly, in my full senses, give my complete and informed consent for microscopic study of surgically resected carcinomatous renal tissue, for the purpose of research.

I hereby confirm that I have been informed (in a language understood by me) that a study is being conducted on "Comprehensive immunophenotypic expression analysis of phospholipid binding proteins in renal organogenesis and in kidney carcinoma". The study has been explained to me in detail. I understand that the information regarding me, collected during the course of this study will remain confidential. I understand that my participation in this study is voluntary. I understand that the records maintained will be used only for research purpose.

The refusal of my participation will not affect my treatment in any way and I may withdraw at any time of the study.

Date:

Patient's signature	Witness signature	Name of doctor
(Name)	(Name)	(Name)

ಶ್ರೀ ಧರ್ಮಸ್ಥಳ ಮಂಜುನಾಥೇಶ್ವರ ವೈದ್ಯಕೀಯ ಮಹಾವಿದ್ಯಾಲಯ ಮತ್ತು ಆಸ್ಪತ್ರೆ ಸತ್ತೂರ
ಧಾರವಾಡ.

ಒಪ್ಪಿಗೆ ಪತ್ರ

ನಾನು _____, ವಯಸ್ಸು _____, ಡಾ ರೋಶನಿ ಎಸ್ ಇವರಿಗೆ ಕಾಂಪ್ರಿಹೆನ್ಸಿವ ಇಮುನೋ–ಫೀನೋ ಟೈಪಿಕ್ ಎಕ್ಸ್‌ಪ್ರೆಶನ್ ಅನಾಲಿಸಿಸ್ ಆಫ್ ಫಾಸ್ಫೊಲಿಪಿಡ್ ಬೈಂಡಿಂಗ ಪ್ರೋಟೀನ್ಸ್ ಇನ್ ರೀನಲ್ ಆರ್‌ಗ್ಯಾನೊಜೆನೆಸಿಸ್ ಅ್ಯಂಡ್ ಇನ್ ಕಿಡ್ನಿ ಕಾರ್ಸಿನೋಮಾ ಎಂಬ ಸಂಶೋಧನೆಯಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಸಂಪೂರ್ಣ ಒಪ್ಪಿಗೆ ಕೊಡುತ್ತೇನೆ. ಶಸ್ತ್ರ ಚಿಕ್ಸಿತ್ಸೆಯಿಂದ ತೆಗೆದ ಕ್ಯಾನ್ಸರ್ ಮೂತ್ರಪಿಂಡದ ತುಣುಕನ್ನು ಸೂಕ್ಷ್ಮದರ್ಶಕದಿಂದ ಸಂಶೋಧನೆಗೆ ಒಳಪಡಿಸಲು ನಾನು ಒಪ್ಪಿರುತ್ತೇನೆ. ಸಂಶೋಧನೆಯ ಬಗ್ಗೆ ನನಗೆ ಅವರು ಪೂರ್ತಿ ಮಾಹಿತಿ ನೀಡಿರುತ್ತಾರೆ.

ಈ ಮೇಲಿನ ಎಲ್ಲಾ ವಿವರಣೆಗಳನ್ನು ಹಾಗೂ ಈ ಸಂಶೋಧನೆಯ ಉದ್ದೇಶವನ್ನು ನಾನು ಓದಿ ಅರ್ಥ ಮಾಡಿಕೂಂಡಿದ್ದೇನೆ ಎಂದು ಖಚಿತ ಪಡಿಸುತ್ತೇನೆ. ಈ ಸಂಶೋಧನೆಯ ಬಗ್ಗೆ ನನಗೆ ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ಅವಕಾಶಮಾಡಿಕೊಟ್ಟಿದ್ದಾರೆ.

ವೈದ್ಯರ ಸಹಿ ವ್ಯಕ್ತಿಯ / ಸಂಬಂಧಿಕರ ಸಹಿ

ವೈದ್ಯರ ಪೂರ್ಣ ಹೆಸರು ವ್ಯಕ್ತಿಯ / ಸಂಬಂಧಿಕರ ಪೂರ್ಣ ಹೆಸರು

ವ್ಯಕ್ತಿ / ರೋಗಿಗೆ ಸಂಬಂಧ

ಶ್ರೀ ಧರ್ಮಸ್ಥಳ ಮಂಜುನಾಥೇಶ್ವರ ವೈದ್ಯಕೀಯ ಮಹಾವಿದ್ಯಾಲಯ ಮತ್ತು ಆಸ್ಪತ್ರೆ ಸತ್ತೂರ ಧಾರವಾಡ.

ಒಪ್ಪಿಗೆ ಪತ್ರ

ನಾನು (ತಾಯಿ) _____, ವಯಸ್ಸು _____,ನನ್ನ ಮೃತ ಭೂಣದ ಮೂತ್ರಪಿಂಡವನ್ನು ಸೂಕ್ಷ್ಮದರ್ಶಕದಿಂದ ಡಾ, ರೋಶನಿ ಎಸ್ ಇವರಿಗೆ ಕಾಂಪ್ರಿಹೆನ್ಸಿವ ಇಮುನೋ–ಫೀನೋ ಟೈಪಿಕ್ ಎಕ್ಸ್‌ಪ್ರೆಶನ್ ಅನಾಲಿಸಿಸ್ ಆಫ್ ಫಾಸ್ಫೊಲಿಪಿಡ್ ಬೈಂಡಿಂಗ ಪ್ರೋಟೀನ್ಸ್ ಇನ್ ರೀನಲ್ ಆರ್‌ಗ್ಯಾನೊಜೆನೆಸಿಸ್ ಆ್ಯಂಡ್ ಇನ್ ಕಿಡ್ನಿ ಕಾರಾಸಿನೋಮಾ ಎಂಬ ಸಂಶೋಧನೆಗೆ ಒಳಪಡಿಸಲು ಸಂಪೂರ್ಣ ಒಪ್ಪಿಗೆ ಕೊಡುತ್ತೇನೆ. ಸಂಶೋಧನೆಯ ಬಗ್ಗೆ ನನಗೆ ಅವರು ಪೂರ್ತಿ ಮಾಹಿತಿ ನೀಡಿರುತ್ತಾರೆ.

ಈ ಮೇಲಿನ ಎಲ್ಲಾ ವಿವರಣೆಗಳನ್ನು ಹಾಗೂ ಈ ಸಂಶೋಧನೆಯ ಉದ್ದೇಶವನ್ನು ನಾನು ಓದಿ ಅರ್ಥ ಮಾಡಿಕೊಂಡಿದ್ದೇನೆ ಎಂದು ಖಚಿತ ಪಡಿಸುತ್ತೇನೆ. ಈ ಸಂಶೋಧನೆಯ ಬಗ್ಗೆ ನನಗೆ ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ಅವಕಾಶಮಾಡಿ ಕೊಟ್ಟಿದ್ದಾರೆ.

ವೈದ್ಯರ ಸಹಿ

ತಾಯಿ ಸಹಿ

ವೈದ್ಯರ ಪೂರ್ಣ ಹೆಸರು

ತಾಯಿ ಸಹಿ / ಪೂರ್ಣ ಹೆಸರು

PROFORMA FOR COLLECTION OF SAMPLE:

Baseline demographic characteristics and medical history information

Name of the patient(Mother):

Maternal age:

IP NO:

OP NO:

Clinical Data:

Hypertension: Yes/No

Diabetes Mellitus: Yes/No

Under medications:

Habits: Smoking/Alchohol/Tobacco

Illnesses in the past:

Basic haematological investigations: Hb, HbAg, HIV

Obstetric history:

Gravida/Para/Abortions

Mode of delivery

USG Details:

Gestational age:

Single/multiple:

Presentation:

Liquor:

Placenta:

Malformations:

Anamoly scanning reports:

Details of the deceased foetus:

Gestational age:

Weight:

Sex:

Cause of death:

Autopsy report of the foetus:

External examination:

Weight/crown rump length/rump to heel/abdominal circumference/head circumference/chest circumference/umbilical cord stump length/right foot/left foot

Internal examination:

Thyroid/thymus/trachea/oesophagus/lungs/heart/git/liver/pancrease/spleen/adrenals/brain/genital organs

KIDNEYS: MACROSCOPIC EXAMINATION

Mass:

Position:

Form:

Microscopic details of renal tissue: Parenchymal differentiation

INSTITUTIONAL PATHOLOGY AUTOPSY REPORT:

DETAILS OF PATIENT DIAGNOSED WITH RENAL CELL CARCINOMA:

Name:

Age:

IP NO:

OP NO:

Detailed clinical history:

Investigations: MRI/CT SCAN/USG

INSTITUTIONAL BIOPSY REPORT:

GADAG INSTITUTE OF MEDICAL SCIENCES

Gadag, Karnataka, INDIA

WORLD CONGRESS OF ANATOMISTS

Host : Karnataka Chapter of Anatomists

Certificate

Dr./Mr/Mrs/Miss ROSHNI SADASHIV bearing Reg.No. 22731A

Registered with _____ Medical Council. Address _____

_____ has been a Delegate/Speaker/Chairperson/

Organizing Committee Member in World Congress of Anatomists, 19th KCA Conference & Post Conference CME held from 22nd to 24th Sept. 2017 & presented a Guest Lecture/Paper/Poster tilted Comparative expression analysis of phospholipid binding protein Annexin A1 in nephrogenesis

Karnataka Medical Council has granted 6 Credit hours for delegates/faculty vide letter No. K.M.C./C.M.E./ 363/2017 Dated : 29-07-2017

Dr. Uma B. R.	Dr. Sanjeev Kolagi	Dr. (Smt) S. Kantha	Dr. Bernard Moxham	Dr. P. S. Bhusaraddi
Zonal Chairman K.M.C. C.M.E. Accreditation Committee	Secretary Karnataka Chapter of Anatomists	Founder President Karnataka Chapter of Anatomists	Past President International Federation of Anatomists	President, World Congress of Anatomists Director, GIMS Gadag

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Organised by

Department of Anatomy AIIMS Rishikesh

Certificate of Participation

This is to certify that

Prof./Dr./Mr./Ms.....Roshni Sadashiv.....

presented a Scientific Research Paper entitled

Annexin A2 Nephrogenesis and Kidney Cancer

at

"National Conference of Anatomical Society of India"

held from 12 -14 November 2018.

7.5 Credit hours for Conference accredited by Uttarakhand Medical Council

Letter no. U.K.M.C./2018-2019/2374

Dr. Kumar Satish Ravi	***Prof. Brijendra Singh***	***Prof. Surekha Kishore***	***Prof. Ravi Kant***
Sub-Dean (Academics)	*Dean (CSR)*	*Dean (Academics)*	*Director & CEO*
Joint-Organising Secretary	*Organising Secretary*	*AIIMS, Rishikesh*	*AIIMS, Rishikesh*
NATCON66	*NATCON66*		

BLDE (DEEMED TO BE UNIVERSITY)

Annexure -I

PLAGIARISM VERIFICATION CERTIFICATE

1. **Name of the Student:** MRS. ROSHNI SADASHIV **Reg No.** 15PHD004
2. **Title of the Thesis:** COMPREHENSIVE IMMUNOPHENOTYPIC EXPRESSION ANALYSIS OF PHOSPHOLIPID BINDING PROTEINS IN RENAL ORGANOGENESIS AND IN KIDNEY CARCINOMA
3. **Department:** ANATOMY
4. **Name of the Guide & Designation:** DR. B. M. BANNUR, PROFESSOR
5. **Name of the Co Guide & Designation:** DR. PRAVEENKUMAR SHETTY, PROFESSOR

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The thesis may be considered for submission to the University. The software report is attached.

18.9.2020

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Signature of Co-Guide
Name & Designation
Dept. of Biochemistry
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KARNATAKA

Signature of Student

Verified by (Signature)
Name & Designation
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Vijayapur.
10/9/2020

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Comparative expression analysis of phospholipid binding protein annexina1 in nephrogenesis and kidney cancer

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Abstract:
Background: The expression in the glomerular mesangial cells, papillary, and collecting duct cells demonstrated annexin A1 (AnxA1)'s role in specific renal functions. With varying concentrations of calcium (Ca^{2+}), it is considered to regulate cellular processes such as cell proliferation, apoptosis, and clearance of apoptotic cells by forming ceramides, a key lipid mediator of apoptosis. It also participates in tumorigenesis based on its location. On account of these features, we investigated the expression of this apoptosis-associated protein in fetal kidneys at different gestational periods, mature kidneys and in kidney cancer tissues in order to localize and possibly characterize its role during nephrogenesis and renal tumors.
Methods: AnxA1 expression was evaluated by an immunohistochemistry technique in "paraffin-embedded" renal tissue sections from autopsied fetuses at different gestational ages, in mature kidneys and renal cancer tissues.
Results: The current study data demonstrated that AnxA1 is expressed in the mesangial cells and podocytes of maturing glomeruli in the developing renal cortex of fetal kidneys at 14 to 19 weeks of gestation. The expression in the mesangial cells declined in later weeks of gestation and persisted into adulthood. AnxA1 expression increased with the progression of clear cell renal cell carcinoma (CCRCC) and also in other cancer types indicating a potential role of the protein in tumorigenesis.
Conclusions: We presume that AnxA1 in the podocytes and mesangial cells play important roles in various signaling pathways in the functioning of the glomerulus. These results and concepts provide a framework to further dissect its biological properties and thereby develop diagnostic, prognostic, and therapeutic strategies targeting the molecule in various renal pathologies.

Keywords: annexinA1, fetal kidney, nephrogenesis marker, phospholipid binding protein, renal cell carcinoma
DOI: 10.1515/jbcpp-2019-0179
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Introduction

The definitive human kidney develops from nephrogenic cord, an unsegmented column of intermediate mesoderm situated behind the embryonic coelom in the dorsal body wall [1], [2]. Three successive kidneys-pronephros (cervical rudimentary kidney), mesonephros (thoracic kidney), and metanephros (functional kidney) develop in the intermediate mesoderm [2]. The first two are transient; it is the metanephros that forms the permanent system of excretion. The mesonephros that functions for a brief period consists of a complex system

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of functional tubules that open into the mesonephric duct [3]. A metanephric diverticulum (ureteric bud) arises from the caudal end of the nephric duct to invade the metanephric blastemal cells. Reciprocal regulatory relation of diverticle and the blastemal cells induce the development of excretory units of adult kidney. However, the collecting units develop from repeated branching of the ureteric bud [1]. Cell proliferation, cell differentiation, apoptosis, angiogenesis, and vasculogenesis are the key processes involved in human renal development [4]. This exciting process of development involves more than 300 genes indexed to date [5]. Numerous studies on elucidating the early molecular events during development have helped in understanding the concepts of mesenchymal epithelial interaction, epithelial cell polarization, branching morphogenesis as well as identifying human genes responsible for renal disease [6]. In this context, two categories of actors have been revealed. The first includes transcription factors (PAX2, LIM1, WT1) and the second is represented by complex signaling pathways (SHH, NOTCH, WNT pathways) [6]. The differences in the embryonic origins of renal cells and various functions of the nephron may contribute to the diversity of morphologic patterns, molecular, and immunohistochemical phenotypes of renal cancers. On account of these features, a panel of immunohistochemical markers is required to substantiate the diagnosis of kidney cancers [7].

AnnexinA1 (AnxA1) belong to a family of 12 annexins, which act as intracellular sensors that identify incoming signals and provide each cell type with a specific function [8]. It is a calcium (Ca^{2+}) sensitive phospholipid binding protein which has the ability to sense changes in pH and to interact specifically with lipid bilayer and protein molecules at the plasma membrane [8]. AnxA1 (calpactin II), a 37 kDa protein like other members of annexin family consists of Ca^{2+} binding sites at its core domain and an unique NH_2 terminal end with 40 residues that engages in specific molecular interactions [8], [9], [10]. It has multiple potential sites that can undergo acetylation, lipidation, tyrosine, serine, and threonine phosphorylation. On exposure to Ca^{2+}, its N- terminal domain is exposed and through its interactions with several other molecules it participates in the regulation of biological processes such as cell proliferation, differentiation, cell death signaling, apoptosis, and phagocytosis [11]. Several studies on the phosphorylation of AnxA1 on serine residues by protein kinase C (PKC) and, phosphorylation on thyrosine residues following the activation of epidermal growth factor (EGF) and hepatocyte growth factor (HGF) receptor-tyrosine kinase have shown the activation of PI3 kinase and extracellular signal-regulated kinase microtubule-associated protein (ERK MAP) kinase glucocorticoid pathway which play important signaling functions in cell proliferation and migration [12]. Another function attributed to AnxA1 is its putative role in facilitating phagocytosis of apoptotic cells and secondary necrotic cells, and recruitment of phagocytes. Several studies support AnxA1 as an endogenous engulfment ligand that involves caspase dependent recruitment, release of intracellular calcium and colocalization with phosphatidyl serine on the cell surface during apoptosis, followed by AnxA1-mediated recognition of the apoptotic population and their engulfment [13]. Several studies document the interaction of AnxA1 with cytoskeletal proteins such as tubulin and actin [12]. In a Ca^{2+}-dependent manner it is found to bind with F actin at the ruffles, cell-cell contacts, and cell surface of various cell types involved in the regulation of biological function such as cytoskeletal reorganization [14]. In addition it is thought to stimulate vascular endothelial growth factor (VEGF)- mediated angiogenesis.

As far as its expression in cells and tissues is concerned, it is present abundantly in the smooth muscle, endothelial cells, and specifically in the digestive and ductal organs [8], [10]. Several authors have detected low levels of AnxA1 in tissues like brain, muscle, and liver [9]. Moreover, a strong AnxA1 immunoreactivity in mesangial cells, epithelial cells of Bowman's capsule, collecting duct cells of normal rabbit and rat kidneys has been reported indicating their role in specific physiological renal functions [10], [15].

In cancers, AnxA1 over expression has been elucidated in esophageal adenocarcinoma, gastric adenocarcinoma, colorectal, pancreatic adenocarcinoma, hepatocellular carcinoma, clear cell carcinoma, hairy cell leukemia, lung adenocarcinoma, and loss of AnxA1 expression in head and neck squamous carcinoma, esophageal squamous carcinoma, breast carcinoma and prostatic adenocarcinoma [16]. The role of AnxA1 protein is still unclear in tumor progression and carcinogenesis due to its inconsistent expression, being upregulated in a few tumors and down regulated in others [11].

Although renal cancers are associated with several etiological factors, one of the causes in the development of cancer is activation of genes which are involved in the biological processes during normal renal development. However, cancer development consists of a cascade of events such as driver mutation (s) and tumorigenesis followed by metastasis. During these transitions, it comes across as a physiological mechanism of apoptosis to eliminate damaged or abnormal cells [16]. In the context of renal development, a programed cell death is an essential component in the establishment of tissue architecture, and helps to both remodel developing structures to their mature form and eliminate unessential cells. An excess of proliferation of cells is associated with renal neoplasms whilst excessive apoptosis that may result in hampered kidney growth. A fine balance between cell proliferation, cell survival, apoptosis, and clearance of apoptotic cells is essential to maintain normal homeostasis in developing renal tissue [17].

The fact that AnxA1 can mediate signals for cell proliferation or apoptosis prompted us to do a systematic study of this protein during various phases of renal development and renal cancers. Although it has been studied in a wide variety of normal tissues and abnormal tumor cells, its function in the developing embryonic tissues remain to be elucidated.

The primary objective of the current study was to localize the protein in the various stages of developmental and pathological (renal cancer) tissues in an attempt to elucidate a possible correlation of the several functional proposals made with respect to this protein. Secondly, to speculate the role of the protein in renal cancer from the perspective of development, anticipating its role as an oncogene. Thus, in the current study we report the expression profile of this apoptotic related protein during renal development at various gestational ages to adult kidney to kidney cancers.

Materials and methods

Fetal, adult, and renal tumor samples

This study was conducted in accordance with the principles of institutional Ethical Committee. Fetal renal tissue from 42 autopsied fetuses (gestational age 14–40 weeks) were received from the Department of Pathology after obtaining written consent from the parents of the deceased fetus for the use of tissue for the purpose of research. The gestational age of the fetus as calculated by obstetrical methods were obtained from clinical records [18]. The ultra sound scanning and anomaly scanning reports from maternal clinical records were reviewed to exclude kidney-associated congenital anomalies. The metanephric stage of kidney development studied included stage 2 (14–22 weeks, n = 15), stage 3 (22–36 weeks, n = 13) and stage 4 (36 weeks onwards that continues to adult life, n = 14) [2]. The process of nephrogenesis is divided into four stages: 7–14 weeks (stage 1), 14–22 weeks (stage 2), 22–36 weeks (stage 3) and 36 weeks to term (stage 4) [2]. Stage 1 fetal kidneys were not included in the current study due to difficulty in obtaining the renal tissue from the aborted fetuses. Mature renal tissues were collected from cadavers (35–85 years of age, n = 15) donated to Department of Anatomy to compare changes in expression of AnxA1 between developing and mature kidneys. The renal samples were fixed in 10% buffered formalin, routinely processed and paraffin embedded. The renal samples were sent for histopathological assessment to exclude microscopic abnormalities. Five micron-thick sections were obtained from the paraffin embedded tissue blocks and stained with hematoxylin and eosin (H&E) to study the histogenesis of fetal kidneys in various phases of fetal development before performing immunohistochemistry. The morphology of glomerular cells was better assessed by subjecting the renal tissues to periodic acid-Schiff (PAS) staining. Thirty-seven renal tumor tissues that included clear cell renal cell carcinoma (CCRCC), papillary RCC, chromophobe RCC, collecting duct carcinoma, and oncocytoma were collected from the archives of the Department of pathology and subjected to immunohistochemistry. Tumors were graded according to Fuhrman et al. [19].

Immunohistochemistry

Three micron-thick sections were obtained from formalin fixed and paraffin embedded renal tissues. Immunohistochemical staining was performed using the avidin biotin (Vector Laboratories, Burlingame, CA, USA) immune peroxidase method [20]. The tissue sections were de-waxed (at 65 °C for 2 h in an incubator followed by immersing the slides in xylene for 10 min, 4 times). The sections were rehydrated before treating them with 0.01 M of citrate buffer (prewarmed for 5min) for 45 min at 100 °C for unmasking the antigen. The endogenous peroxidase activity was blocked by incubating the renal tissue with 0.3% hydrogen peroxide (H_2O_2) [1:100 dilutions in methanol (CH_3OH)]. Nonspecific binding sites were blocked by incubating the sections with normal horse serum (Vector Laboratories, Burlingame, CA, USA). The sections were incubated overnight with primary antibody against AnxA1 (DIL 1:100, Santa Cruz Biotechnology Inc; SantaCruz, CA, USA; catalog no. 12740). As a negative control, anti-mouseIgG whole molecule (Sigma-Aldrich) was used at 1:1000 dilution. This was followed by sequentially incubating the sections with biotynylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) and avidin biotin horse raddish peroxidase complex (ABC; Vector) [21]. The antigen of interest was detected by use of a 3, 3^1-diamino benzidine (DAB) chromogen and by counterstaining with hematoxylin. The tissues were evaluated under a light microscope with a Lieca Image Centre. Immunohistochemistry (IHC)-stained samples were evaluated by two pathologists and all thesamples were blinded.

The localization of AnxA1 protein was counted in 10 random fields across the cortex and medulla of renal tissues. Tissue sections stained with anti-AnxA1 antibody were graded based on (a) patterns of their expression as: M—membranous, C—cytoplasmic, N—nuclear, Mx—mixed (cytoplasmic and membranous) [22]. (b) percentage of immunoreactive cells (0 = no staining, 1–10% as 1, 11–50% as 2, 51–70% as 3, 71 to −100% as 4) [9], (c)

intensity of staining 0 = no color, (1+) weak brown, (2+) moderate brown, (3+) dense brown, strong expression [9]. The intensity and percentage scores were multiplied. A score of more than 3 was considered significant.

Statistical analysis

The chi-square ($\chi 2$) test was employed to determine the significance of differences between groups for categorical data by SPSS software. The statistical significance was set at 5% level of significance ($p \leq 0.05$, $\alpha < 0.05$). The expression of AnxA1 in the cells of mature glomeruli at various gestational ages of fetus and mature adult kidney were compared.

Results

The evaluation of AnxA1 immunostaining in the cortical and medullary compartments of the developing kidney was based on the histomorphologic features of renal tissues stained with H&E.

Validation of anti AnxA1 antibody for current immunohistochemistry study

To verify the antigen specificity of anti-AnxA1 monoclonal antibody (DIL 1:100, Santa Cruz Biotechnology Inc; SantaCruz, CA, USA; catalog no. 12740), we used two different prostate cancer cell lines: the hormone-dependent prostate cancer AnxA1 null LnCaP cell line and the AnxA1 abundant metastatic prostate cancer PC-3 cell lines. We confirmed the expression using Western blot analysis (Figure 1A) and immunohistochemistry of these cell lines that showed +3 expression in PC-3 cells and null in LnCaP cells (Figure 1B).

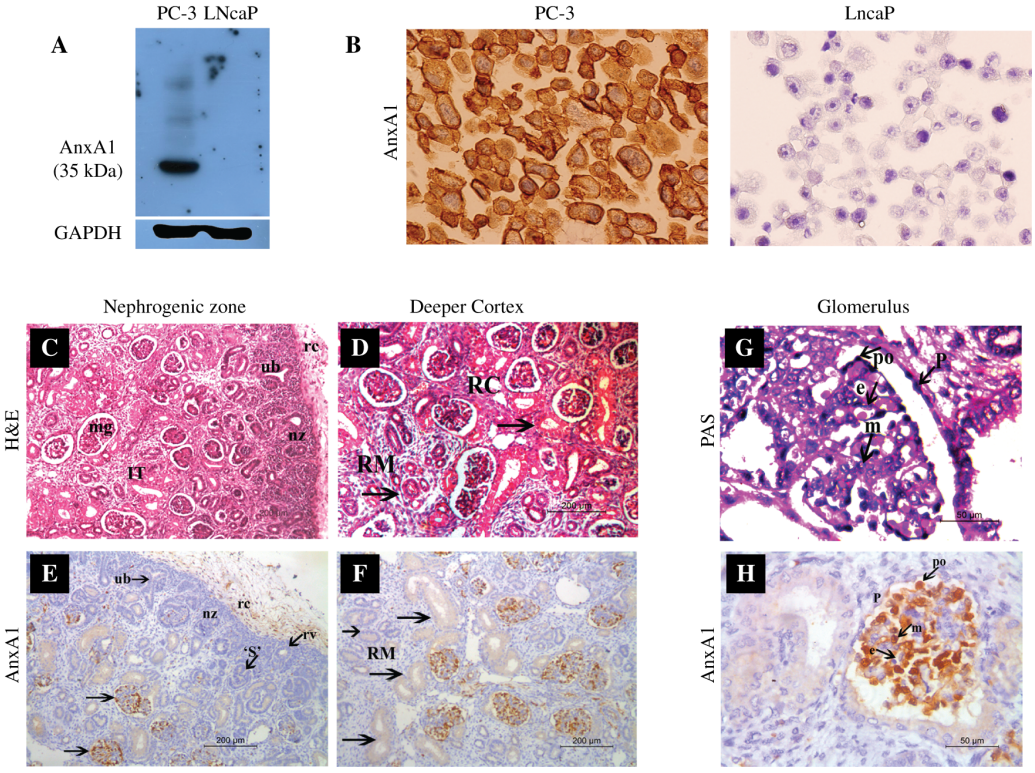

Figure 1: AnxA1 expression pattern in developing human kidney at 14 weeks' gestational age.
Antigen specificity of anti ANXA1 monoclonal antibody was verified using two different prostate cancer cell lines. Hormone dependent prostate cancer ANXA1 null LnCaP cell line and AnxA1 abundant metastatic prostate cancer PC-3 cell lines. The correlation of altered expression was demonstrated by Western blot analysis (A) and immunohistochemistry of these cell lines that showed +3 expression in PC-3 cells and null in LnCaP cells (B). (C) H&E staining showing differentiating outer cortex and inner medulla and general structural features. Cortex showing nephrogenic zone (nz) beneath the renal capsule (rc). Ureteric bud (ub) capped by blastemal cells. Mature glomeruli (mg) in the deeper part of cortex. Immature tubules (IT) between the glomerulus. (D) H&E of deeper cortex with maturing glomeruli and immature tubules in renal cortex (RC) and differentiating renal medulla (RM). (E) Embryonic kidney showing strong AnxA1 immunoreactivity in the glomerular cells (arrows), with lack of staining in the nephrogenic zone, immature forms of glomeruli renal vesicle (rv), S-shaped bodies 'S', and ureteric bud. (F) Absence of staining in the immature tubules of renal cortex and medulla (RM) (arrows). (G) Glomerular cellular details are better appreciated using PAS staining, demonstrating various glomerular cells podocyte (p), mesangial cell (m), endothelial cell (e) and parietal cell (P). (H) Mature glomerulus showing strong AnxA1 expression in the mesangial cells (m), podocytes (p) and endothelial cell (e). Magnification 10×, bar = 200 μm and 40×, bar = 50 μm.

Mesangial cells demonstrate increased expression of AnxA1 at 14–22 weeks of gestational age

The second stage of nephrogenesis is a period of nephron arcade formation and cessation of ureteric bud branching [2]. H&E sections of fetal kidney revealed densely stained undifferentiated mesenchyme cells just beneath the renal capsule and growing ureteric bud lined with cuboidal cells, capped with nephrogenic cells [23]. These cells continued to add newer developing glomeruli, such that the older mature glomeruli were located deeper in the cortex and newer immature forms were located under the capsule [24] (Figure 1C). In between the developing glomeruli within the connective tissue, the developing tubules with eosinophilic cytoplasm were observed. The medulla showed groups of tubules indicating the formation of collecting tubules. A few primitive blood vessels lined with squamous epithelium were appreciated [23] (Figure 1D).

As shown in Figure 1, an overall expression of AnxA1 in fetal kidneys was observed more in the cytoplasm with little membranous staining than observed in the nucleus in fewer cell types. Immature forms of glomeruli such as the renal vesicle (rv), 'S' shaped 'C' capillary loop structures in the nephrogenic zone demonstrated weak expression of AnxA1 in renal tissues from 14 weeks to 22 weeks of gestation. There was no immunostaining in the ureteric bud and cap mesenchymal cells overlying them (Figure 1E). AnxA1 was negative in immature and maturing tubular structures of the cortex and medulla (Figure 1F). A striking strong AnxA1 immunoreactivity was observed in the cells of maturing glomeruli in the deeper parts of the cortex. These cells were the mesangial cells, podocytes, and endothelial cells as delineated by PAS staining (Figure 1G). Although H&E stained renal sections enabled the recognition of the glomerular cells such as mesangial, podocytes, parietal epithelial cells, and endothelial cells, we used PAS staining that permitted precise resolution of relationship between cells and matrix as it accentuates the matrix and basement membrane constituents [25]. The intra glomerular mesangial cells were found to be embedded within the mesangium, the podocytes were found protruding into the capsular space of Bowman's capsule, investing the outer surface of mesangium and the endothelial cells with oval nuclei lined the inner surface of glomerular capillaries [26], [25] (Figure 1G, H). Strong expression of AnxA1 was observed in the mesangial cells and podocytes during the early phase from 14 to 19weeks of gestation. Though there was faint AnxA1 expression in the parietal cells of the Bowman's capsules of most of the glomeruli, few glomeruli also demonstrated moderate expression of AnxA1 in the parietal cells. However, the staining intensity declined from 20 weeks onwards. Of the 15 cases studied under this age group, 10 cases showed strong positivity (+3) for AnxA1 in the mesangial cells and podocytes ($p < 0.0001$), only four cases showed mild to moderate AnxA1 reactivity (+2) in the glomerular endothelial and parietal epithelial cells, respectively (Figure 2).

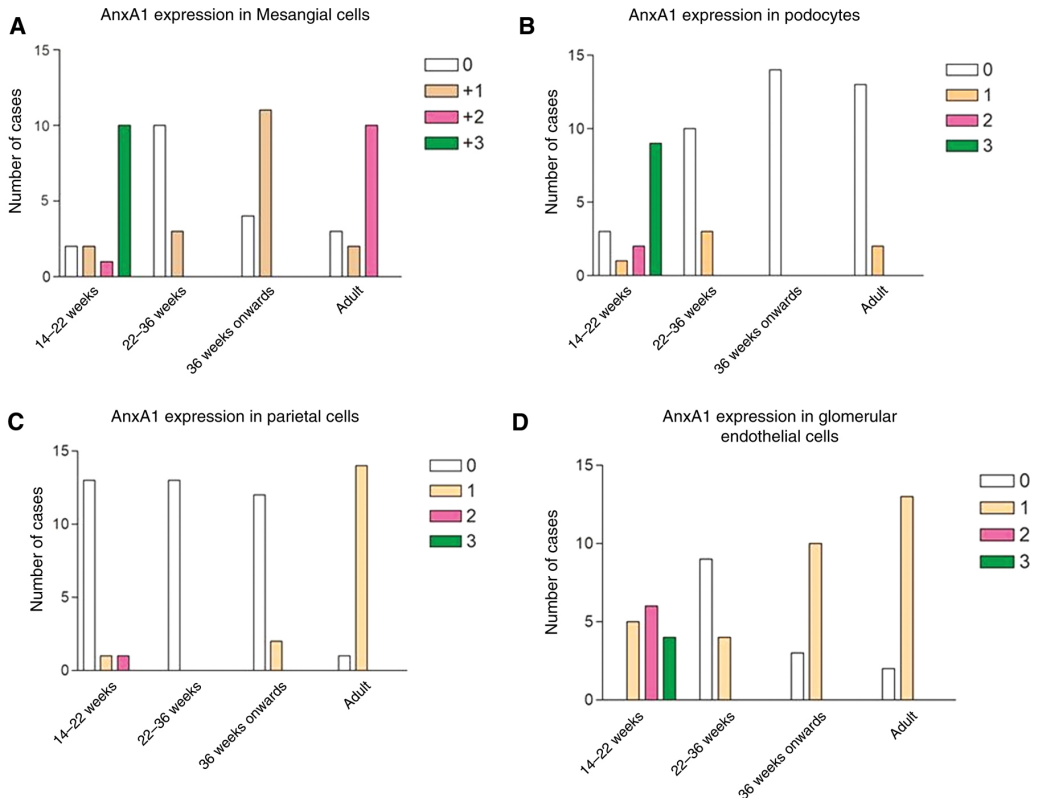

Figure 2: Semiquantitative expression analysis of AnxA1 by IHC.
Data shown are representative of number of cases immunoreactive for AnxA1 in glomerular cells at various gestational periods of renal development and in adult kidney. (A) mesangial cells, $p < 0.0001$; (B) podocytes, $p < 0.0001$; (C) parietal cells, p = NS; (D) glomerular endothelial cells, $p < 0.0001$.

Decline in the expression of AnxA1 in the glomerular cells

During 22–36 weeks of nephrogenesis, the cortex appeared more mature with an increase in the number of mature glomeruli and fewer immature forms. Proximal convoluted tubules (PCTs) and distal convoluted tubules (DCTs) could be easily identified. PCTs were lined by cuboidal cells with acidophilic cytoplasm and an apical brush border. They were larger in cross sections unlike the fewer, smaller DCTs lined by cuboidal cells with less abundant paler cytoplasm. Ureteric buds, derivatives of the Wolffian duct invaded the mesenchymal cells. The cells of the ureteric buds appeared cuboidal with centrally located nuclei. There was no further branching of ureteric bud. In the deeper medulla, thin and thick loops of Henle (LOH) were lined by simple squamous and simple cuboidal epithelium, respectively, and appeared as regularly round in cross section [26]. The collecting tubules derived from the ureteric buds resembled the thick LOH but were less regular in shape. A collecting duct was clearly identified by a larger diameter and tall columnar cell lining [26] (Figure 3A, B). The expression of AnxA1 increased in the glomerular cells during early development (stage 2) but decreased during the later stage (stage 3). The podocytes, parietal, and endothelial cells were negative for AnxA1 in most of the cases. A few cases in this group demonstrated mild immunoreactivity (+1) in mesangial cells and podocytes (Figure 2). There was no immunostaining for AnxA1 in either mesonephric duct-derived ureteric buds or metanephric blastema-derived early structures such as vesicles, S-shaped or C-shaped bodies (Figure 3C, E). No significant immunostaining was detected in maturing tubules, stroma or vascular structures of fetal renal compartments at this growth phase (Figure 3C–F).

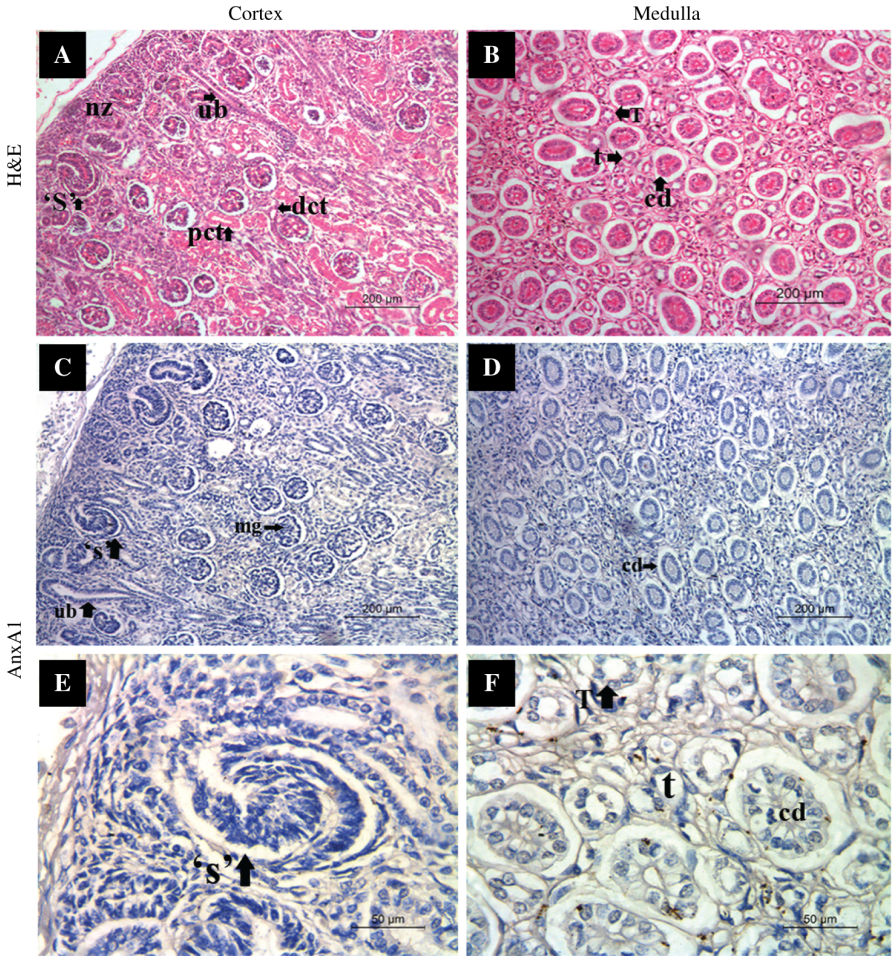

Figure 3: AnxA1 immunohistochemical staining of developing human kidney at 23 weeks of gestation. (A) H&E staining of fetal renal cortex showing nephrogenic zone, ureteric bud, immature glomeruli (S-shaped), mature glomeruli (mg) and clearly discernable PCTs with intense eosinophilic cytoplasm and centrally located nuclei and DCTs, less numerous and with scanty cytoplasm. (B) H&E staining of fetal renal medulla, displays the developing collecting ducts (cd), thick LOH (T), thin LOH (t), and blood capillaries. Cortex (C, E) and medulla (D, F) showing absence of AnxA1 staining in all renal tubular structures. Magnification 10×, bar = 200 µm and 40×, bar = 50 µm.

Lack of AnxA1 expression at 36 weeks of gestation

During the fourth stage of kidney development, no new nephrons are formed. The volume density of glomeruli increases. The LOH continue to increase in length and convoluted tubules become longer and tortuous [2]. The thickness of the cortex and medulla; renal vascularity increases with the increase in gestational age whereas the size of the nephrogenic zone decreases with increase in fetal age as shown in Figure 3 [23]. A decrease in the expression of AnxA1 was observed in the mesangial cells and endothelial cells of the renal corpuscle. The podocytes and parietal cells of Bowman's capsule, cortical, and medullary tubules, demonstrated an absence of AnxA1 staining (Figure 4C, E). Renal interstitium and blood vessels exhibited low expression of AnxA1 (Figure 4C–F).

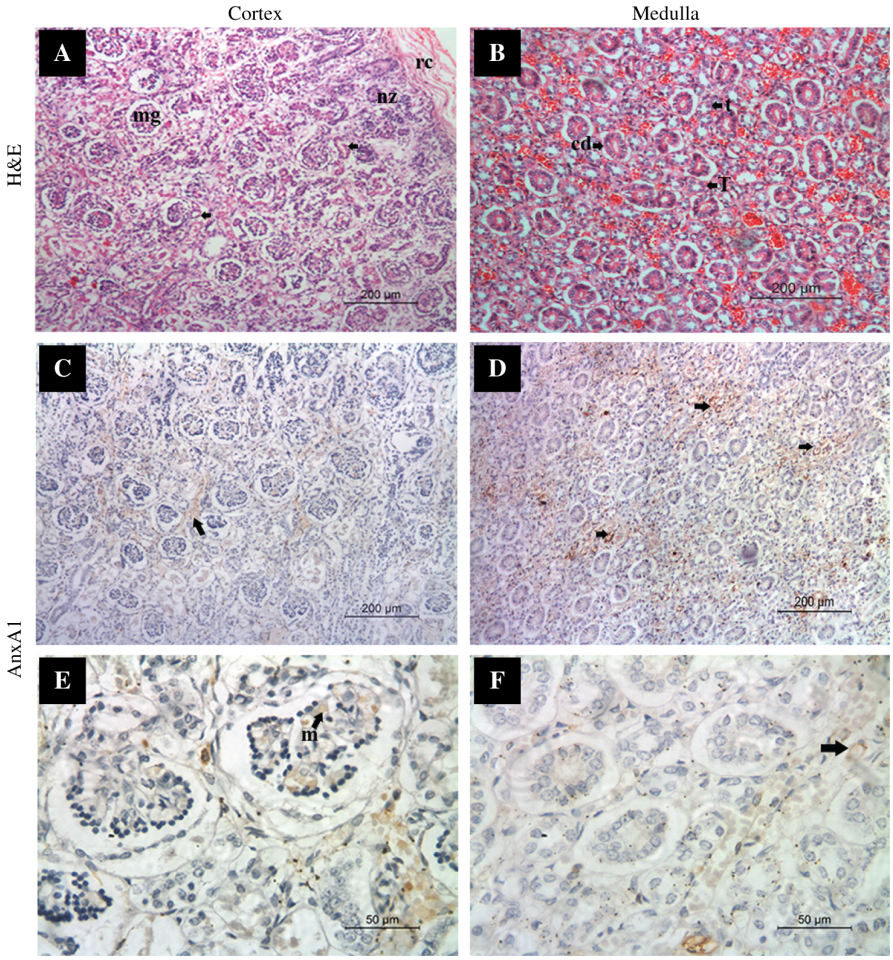

Figure 4: AnxA1 immunostaining of developing human kidney cortex and medulla at 36 weeks of gestational age. (A) H&E section of fetal renal cortex showing decrease in the thickness of nephrogenic zone with numerous maturing tubules (arrows) and glomeruli (mg). (B) H&E section of fetal renal medulla showing abundant tubules representing collecting ducts, thick and thin LOH, and increased vascularity. (C–F) Mild expression of AnxA1 can be appreciated in the cortical and medullary interstitium and blood vessels (arrows). (E) low expression of AnxA1 staining in the mesangial cells (m) of mature glomeruli and (F) absence of staining in the medullary tubules. Magnification 10×, bar = 200 µm and 40×, bar = 50 µm.

Moderate expression of AnxA1 in mesangial cells of adult kidneys

The adult kidney shows a well-demarcated outer cortex and inner medulla. It is composed of numerous uriniferous tubules bound by connective tissue stroma. The cortex is mainly occupied by renal corpuscle, PCT and DCT [27], [28]. The renal corpuscle consists of glomerular plexus of capillaries invaginating into Bowman's capsule. The vascular mesentery consists of mesangial cells. The capsule consists of an outer parietal layer lined with flattened epithelium and an inner visceral layer that is lined by large polyhedral cells. Both layers are separated by a capsular space. The PCTs are numerous, with smaller lumen when compared with fewer, shorter DCTs devoid of microvilli with a faint eosinophilic stain (Figure 5A). The medulla contains straight portions of tubules, segments of LOH and collecting duct. The thin segments of LOH are lined by simple squamous epithelium and resemble the capillaries and are distinguished from the latter by the absence of blood cells in the lumina and thicker epithelial lining [28], as shown in Figure 5B. Our immunohistochemical data revealed AnxA1 staining in the adult kidney was consistent in the mesangial cells of the renal corpuscle and few blood capillaries in the medulla as demonstrated in Figure 5C–E. Adult renal tissues demonstrated moderate immunostaining for AnxA1 in the mesangial cells (Figure 5E). In addition the endothelial lined capillary loop, parietal epithelial cells of renal corpuscle, also demonstrated a moderate expression of AnxA1 in a few cases. There was an absence of staining in the cortical tubules. No staining was seen in the medullary collecting ducts and segments of LOH (Figure 5C, F). A summary of the observed immunostaining in various renal structures is presented in Table 1.

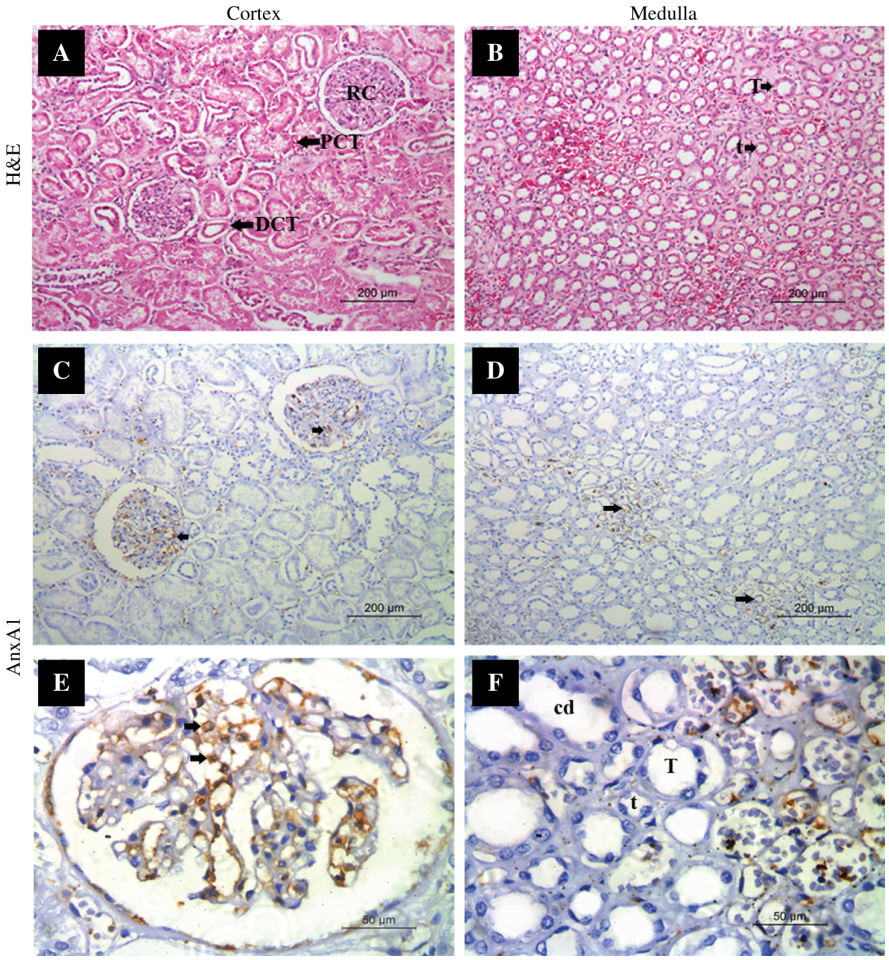

Figure 5: AnxA1 immunostaining of mature adult kidney.
(A) H&E section of kidney cortex occupied by numerous mature PCT, few pale staining DCT lined with cuboidal cells and renal corpuscles (RC). (B) H&E section of kidney medulla demonstrating mature tubules. (C, E) Immunohistochemical expression of AnxA1 in adult renal cortex shows positive moderate expression in the mesangial and capillary loop of renal corpuscle. (D, F) AnxA1 staining in the medulla is restricted to blood capillaries (arrows) and negative in the tubules. Magnification 10×, bar = 200 µm and 40×, bar = 50 µm.

Table 1: Expression of AnxA1 in normal human fetal and adult kidney.

Renal structures	**14**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**22–36 weeks**	**36 weeks onwards**	**Mature adult**
n = no of cases	**n = 4**	**n = 2**	**n = 1**	**n = 3**	**n = 1**	**n = 1**	**n = 1**	**n = 2**	**n = 13**	**n=14**	**n = 15**
Immature forms of glomeruli	+	+	0	+	+	0	+	0	0	mv	mv
Ureteric bud	0	0	0	0	0	0	0	0	0	mv	mv
Immature tubules (IT)	+	+	+	mv	mv	mv	mv	mv	mv	mv	mv
Mature glomeruli											
Mesangial cells	+++	+++	++	+++	+++	+	+	+	+	+	++
Podocytes	+++	+++	++	+++	++	0	+	0	0	0	0
Parietal cells	0	0	0	0	++	0	+	0	0	0	+
Endothelial cells	+++	++	++	++	+	+	+	+	0	0	+
PCT	mv	mv	mv	0	0	0	0	0	0	0	0
DCT	mv	mv	mv	0	0	0	0	0	0	0	0
Thick LOH	mv	mv	mv	0	0	0	0	0	0	0	0
Thin LOH	mv	mv	mv	0	0	0	0	0	0	0	0
CD	mv	mv	mv	0	0	0	0	0	0	0	0

CD, collecting duct; DCT, distal convoluted tubule; LOH, loop of Henle; mv, missing value; PCT, proximal convoluted tubule.
Immunohistochemical staining for AnxA1,0 = no staining, 1–10% as 1+, 11–50% as 2+, 51–70% as 3+, 71–100% as 4+.

Upregulation of AnxA1 in kidney cancer

Immunohistochemical staining of the normal parts of adult kidneys showed an absence of AnxA1 staining of proximal tubular cells as shown in Figure 6C, D. Our study comprised predominantly CCRCC and were graded based on Fuhrman nuclear grades. The tumor cells either presented with clear cytoplasm or admixed with eosinophilic or granular cytoplasm [29]. Evaluating the expression of AnxA1 in the tumor cells of CCRCC, grade 1 tumors showed mild positivity for AnxA1 (Figure 6G, H), the staining intensity increased in grade 2 and grade 3 CCRCC (+2) with tumor progression (Figure 6K, L, O, P). AnxA1 expression was tested on non-neoplastic tissue and CCRCC. Data were calculated as average percentage of AnxA1 expressing cells in the proximal tubular cells of non-neoplastic adult tissues against tumor cells of CCRCC at different tumor grades. The percentage of tumor cells staining positive for AnxA1 increased with increasing tumor grade (Figure 6B) ($p < 0.0001$).

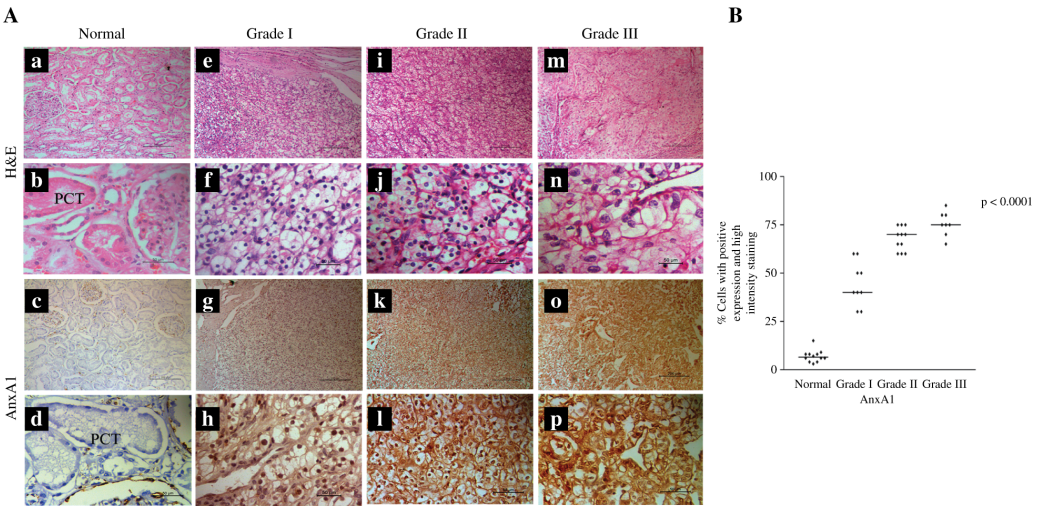

Figure 6: AnxA1 immunostaining of CCRCC tissues.
H&E section of normal adult kidney cortex (A, B). Immunohistochemical expression of AnxA1 in adult renal cortex showing absence of AnxA1 staining in proximal tubular cells (C, D). H&E section of Fuhrman histological grade I CCRCC showing clear cells with small round uniform nuclei and absence of nucleoli (E, F). Immunohistochemical expression of AnxA1 grade I CCRCC showing mild membranous staining in tumor cells (G, H). H&E section of Fuhrman histological grade II CCRCC showing clear cells with relatively larger irregular nuclei and presence of nucleoli (I, J). Immunohistochemical expression of AnxA1 grade II CCRCC showing moderate staining in tumor cells (K, L). H&E section of Fuhrman histological grade III CCRCC showing clear cells with larger irregular nuclei and presence of prominent nucleoli (M, N). Immunohistochemical expression of AnxA1 grade III CCRCC showing moderate staining in tumor cells (O, P). Magnification 10×, bar = 200 μm and 40×, bar = 50 μm. B: AnxA1 vs. tumor grade: Comparison of AnxA1 expression in adult proximal tubular cells against tumor cells in CCRCC relative to tumor grade, $p < 0.0001$.

The growth pattern of papillary RCC was either papillary or tubule-papillary type [29]. Figure 7A, B shows type 2 tumor cells having eosinophilic cytoplasm, high-grade nuclei with pseudo-stratification [30]. In all the cases of papillary RCC studied, tumor cells were immunopositive for AnxA1 as demonstrated in Figure 7C, D. H&E staining of collecting duct carcinoma was characterized by glandular or papillary growth pattern (Figure 7E, F) [29]. The tumor cells of collecting duct carcinoma showed moderate cytoplasmic and nuclear AnxA1 immunostaining (+2) as depicted in Figure 7G, H. Chromophobe RCC consisted of large cells with reticulated cytoplasm and small cells with granular cytoplasm in H&E tumor sections (Figure 7I, J) [29]. Chromophobe RCC sections revealed strong immunostaining for AnxA1 (+3) as shown in Figure 7K, L. Oncocytomas largely mimic chromophobe RCC histologically, however, these tumor cells consisted of deeply eosinophilic cytoplasm, centrally located nuclei (Figure 7M, N) [30]. In all cases of oncocytoma studied, tumor cells demonstrated moderate immunopositive staining for AnxA1 (+2) as depicted in Figure 7O, P.

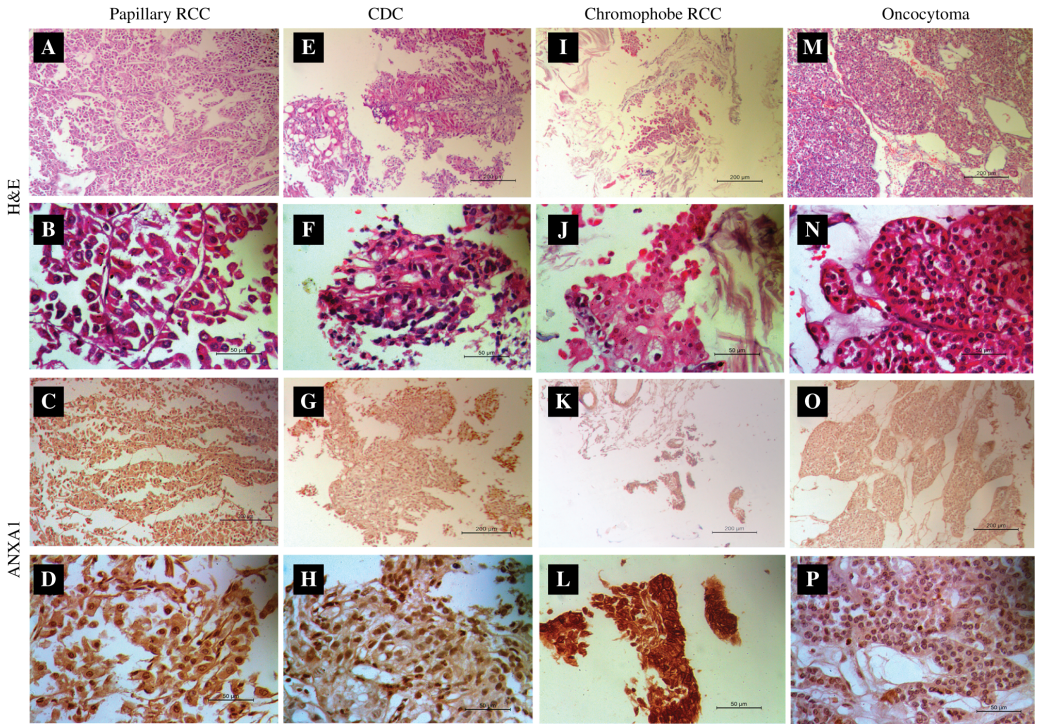

Figure 7: AnxA1 immunostaining in other types of renal cell carcinomas.
H& E sections of papillary renal cell carcinoma, chromophobe renal cell carcinoma, collecting duct CDC and oncocytoma (A–H). Immunopositive AnxA1 staining was detected in papillary RCC, chromophobe RCC, collecting duct RCC and oncocytoma (I–F).

Discussion

The first human metanephric glomerulus is formed in the 9th week and the nephrogenic zone is not seen until the 36th week of gestation. The complex developmental and functional architecture of the human kidney is regulated by molecular factors such as glial cell-derived neurotrophic factor (GNDF) and Wilms' tumor suppressor gene 1 (*WT1*), which stimulates the branching of the ureteric bud to form the collecting component of the kidney. Fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 7 (Bmp7) block apoptosis and stimulate proliferation of metanephric mesenchyme, the paired box gene 2 (*pax2*) and the wingless related gene (*WNT4*) participate in the differentiation of mesenchyme to nephron epithelium [31]. The present study demonstrates the expression of AnxA1 in podocytes, mesangial, parietal epithelial cells, and endothelial cells of developing mature glomeruli during the early phase of nephrogenesis. The expression of AnxA1 in the mesangial cells persists into adulthood. These results suggested a probable correlation of the physiological roles of AnxA1 and certain functions of glomerular cells.

Mesangial cells occupy a central position in the renal glomerulus, mostly found in the stalk of the glomerular tuft. They may originate from mesenchymal cells that develop from other cells of the nephron or develop from extra renal components such as hematopoietic lineages [32], [33]. Several vasoactive agents such as angiotensin II and vasopressin are thought to influence the glomerular mesangial function by activation of phospholipase C for phosphotidyl-inositol 4, 5- bisphosphate (PIP_2) which in turn results in the formation of diacylgycerol (DAG) and inositol triphosphate (IP_3) [32].

AnxA1 acts as a sensor for lipid second messengers such as PIP_2 and DAG and further increases in Ca^{2+} levels promotes membrane binding of AnxA1 [8]. On activation of PKC by its major activator DAG, PKC participates in various signaling pathways by phosphorylating target proteins. In the mesangial cells, PKC has a role in hormone-induced prostaglandin formation and acts as a negative feedback regulator of the inositol lipid signaling cascade, having a major role in regulating the glomerular filtration rate [34]. Upon elevation of Ca^{2+}, both PKC and AnxA1 are known to translocate from cytosol to the plasma membrane to interact with each other [8]. *In-vivo* and *in-vitro* studies on rat mesangial cells demonstrated AnxA1 phosphorylation catalyzed by PKC required the presence of Ca^{2+}, phospholipids, and PKC activators such as angiotensin II and vasopressin and the phosphorylation occurred at serine and tyrosine residues of the NH_2 terminal end of AnxA1 [35]. This interaction brings about wide variety of cellular responses. An increase in intracellular Ca^{2+} also activates phospholipase A_2 and arachidonic acid release from membrane phospholipids and thus, local prostaglandin

formation in mesangial cells contributing to its various functions [32]. AnxA1 together with cytosolic phospholipase A_2 ($cPLA_2$) is translocated to the plasma membrane. Although cleaved AnxA1 maintains its interaction with $cPLA_2$, it fails to inhibit the phosphorylation of $cPLA_2$, thus leading to the release of arachidonic acid [36]. The association between AnxA1 and PKC, and AnxA1 and PLA_2 are therefore critical determinants in the regulation of the glomerular functions by the mesangial cells.

Although GFR is established during intrauterine life, it is barely significant, as the fetal kidneys do not primarily function in regulating water and fluid electrolytes. A gradual increase in blood flow during nephrogenesis suggests that the kidneys are able to autoregulate early in life but with relatively lower efficiency [31].

In the present study, we found a mild positivity for AnxA1 in the mesangial cells at later stages of gestation. We predict that AnxA1 may aid in autoregulation, however, at a lower base line.

Ka et al. demonstrated that full length AnxA1 and its cleaved form are released by mesangial cells [37]. However, the mode of secretion of AnxA1 protein remains an unsolved mystery. A new mode of secretion of AnxA1 through the release of microparticles or microvesicles from its various cell sources has been hypothesized. These exosomes are shed from activated cells by a flippase-scramblase mechanism, where there is a reversing of its lipid bilayer exposing phosphotidyl serine to the exterior [38].

A recent study, demonstrated the involvement of AnxA1 in Ca^{2+}-dependent production and clustering of ceramides on the plasma membrane of cells. Ceramide is a key lipid mediator for apoptosis [8]. Under the influence of calcium, AnxA1 is known to bind to negatively charged phospholipids such as phosphatidylserine [39]. Evidence suggests that ceramides along with phosphatidyl serine promote binding of AnxA1 to apoptotic cells and mediate their clearance followed by AnxA1 induced apoptosis of surrounding cells [8]. In humans, apoptosis occurs both in developing and adult kidneys but the rate of apoptosis is particularly intense in early phases of nephrogenesis [4], [40]. Glomerular mesangial cells behave as semi-professional phagocytes. They deploy various mechanisms for the phagocytosis and clearance of apoptotic bodies in order to eliminate unwanted cells, so as to fine tune the number of renal cells and also prevent a local inflammatory insult due to secondary necrosis of uncleared apoptotic cells [41]. The strong expression of AnxA1 as observed in our study, in the mesangial cells of fetal kidneys of early gestational weeks could be correlated to this function of mesangial cells and the expression of AnxA1 could be speculated much earlier.

Angiogenesis (formation of new capillaries from existing preformed capillaries) is essential for enhancing the vascular network in normal processes. VEGF is the major promoter that increases the interaction between AnxA1 and actin which is essential for cytoskeleton remodeling into lamellipodia and thus migration and proliferation of capillary endothelial cells. Thus, AnxA1 is an important regulator in VEGF-mediated angiogenesis [12]. Immunopositive AnxA1 expression as demonstrated in the endothelial lined capillary loops of fetal and adult glomeruli in the current study may presumably be attributed to this function of AnxA1.

Podocytes and parietal epithelial cells have been implicated in maintaining the molecular sieve-like structure of glomerulus and its functions. They aid in the maintenance of the filtration barrier and transport, respectively. If the podocyte structure is compromised, it leads to the development of proteinuria and progressive glomerulosclerosis [24]. A proteomic study on cultured podocytes showed abundant actin cytoskeletal proteins, annexins, and VEGF and they also expressed glucocorticoid receptors [42]. Although AnxA1 promoter contains glucocorticoid response elements it is thought to act as a second messenger in glucocorticoid-induced cellular responses which is required for both kidney development and recovery from physiological stress. Intracellular AnxA1 is also found to be associated with the endosomes at 1–26 residues of the NH_2 terminal domain indicating its involvement in endocytotic processes [10]. The present investigation shows that AnxA1 is expressed in human fetal podocytes, and fewer parietal epithelial cells of maturing glomerulus which suggest a significant role in the biology of renal corpuscle. Perhaps the expression in both the cell types could be due to the fact that both cells have a common mesenchymal origin and are the result of divergent differentiation during embryogenesis [43]. Furthermore, a study on Adriamycin-induced glomerulopathy in a mouse model demonstrated high levels of urinary AnxA1and its signals were appreciated in the renal podocytes. Their data suggested that the urinary AnxA1were derived from the apoptotic renal tissues [37].

RCC accounts for 3% of adult malignancies 90% of which arise from the renal tubules. The histological subtypes of RCC such as the clear cell and papillary RCC are thought to be derived from proximal tubular cells and chromophobe and collecting duct from the distal portions of nephron and renal pelvis, respectively [29]. Zimmermann et al. demonstrated the upregulation of AnxA1 expression in the conventional RCC and correlated to the Fuhrman grade, clinical outcome and metastatic potential [44]. A recent study by Yamanoi et al. showed the expression of AnxA1 in the membranes of RCC cells received from 27 patient specimens with evidence suggesting its positive correlation with respect to patients' malignant outcome [45]. Our findings are in accordance with their study with a strong expression for AnxA1 in the membranes of CCRCCs which increased with the progression of the tumor. The absence of immunostaining in the proximal tubular cells of normal adult kidney indicates malignant transformation of these cells. Our findings also support the observation that low AnxA1 expression in normal tissues increases during tumor transformation [11].

The current study reports the increased expression status in other histological subtypes such as chromophobe, papillary, collecting duct RCC, and oncocytoma. Zimmermann et al. emphasized the expression of AnxA1 as a feature of eosinophilic cells in conventional RCC. The latter indicating the degree of tumor differentiation [44]. Renal neoplasms with granular or eosinophilic cells also includes oncocytoma, eosinophilic variant of chromophobe RCC, papillary RCC type 2, and collecting duct carcinoma speculating AnxA1 expression in these neoplasms. Contrary to normal cells the tumor cells are under constant oncogenic stress, genomic instability and cellular hypoxia. Under the influence these stimuli it activates the intrinsic pathway of apoptosis triggering the release of pro-apoptotic proteins which could be disabled by the tumor cells by destabilizing the latter [16]. Some researchers are of the opinion that AnxA1 is a proapoptotic factor while others suggest it to be antiapoptotic. The exact mechanism still remains unclear [11]. AnxA1 behaves differently in different tumor types. For example, in cancers such as breast, bladder, gastric, and leukemia it could act both like a tumor suppressor and as an oncogene [46]. The subcellular localization of AnxA1 (cytosol, nuclear, membrane) is suggested to play important role in tumorigenesis through its interaction with various cognate partners. Several studies have indicated the role of AnxA1/FPR (formyl peptide receptors) in cancer progression. A study on keratinocyte proliferation in squamous cancer cells showed that AnxA1 interaction with $cPLA_2$ required the formation of S100A11/AnxA1 complex and on proteolytic cleavage AnxA1 lost its capacity to bind to S100A11 thus, $cPLA_2$ maintained its active state enabling keratinocyte proliferation. In a few cancers, the disruption of the S100A11/AnxA1 complex and activation of EGFR downstream signaling pathways enabling enhanced migration has been established [36]. AnxA1 is a protein that inhibit inflammation while stimulate VEGF-mediated angiogenesis.

To date, several studies have dealt with immunohistochemical markers expressed by renal cells of nephron lineage with little attention to markers of non-nephron lineage such as those originating from cortical, medullary interstitium, or mesangium [47]. The expression of AnxA1 in the human kidney appears to be linked to several physiological functions of the glomerulus. It restores intracellular homeostasis by participating in specific pathways and receptor interactions, thus, making it an accessible target for therapy in altered physiology [8], [35]. This biomarker may constitute the key to uncover early molecular events during nephrogenesis and may be used as a strategy to deal with further renal pathogenesis.

Seventy percent of RCCs are clear cell carcinomas and are thought to be derived from proximal convoluted cells [48]. As the expression of AnxA1 is observed to be absent in the fetal and adult proximal tubular cells, their expression in the renal tumor cells shows malignant transformation of the normal cells indicating an aberrant expression pattern unlikely to be due to the reactivation of a repressed gene in the process of normal embryonic development. Further, its interaction with formyl peptide receptors on the cell surface of tumor cells makes it a potential target to be assessed in therapy as the formyl peptide receptors can be activated or silenced by specific ligands [36]. In addition to this, AnxA1 is a known anti-inflammatory upstream regulatory molecule for all the downstream devastation of cytokines like tumor necrosis factor (TNF) α, interleukin (IL)-6, IL-17, etc. Correlating the level of this critical anti-inflammatory molecule during nephrogenesis, normal adult functional kidney, and further during various clinical conditions will be ideal to know as in most of these diseased renal conditions, inflammation is the leading complication.

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Author contributions: Conceived the idea and designed the experiments: RS and PKS; helped in clinical sample collection and correlated the clinical relevance to the study: USD, BMB and SKD; Performed the experiments: RS, SE, AB, and KR; analyzed the data (pathology): USD. Analyzed the data (statistical analysis): RS, PKS, JKV, and AB; manuscript preparation: RS and PKS; revised the manuscript: PKS and JKV; supervised the overall study: PKS and BMB.

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Informed consent: Informed consent was obtained from all individuals included in this study.

Ethical approval: Research involving human subjects compliedwith all relevant national regulations, institutional policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors' Institutional Review Board or equivalent committee.

References

[1] Moore KL, Persaud TV, editors. The developing human: clinically oriented embryology, 8th ed. Philadelphia, PA: Saunders, 2008:244–60.
[2] Lacson A, Bernstein J, Risdon RA, Gilbert-Barness E. Renal system. In: Gilbert-Barness E, editor. Potter's pathology of the fetus, infant and child. 2nd ed. Philadelphia, PA: Elsevier Mosby, 2007:1281–344.
[3] Alexander DP, Nixon DA. The foetal kidney. Br Med Bull 1961;17:112–7.
[4] Jeon YJ, Kim J, Chae JI. Comparative proteomic analysis of kidney development-related proteins in the pig. Vitr Cell Dev Biol Anim 2013;49:315–23.
[5] Leroy X, Devisme L, Buisine MP, Copin MC, Aubert S, Gosselin B, et al. Expression of human mucin genes during normal and abnormal renal development. Am J Clin Pathol 2003;120:544–50.
[6] Dormoy V, Jacqmin D, Lang H, Massfelder T. From development to cancer: lessons from the kidney to uncover new therapeutic targets. Anticancer Res 2012;32:3609–17.
[7] Tong GX, Yu WM, Beaubier NT, Weeden EM, Hamele-Bena D, Mansukhani MM, et al. Expression of PAX8 in normal and neoplastic renal tissues: an immunohistochemical study. Mod Pathol 2009;22:1218–27.
[8] Monastyrskaya K, Babiychuk EB, Draeger A. The annexins: spatial and temporal coordination of signaling events during cellular stress. Cell Mol Life Sci 2009;66:2623–42.
[9] Kang WY, Chen WT, Huang YC, Su YC, Chai CY. Overexpression of annexin 1 in the development and differentiation of urothelial carcinoma. Kaohsiung J Med Sci 2012;28:145–50.
[10] Markoff A, Gerke V. Expression and functions of annexins in the kidney. Am J Physiol Renal Physiol 2005;289:F949–56.
[11] Biaoxue R, Xiguang C, Shuanying Y. Annexin A1 in malignant tumors: current opinions and controversies. Int J Biol Markers 2014;29:e8–20.
[12] Pin AL, Houle F, Fournier P, Guillonneau M, Paquet ÉR, Simard MJ, et al. Annexin-1-mediated endothelial cell migration and angiogenesis are regulated by vascular endothelial growth factor (VEGF)-induced inhibition of miR-196a expression. J Biol Chem 2012;287:30541–51.
[13] Arur S, Uche UE, Rezaul K, Fong M, Scranton V, Cowan AE, et al. Annexin I is an endogenous ligand that mediates apoptotic cell engulfment. Dev Cell 2003;4:587–98.
[14] Belvedere R, Bizzarro V, Popolo A, Dal Piaz F, Vasaturo M, Picardi P, et al. Role of intracellular and extracellular annexin A1 in migration and invasion of human pancreatic carcinoma cells. BMC Cancer 2014;14:1–15.
[15] Tribolo S, Maroux S, Massey-Harroche D. Kidney proximal tubule cells: epithelial cells without EGTA-extractable annexins? Biochem Cell Biol 2000;78:495–502.
[16] Fernald K, Kurokawa M. Evading apoptosis in cancer. Trends Cell Biol 2013;23:620–33.
[17] Woolf AS, Jenkins D. Development of the kidney. In: Jennette JC, Olson JL, Silva FG, D'Agati VD, editors. Heptinstall's pathology of the kidney, 7th ed. Philadelphia, PA: Wolters Kluwer Health, 2015:67–89.
[18] Chikkannaiah P, Roy M, Kangle R, Patil PV. Glomerulogenesis: can it predict the gestational age? a study of 176 fetuses. Indian J Pathol Microbiol 2012;55:303–7.
[19] Eble NJ, Grignon JD, Young HR. Tumors of the urinary tract. In: Fletcher MDC, editor. Diagnostic histopathology of tumors, 4th ed. Philadelphia, PA: Elsevier Saunders, 2013:559–601.
[20] Cheng CW, Rifai A, Ka SM, Shui HA, Lin YF, Lee WH, et al. Calcium-binding proteins annexin A2 and S100A6 are sensors of tubular injury and recovery in acute renal failure. Kidney Int 2005;68:2694–703.
[21] Hudkins KL, Giachelli CM, Cui Y, Couser WG, Johnson RJ, Alpers CE. Osteopontin expression in fetal and mature human kidney. J Am Soc Nephrol 1999;10:444–57.
[22] Lacunza E, Ferretti V, Barbeito C, Segal-Eiras A, Croce MV. Immunohistochemical evidence of Muc1 expression during rat embryonic development. Eur J Histochem 2010;54:e49.
[23] Tank KC, Saiyad SS, Pandya AM, Akbari VJ, Dangar KP. A study of histogenesis of human fetal kidney. Int J Biol Med Res 2012;3:1315–21.
[24] Bertelli E, Regoli M, Fonzi L, Occhini R, Mannucci S, Ermini L, et al. Nestin expression in adult and developing human kidney. J Histochem Cytochem 2007;55:411–21.
[25] Bonsib FM. Renal anatomy and histology. In: Jennette JC, editor. Heptinstall's pathology of the kidney, 7th ed. Philadelphia, PA: Wolters Kluwer Health, 2015:1–65.
[26] Young B, Lowe JS, Stevens A, Heath JW. Wheater's functional histology: a text and color atlas, 5th ed. Philadelphia, PA: Churchill Livingstone Elsevier Ltd, 2009:302–27.
[27] Junqueira LC, Jose C, editors. Basic histology: text and atlas, 11th ed. New York, NY: McGraw-Hill, 2005:373–91.
[28] Eroschenko VP. Urinary system. In: Eroschenko VP, editor. Di Fiore's atlas of histology with functional correlations. Philadelphia, PA: Lippincott Williams & Wilkins, 2005:309–27.
[29] Truong LD, Shen SS. Immunohistochemical diagnosis of renal neoplasms. Arch Pathol Lab Med 2011;135:92–109.
[30] Kuroda N, Tanaka A, Ohe C, Nagashima Y. Recent advances of immunohistochemistry for diagnosis of renal tumors. Pathol Int 2013;63:381–90.
[31] Sulemanji M, Vakili K. Neonatal renal physiology. Semin Pediatr Surg 2013;22:195–8.
[32] Schlöndorff D. The glomerular mesangial cell: an expanding role for a specialized pericyte. FASEB J 1987;1:272–81.
[33] Quaggin SE, Kreidberg JA. Development of the renal glomerulus: good neighbors and good fences. Development 2008;135:609–20.
[34] Pfeilschifter J. Regulatory functions of protein kinase c in glomerular mesangial cells. Klin Wochenschr 1990;68:1134–7.
[35] D'Acunto CW, Gbelcova H, Festa M, Ruml T. The complex understanding of Annexin A1 phosphorylation. Cell Signal 2014;26:173–8.
[36] Boudhraa Z, Viallard B, Bouchon C, D'Incan M, Degoul F. Annexin A1 localization and its relevance to cancer. Clin Sci 2016;130:205–20.
[37] Ka SM, Tsai PY, Chao TK, Yang SM, Hung YJ, Chen JS, et al. Urine annexin A1 as an index for glomerular injury in patients. Dis Markers 2014:1–12.

[38] Perretti M, Dalli J. Exploiting the Annexin A1 pathway for the development of novel anti-inflammatory therapeutics. Br J Pharmacol 2009;158:936–46.
[39] Weyd H, Abeler-Dörner L, Linke B, Mahr A, Jahndel V, Pfrang S, et al. Annexin A1 on the surface of early apoptotic cells suppresses CD8+ T cell immunity. PLoS One 2013;8:e62449.
[40] Sanz AB, Santamaria B, Ruiz-Ortega M, Egido J, Ortiz A. Mechanisms of renal apoptosis in health and disease. J Am Soc Nephrol 2008;19:1634–42.
[41] Cortes-Hernandez J, Fossati-Jimack L, Carugati A, Potter PK, Walport MJ, Cook HT, et al. Murine glomerular mesangial cell uptake of apoptotic cells is inefficient and involves serum-mediated but complement-independent mechanisms. Clin Exp Immunol 2002;130:459–66.
[42] Schnenberger E, Ehrich JH, Haller H, Schiffer M. The podocyte as a direct target of immunosuppressive agents. Nephrol Dial Transplant 2011;26:18–24.
[43] Miesen L, Steenbergen E, Smeets B. Parietal cells – new perspectives in glomerular disease. Cell Tissue Res 2017;369:237–44.
[44] Zimmermann U, Woenckhaus C, Teller S, Venz S, Langheinrich M, Protzel C, et al. Expression of annexin AI in conventional renal cell carcinoma (CRCC) correlates with tumour stage, Fuhrman grade, amount of eosinophilic cells and clinical outcome. Histol Histopathol 2007;22:527–34.
[45] Yamanoi M, Yamanoi K, Fujii C, Fukuda MN, Nakayama J. Annexin A1 expression is correlated with malignant potential of renal cell carcinoma. Int J Urol 2019;26:284–90.
[46] Sheikh MH, Solito E. Annexin A1: uncovering the many talents of an old protein. Int J Mol Sci 2018;19:1–20.
[47] Gerosa C, Fanni D, Nemolato S, Di Felice E, Iacovidou N, Eyken PV, et al. Shift of galectin-3 expression in the human kidney during development. J Pediatr Neonat Individual Med 2013;2:1–6.
[48] Thedieck C, Kuczyk M, Klingel K, Steiert I, Müller CA, Klein G. Expression of Ksp-cadherin during kidney development and in renal cell carcinoma. Br J Can 2005;92:2010–7.

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Differential expression pattern of annexin A2 during nephrogenesis and kidney carcinoma

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Abstract

The creation of a cancer cell could be due to reactivation of repressed gene in the process of normal embryonic development. The differences in embryonic origins and functions of various components of nephron may contribute to the diversity of morphological patterns, molecular and immunohistochemical phenotypes of common renal neoplasms. Renal cell carcinomas (RCCs) are the most common amongst the genitourinary cancers. Annexin A2 (AnxA2) is a multifunctional calcium-regulated phospholipids-binding protein found in a subset of renal neoplasms. Since the tumor cells usually recapitulate embryonic cells, we studied the ontogeny of AnxA2 in developing renal tissues and compared it with those of normal adult RCCs, to better understand their role in renal development and tumorigenesis. AnxA2 immunoexpression was evaluated by immunohistochemistry from various autopsied fetuses, mature kidney and renal cancer tissue specimens. The study showed moderate membranous AnxA2 immunoexpression in the ureteric buds and collecting tubules of fetal kidneys (in all gestational ages) and in the collecting ducts of adult normal renal tissues. It is not often expressed in the proximal convoluted tubules of normal adult kidney; however, younger fetal kidneys show moderate AnxA2 immunoexpression in the proximal convoluted tubules (thought to be the origin of RCC) and the reappearance of strong membranous AnxA2 immunoexpression in the clear cell carcinoma is suggesting a deregulation of the gene during tumorigenesis. The understanding of the AnxA2 molecular immunoexpression pattern during development, its specific function and deregulated immunoexpression in different renal carcinoma types indicates the decisive role of AnxA2 in the cancer progression.

Keywords: annexin A2, kidney, nephrogenesis marker, phospholipid-binding protein, renal cell carcinoma.

Introduction

The pre-embryonic, embryonic and fetal periods of human renal development are characterized by three successive primitive forms of excretory system, the pronephros, mesonephros and metanephros. The metanephros (primordial layer of permanent kidney) develops from the nephrogenic cord of intermediate mesoderm by reciprocal inductive interactions between the ureteric bud and metanephric blastema, with the formation of filtration units (nephrons) of adult kidney [1, 2]. The metanephric blastemal cell condenses to form nephrogenic vesicles, S-shaped bodies. The invagination of capillaries (*in situ* formed) into the nephrogenic vesicles helps in the formation of glomerulus; the elongation of the limbs of nephrogenic vesicles differentiate into proximal and distal tubules and loop of Henle (LOH). The ureteric bud progresses to form the collecting tubules, ureters and renal pelvis [3]. This process of tubulogenesis and branching morphogenesis is under the influence of many growth factors and signaling molecules, involving more than 300 genes [4]. The Wilm's tumor suppressor gene 1 (*WT1*) stimulates the ureteric bud to induce differentiation and branching by regulating the production of glial cell line-derived neurotrophic factor (GDNF) and hepatocyte growth factor (HGF). Fibroblast growth factor 2 (FGF2) and bone morphogenetic protein 7 (BMP7) are known to block apoptosis and further aid in the proliferation of the metanephric mesenchyme. Paired-box 2 (*PAX2*) gene and wingless-related (*WNT4*) upregulation promotes the differentiation of mesenchyme into nephron epithelium [5].

Annexin A2 (AnxA2, p36) belongs to a family of calcium (Ca^{2+})-sensitive phospholipid-binding proteins. It has been recognized to play roles in a wide range of biological processes, such as: angiogenesis, proliferation, apoptosis, cell migration, invasion, adhesion, exocytosis, endocytosis, membrane organization, ion channel conductance and linkage of F-actin cytoskeleton to the plasma membrane, based on its localization [6].

Dysregulation and atypical expression of this 36-kDa protein has been linked to a wide spectrum of cancers, emphasizing its participation in tumor cells: adhesion, proliferation, invasion, metastasis and neovascularization [7].

Renal cell carcinoma (RCC) accounts for approximately 3% of all human malignancies and remains a major health issue due to an increase in incidence and mortality rates [8]. Its heterogeneous nature is due to the overlapping of its histomorphological features among its variants [*e.g.*, chromophobe RCC, oncocytoma, granular variant of clear cell RCC (CCRCC), collecting duct RCC] [9]. Diagnosis and treatment of RCC still remain a challenging tasks to healthcare personnel and biomarkers could be helpful in achieving these goals. Currently, imaging biomarkers [*e.g.*, positron emission tomography/computed tomography (PET/CT) with radiotracers, magnetic resonance imaging (MRI) perfusion and MRI diffusion], texture analysis, radiomics, serum biomarkers [*e.g.*, vascular endothelial growth factor (VEGF), interleukin 6, urine biomarkers, neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1)], tissue biomarkers [*e.g.*, PAX8 and PAX2 transcription factors, vimentin, cytokeratin 7 (CK7), c-kit, cathepsin K] are being studied in order to understand the characteristics of RCC [10].

Differential expression of many genes have been explored: neurogenic locus notch homolog protein (*NOTCH*), sonic Hedgehog (*SHH*), wingless-type (*WNT*), *WT1*, *PAX2* and LIM homeobox protein 1 (*LIM1*) have been identified in the past as participants in nephrogenesis molecular pathways/networks, with their re-expression in human renal tumors [11].

Although AnxA2 has been studied in a wide variety of tissues, limited data is provided on its localization in the human fetal and adult kidney [6, 7, 12]. Belonging to the Ca^{2+}-dependent phospholipid-binding protein family, AnxA2 has cell adhesive property and largely participates in the transport across ion channels [7, 13]. These properties of AnxA2 are presumably essential for both the development and functioning of normal adult kidney.

Since the behavior and molecular basis of cancer largely mimics the development of embryo, we tried to study the expression pattern of this Ca^{2+}-dependent phospholipid-binding protein AnxA2 in the renal development, normal functioning and during cancer progression, in an attempt to elucidate its potential role during development and thus provide an insight into kidney tumorigenesis and metastasis. Understanding RCC from the perspective of developmental biology could pave the way to newer therapeutic avenues and may further strengthen the support in making major clinical decisions.

Materials and Methods

Sample collection

This work was approved by the institutional Ethics Committee, Shri Dharmasthala Manjunatheshwara College of Medical Sciences and Hospital, Dharwad, India.

Paraffin-embedded renal tissues were collected from the Archives of the Department of Pathology, and comprised 42 fetal renal tissue samples of gestational age (ranging from 14 to 40 weeks) and 37 renal cancer tissue samples. Thirty-seven normal adult renal tissues were collected by cadavers donated to the Department of Anatomy and were subjected to tissue processing. Five-μm thick sections were obtained from the paraffin-embedded tissue blocks and were stained with Hematoxylin–Eosin (HE) to study: (*a*) the histogenesis of fetal kidney in various phases of fetal development; (*b*) the normal histological architecture of mature kidney; (*c*) the types of RCC, to confirm the original diagnosis, before performing immunohistochemistry. All the fetal and adult renal tissues underwent histopathological assessment to exclude any microscopic abnormalities and affirm the histological diagnosis of tumors. Tumors were graded according to Fuhrman *et al.* [14].

The immunohistochemical (IHC) detection of AnxA2 was performed as described previously [15]. Primary antibody against AnxA2 (1:100 dilution) was purchased from Santa Cruz Biotechnology Inc. (SantaCruz, CA, USA, Catalogue No. Annexin II (H-50): sc-9061]. Anti-AnxA2 primary antibody was replaced by anti-rabbit immunoglobulin G (IgG) whole molecule (Sigma-Aldrich, USA, Catalogue No. A0545), at 1:1000 dilution, as negative control, while triple negative breast cancer cell sections were used as positive controls [16]. Tissue sections immunostained with anti-AnxA2 antibody were graded based on: (*a*) the patterns of their immunoexpression (M – membranous, C – cytoplasmic, N – nuclear, Mx – mixed: cytoplasmic and membranous) [17]; (*b*) the percentage of immunoreactive cells (0 – no staining, 1–10% as 1, 11–50% as 2, 51–70% as 3, 71–100% as 4); (*c*) intensity of staining [0 – no color, (1+) weak brown, (2+) moderate brown, (3+) dense brown, strong immunoexpression] [18]. The intensity and the percentage scores were multiplied. A score of more than (3) was considered significant. Pathology scoring of AnxA2 immunostaining pattern was performed by two independent observers.

Statistical analysis

Using GraphPad Prism7 software, the χ^2 (*chi*-square) test was employed to determine the significance of differences between groups for categorical data. The statistical significance was set at 5% level ($p \leq 0.05$, $\alpha < 0.05$). AnxA2 immunoexpression in the collecting ducts, the collecting tubular cells of fetal kidney at various gestational ages of fetus and mature adult kidney were compared. AnxA2 immunoexpression in the proximal tubular cells of fetal kidney was also compared to the immunoexpression status in that of adult and tumor cells of CCRCC using one-way analysis of variance (ANOVA).

Results

In the current study, we divided the fetal kidneys into three developmental groups. The first group comprised fetal kidneys in stage 2 (14–22 weeks, n=15), during which the ureteric bud ceases to branch and each ampulla induces the formation of six to eight nephrons; this is a period of nephron arcade formation. The second group comprised fetal kidneys in stage 3 (22–36 weeks, n=13), that showed the last formed nephrons in the nephrogenic zone of fetal kidney with less than 36 weeks. The third group comprised fetal kidneys in stage 4 (36 weeks

onwards to adult life, n=14), a stage of pure growth, with no new formation of nephrons [3]. Fetal kidneys in the first stage of development (7 to 14 weeks), in which the ureteric bud branches for six to eight generation, inducing the formation of new nephrons, were not included, due to the difficulty in obtaining the renal tissue from the aborted fetuses. This classification was followed to check the changes in the AnxA2 immunoexpression pattern at various stages of nephronogenesis.

AnxA2 in ureteric buds and collecting ducts of fetal kidneys

The nephrogenic zone (nz) was comprised of primitive forms of nephrons, such as renal vesicles (rv) and S-shaped bodies (S), capillary loop (C) and also the ureteric bud (ub) (lined by cuboidal cells forming the collecting component of mammalian kidney) [19] (Figure 1a). The cortex demonstrated mature glomeruli (mg), developing proximal tubular cells (pt) and distal tubular cells (dt) and a differentiating medulla showing 'ub' derived developing collecting duct cells (cd) (Figure 1b). AnxA2 immunostaining of renal cortex of fetal kidneys from 14 to 22 weeks of gestation (nine out of 15 cases) revealed moderate cytoplasmic positivity in the developing 'pt' (p<0.0001) (Figure 1c). At all-time points, a moderate to strong AnxA2 immunoexpression was observed in the membranes and cytoplasm of 'ub' (as recognized up to 32 weeks of gestation), developing collecting tubules (ct) and 'cd', endothelial cell lining blood vessels of all fetal renal tissues (Figure 1, c and d). As shown in Figure 1 (e and f), the immunoexpression was stronger in the membrane facing the lumens of 'ub' and 'cd'. AnxA2 immunolocalization was rarely seen in the immature forms of glomeruli derived from metanephric blastema.

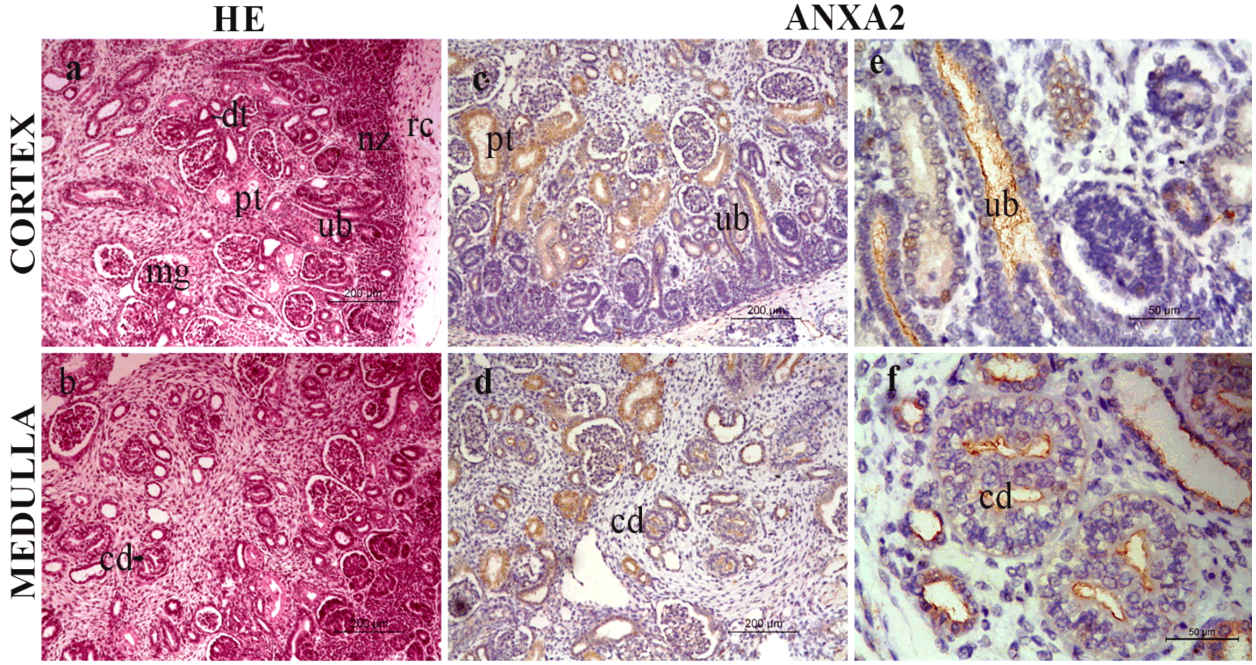

Figure 1 – ***AnxA2 immunoexpression pattern in developing human kidney at 14-week gestational age: (a) HE staining showing a renal capsule (rc), nephrogenic zone (nz) and differentiating outer cortex. Condensations of metanephric blastemal cells with slit-like cavity (renal vesicles – rv) besides the dilated tips of ureteric bud (ub) are seen in the nephrogenic zone. Developing proximal tubular cells (pt), distal tubular cells (dt) and mature glomeruli (mg) in the deeper part of cortex; (b) HE staining of differentiating renal medulla showing developing collecting duct cells (cd) lined by tall columnar epithelium; (c) Embryonic kidney showing moderate AnxA2 immunoreactivity in the 'ub' (membranous), cytoplasmic immunoreactivity in 'pt'; (d) AnxA2 immunoreactivity seen in developing 'cd'; (e) Moderate (2+) immunostaining for AnxA2 observed in the membranes of the cells lining the 'ub'; (f) 'cd' showing strong AnxA2 immunoexpression in the membranes facing the lumen. HE staining: (a and b) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (c and d) ×100 – Scale bar = 200 µm; (e and f) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; HE: Hematoxylin–Eosin.***

Decline of the AnxA2 immunoexpression in proximal tubular cells

A single section of fetal kidney at third stage of development shows the nephrogenic zone (nz) with: 'rv', 'S', 'ub', developing 'mg' – at various stages of maturation –, 'pt', 'dt', LOH, thick limb of LOH (T) and thin limb of LOH (t), and 'cd' (Figure 2, a and b). 'ub' and 'cd' continued to express AnxA2, as shown in Figure 2 (c–f); however, a decline of the AnxA2 immunoexpression in 'pt' was observed in fetal kidneys in third and fourth stages of nephron development (Figure 2c). The primitive forms of immature glomerulus, such as the 'rv', 'S', in the nephrogenic zone showed either mild AnxA2 immunostaining or absence of AnxA2, as shown in Figure 2c.

Unswerving immunoexpression of AnxA2 in the collecting tubular and duct cells

At the fourth stage of nephron development, the cortex appeared more mature with an increase in volume of glomeruli and an increase in tortuosity of 'pt' and 'dt' (Figure 3a), as well as a differentiated medulla, as shown in Figure 3b [3]. IHC staining of these sections of fetal

kidney showed a consistent AnxA2 immunoexpression in the 'ct' and 'cd' (Figure 3, c–f) (p=0.0001). The cells of renal corpuscles, 'dt', LOH showed weak to moderate AnxA2 immunoexpression in fewer cases.

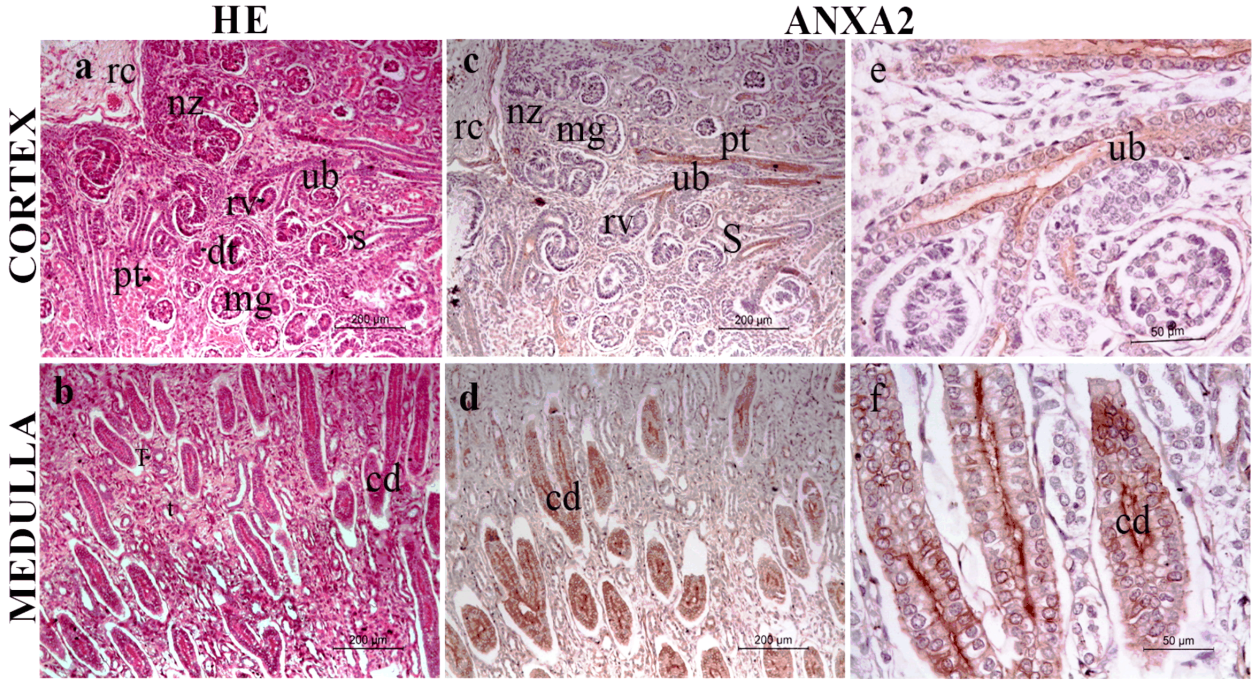

Figure 2 – ***AnxA2 immunostaining of developing human kidney at 24 weeks of gestation: (a) HE staining of embryonic kidney showing outer renal cortex with renal capsule (rc), nephrogenic zone (nz), ureteric bud (ub), renal vesicle (rv), S-shaped bodies (S), mature glomeruli (mg), proximal tubular cells (pt), and distal tubular cells (dt); (b) HE staining of inner renal medulla, demonstrating the developing collecting duct cells (cd), thick LOH (T), thin LOH (t), and blood capillaries; (c and e) AnxA2 mild immunostaining (1+) in the 'pt' and moderate (2+) immunostaining in the 'ub' cells; (d and f) 'cd' in the medulla showing moderate to strong AnxA2 immunostaining. HE staining: (a and b) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (c and d) ×100 – Scale bar = 200 µm; (e and f) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; HE: Hematoxylin–Eosin; LOH: Loop of Henle.***

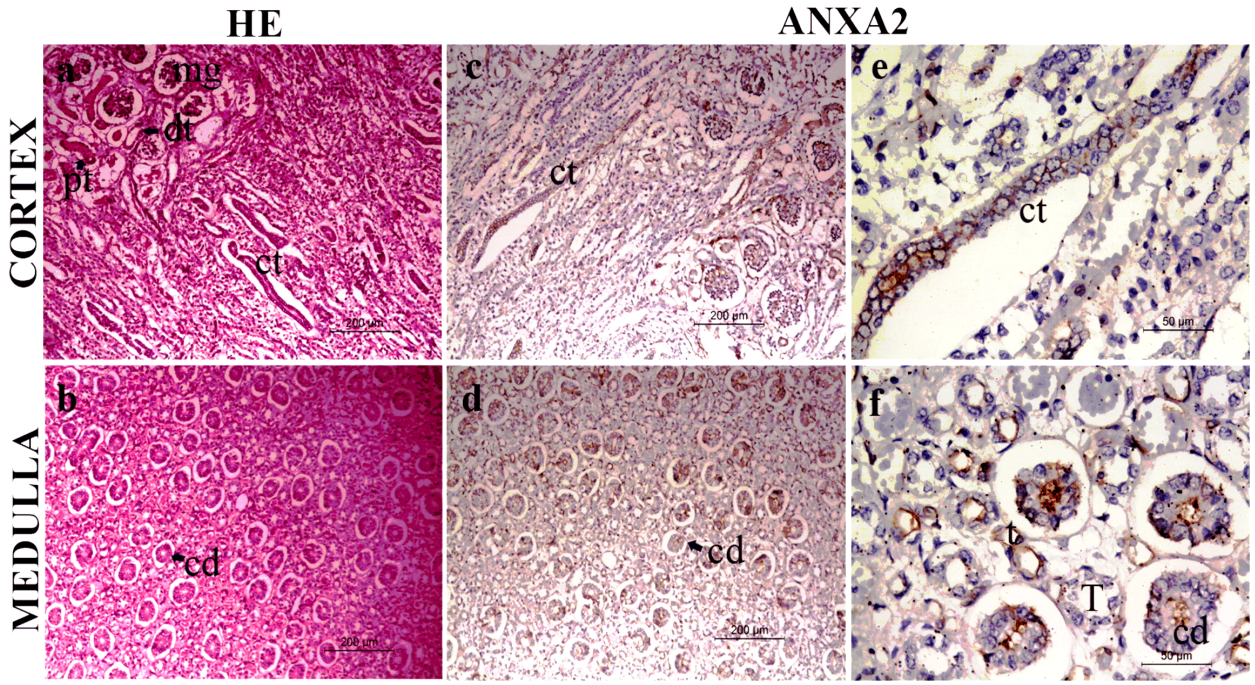

Figure 3 – ***AnxA2 immunostaining of developing human kidney at 36-week gestational age: (a) HE staining of fetal renal cortex showing numerous maturing tubules, proximal tubular cells (pt), distal tubular cells (dt) and mature glomeruli (mg); (b) HE staining of well-differentiated fetal renal medulla showing numerous tubules, such as collecting duct cells (cd), thick LOH (T) and thin LOH (t); (c and e) Moderate immunoexpression of AnxA2 can be appreciated in the cortical collecting tubules (ct); (d and f) Moderate AnxA2 immunostaining in the membranes of 'cd' and 't'. HE staining: (a and b) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (c and d) ×100 – Scale bar = 200 µm; (e and f) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; HE: Hematoxylin–Eosin; LOH: Loop of Henle.***

Lack of AnxA2 immunoexpression in proximal tubules of adult kidneys

The renal cortex is identified by the presence of renal corpuscles (RC), proximal convoluted tubules (PT) and distal convoluted tubules (DT), as depicted in Figure 4a. PT, usually found as a continuation of renal corpuscle, is a coiled tube lined by cuboidal brush-bordered epithelium and intensely stained cytoplasm, whereas the DT is a continuation of ascending limb of LOH, found amongst the PT, and clearly differentiated from the latter by the absence of brush border and a defined lumen, as depicted in Figure 4a. Medulla is distinguished by the presence of collecting tubules (lined by cuboidal epithelium, and a less regular lumen), collecting ducts (lined by columnar epithelium), the thick limb of LOH (T) and thin limb of LOH (t) (lined by cuboidal and squamous epithelium, respectively) and blood vessels (Figure 4b) [20]. All of the cases treated with anti-AnxA2 antibody showed either no staining or very little staining of PT epithelial cells (1+) (Figure 4, c and e). The cells of DT, LOH exhibited AnxA2 immunopositivity (2+) in fewer cases. Out of the 37 normal adult kidneys immunostained, AnxA2 immunoexpression remained constant in the membranes and cytoplasm of collecting ducts and endothelial cells of arterioles and blood capillaries (2+) (Figure 4, d and f). AnxA2 was detected in the mesangial cells, podocytes and parietal cells of RC (2+), in fewer normal renal tissues.

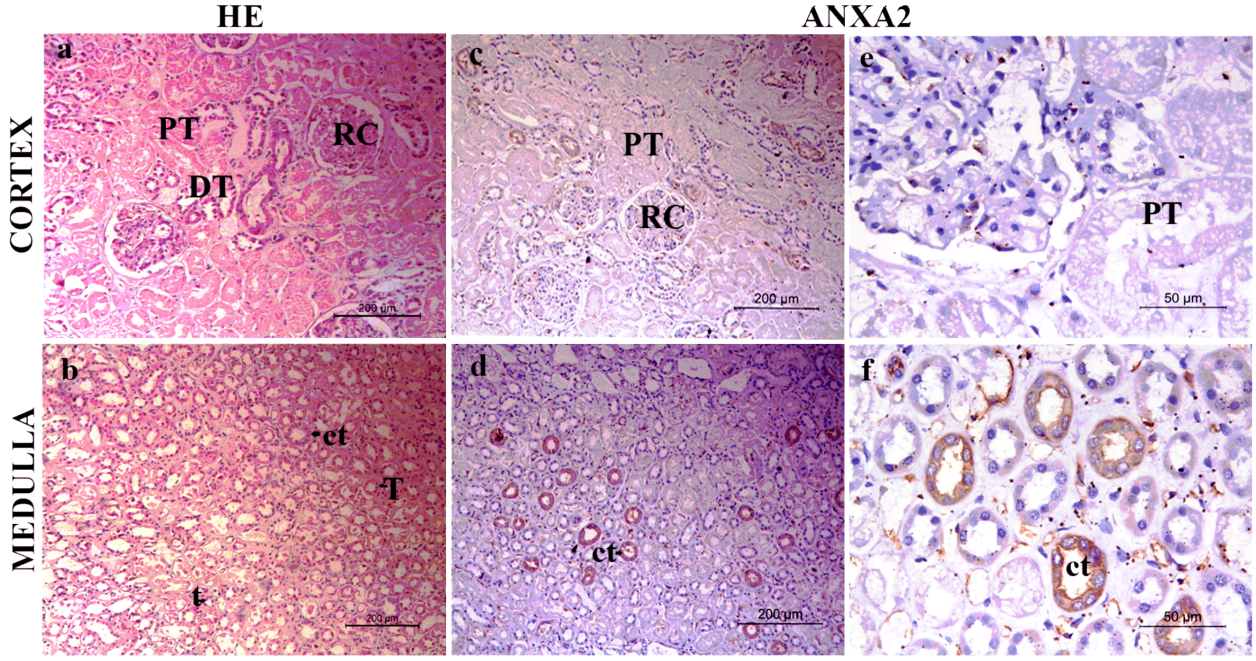

Figure 4 – ***AnxA2 immunostaining of mature adult kidney: (a) HE staining of adult kidney cortex occupied by many proximal tubular (PT) cells lined by cuboidal cells, strongly eosinophilic cytoplasm and presence of microvili. Few pale staining distal tubular (DT) cells lined with cuboidal cells and absence of microvilli amidst the renal corpuscles (RC); (b) HE staining of adult kidney medulla showing mature tubules, such as the collecting tubule (ct), thin limb of LOH (t) and thick limb of LOH (T); (c and e) AnxA2 immunoexpression in adult renal cortex shows absence of immunostaining in the PT cells and mild to moderate immunostaining in fewer DT cells and blood vessels; (d and f) AnxA2 moderate immunostaining in the medulla is restricted to 'ct' cells. HE staining: (a and b) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (c and d) ×100 – Scale bar = 200 µm; (e and f) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; HE: Hematoxylin–Eosin; LOH: Loop of Henle.***

AnxA2 immunoexpression increases with the progression of CCRCC

IHC staining of normal parts of adult kidneys showed either absence or mild AnxA2 immunostaining of PT cells, as shown in Figure 5 (b and c). However, our study comprised predominantly CCRCC cells. The growth patterns of CCRCC varied from typical solid, acinar to tubular, cystic and pseudopapillary type. The tumor cells either presented themselves with clear cytoplasm or admixed with eosinophilic or granular cytoplasm (Figure 5, d, g, and j) [21]. Evaluating AnxA2 immunoexpression in CCRCC tumor cells showed primarily membranous, nuclear and cytoplasmic reactivity. While grade I tumors showed mild AnxA2 immunopositivity (Figure 5, e and f), the immunostaining intensity increased in grade II and grade III CCRCCs (2+) with tumor progression (Figure 5, h, i, k and l).

AnxA2 immunoexpression in variants of RCC

Adult normal kidney collecting tubule demonstrated moderate AnxA2 immunostaining and null immunoexpression in the PT (Figure 6, b and c). The respective immunoexpression was compared with the tumor cells of papillary RCC. The growth pattern of papillary RCC is either papillary or tubulopapillary type [21]. It is histologically differentiated as type I, tumor cells presents with basophilic cytoplasm, low-grade nuclei and absence of nuclear pseudostratification. Type II tumor cells having eosinophilic cytoplasm, high-grade nuclei with pseudostratification, as shown in Figure 6d [22]. In all the cases of

papillary RCC studied, tumor cells were AnxA2 immunonegative, as demonstrated in Figure 6 (e and f).

The adult kidney collecting duct cells showed moderate cytoplasmic and membranous AnxA2 immunostaining, as demonstrated in Figure 7 (a–c). HE staining of collecting duct carcinoma was characterized by glandular or papillary growth pattern (Figure 7d) [21]. Tumor cells of collecting duct carcinoma showed strong AnxA2 immunostaining (3+), as depicted in Figure 7 (e and f). Chromophobe RCC consisted of either solid sheets of cells separated by hyalinized blood vessels, or of large cells with reticulated cytoplasm and small cells with granular cytoplasm. The tumor cells exhibited nuclear wrinkling and distinct cell membranes in HE-stained tumor sections (Figure 7g) [21]. Chromophobe RCC revealed moderate to intense AnxA2 immunostaining (3+), as shown in Figure 7 (h and i). Oncocytomas largely mimic chromophobe RCC histologically, however these tumor cells consisted of deeply eosinophilic cytoplasm, centrally located nuclei with smooth margin (Figure 7j) [22]. In all studied cases of oncocytoma, tumor cells demonstrated mild AnxA2 immunostaining (2+), as depicted in Figure 7l.

We retrospectively compared the immunoexpression of AnxA2 in the PT of fetal kidney at different stages of development with the AnxA2 immunostaining of increasing grades CCRCC tumor cells (Figure 8A, a–f). The expression of the protein in the PT cells of fetal kidneys at earlier gestational weeks was obvious and the further reappearance of AnxA2 in the tumor cells supports the precedential theory. Though both CCRCC and papillary RCC have similar embryonic origin (PT cells), *AnxA2* gene mutation could be speculated only for CCRCC, in papillary RCC an inactivation of *AnxA2* gene may be most probably implicated.

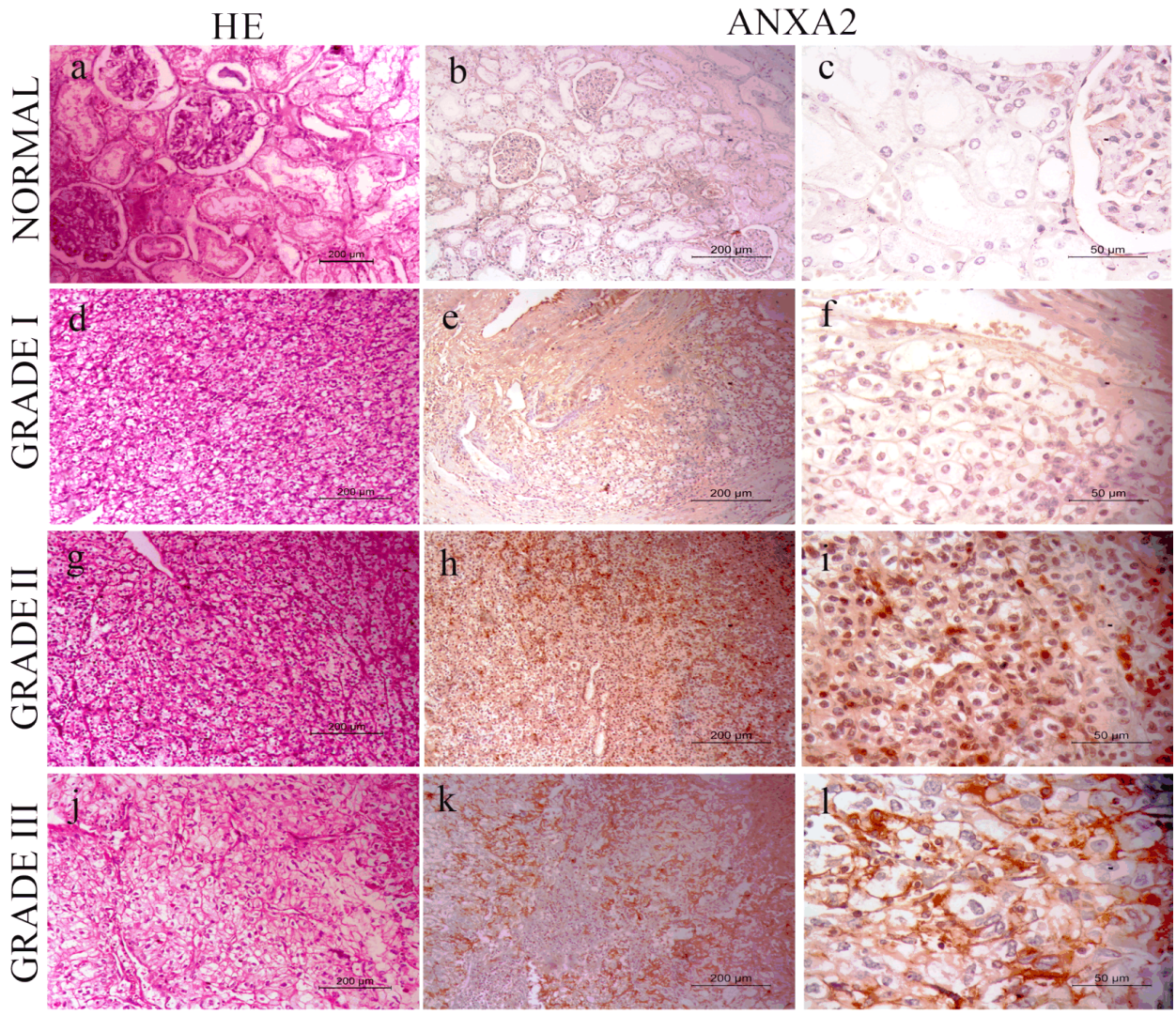

Figure 5 – ***AnxA2 immunostaining of CCRCC: (a) HE staining of normal adult renal cortex showing renal corpuscles (RC), proximal tubular (PT) cells and distal tubular (DT) cells; (b and c) AnxA2 immunostaining of normal adult renal cortex demonstrating lack of immunostaining in the PT cells; (d) HE staining of grade I CCRCC; (e and f) Mild AnxA2 immunostaining in the tumor cells and blood vessels of grade I CCRCC; (g) HE staining of grade II CCRCC; (h and i) Moderate to strong AnxA2 immunoexpression can be appreciated in the tumor cells of grade II CCRCC; (j) HE staining of grade III CCRCC; (k and l) Moderate to strong AnxA2 immunoexpression demonstrated in the tumor cells of grade III CCRCC. HE staining: (a, d, g and j) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (b, e, h and k) ×100 – Scale bar = 200 µm; (c, f, i and l) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; CCRCC: Clear cell renal cell carcinoma; HE: Hematoxylin–Eosin.***

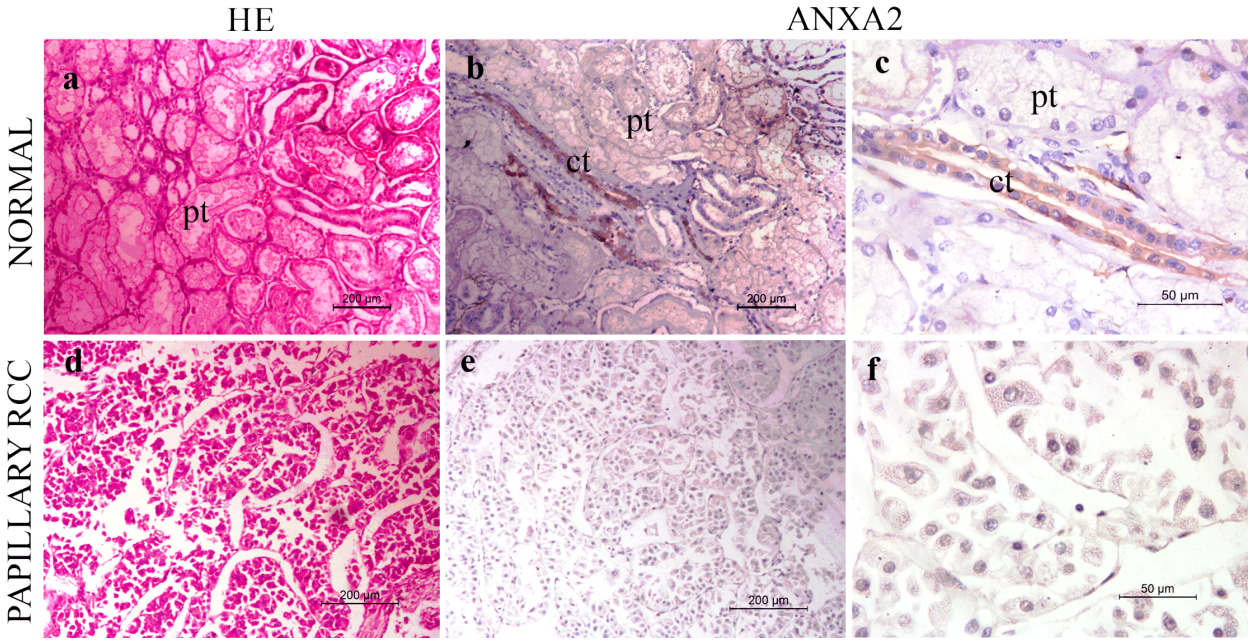

Figure 6 – ***AnxA2 immunostaining of papillary RCC: (a) HE staining of normal adult renal cortex showing numerous proximal tubular (PT) cells; (b and c) AnxA2 immunostaining of normal adult renal cortex demonstrating lack of immunostaining in the PT cells and moderate immunostaining in the collecting tubular cells; (d) HE staining of papillary RCC; (e and f) Absence of AnxA2 immunostaining in the tumor cells of papillary RCC. HE staining: (a, and b) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (c and d) ×100 – Scale bar = 200 µm; (e and f) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; RCC: Renal cell carcinoma; HE: Hematoxylin–Eosin.***

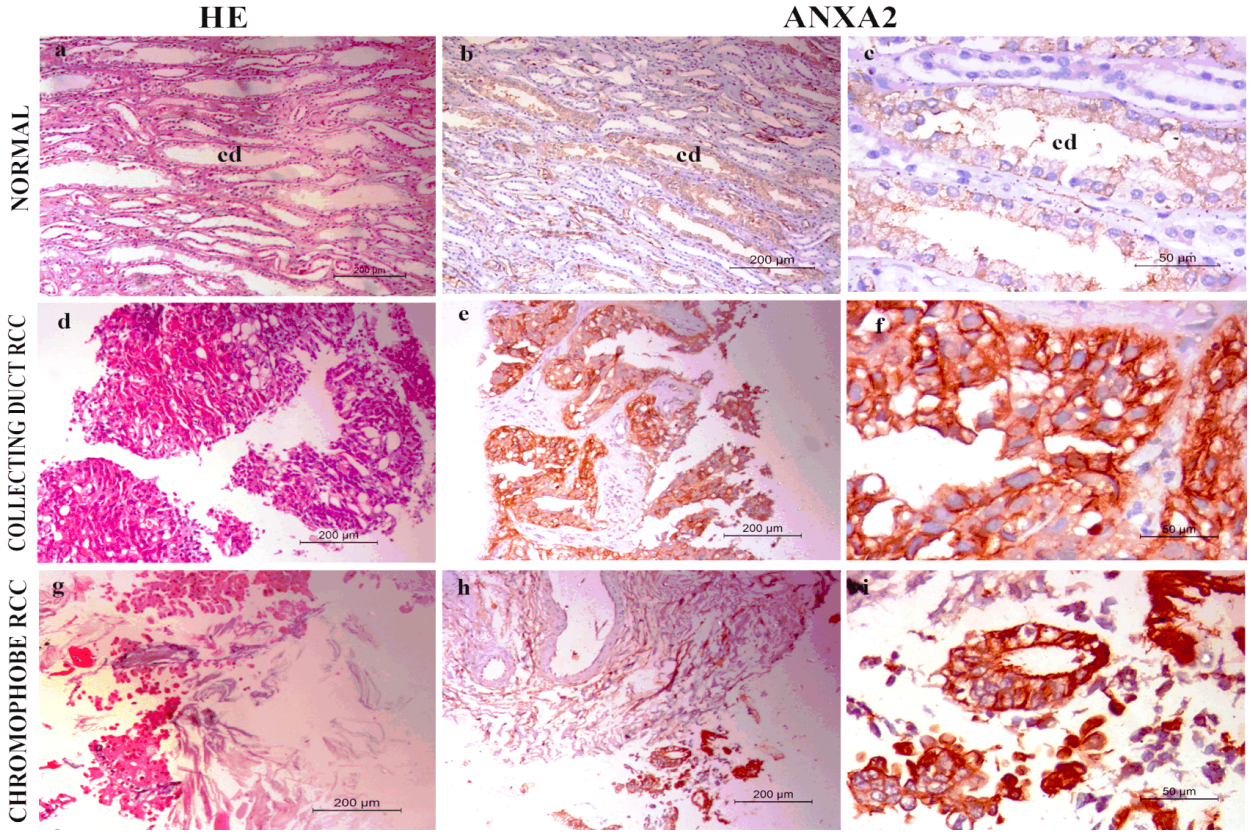

Figure 7 – ***AnxA2 immunostaining in variants of RCC: (a) HE staining of normal adult renal medulla showing mature tubules with collecting duct cells (cd); (b and c) AnxA2 immunostaining of normal adult renal medulla demonstrating moderate membranous and cytoplasmic immunostaining in 'cd'; (d) HE staining of collecting duct RCC; (e and f) Strong immunostaining for AnxA2 in the tumor cells of collecting duct RCC; (g) HE staining of chromophobe RCC; (h and i) Strong immunoexpression of AnxA2 can be appreciated in the tumor cells of chromophobe RCC;. HE staining: (a, d, g and j) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (b, e, h and k) ×100 – Scale bar = 200 µm; (c, f, i and l) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; RCC: Renal cell carcinoma; HE: Hematoxylin–Eosin.***

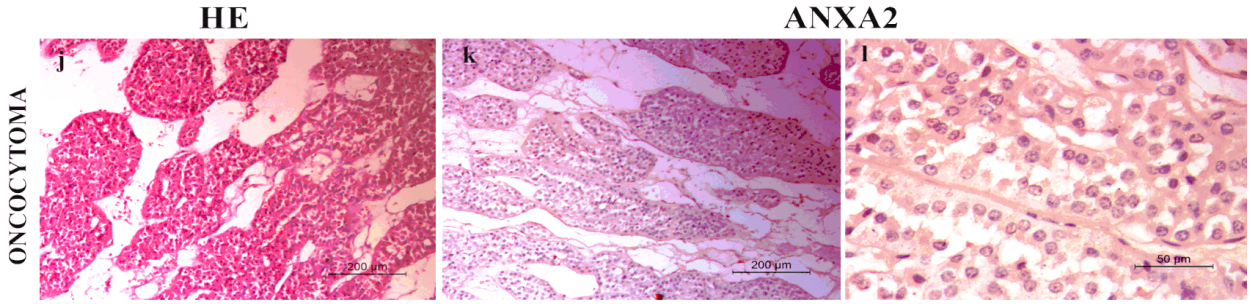

Figure 7 (continued) – ***AnxA2 immunostaining in variants of RCC: (j) HE staining of oncocytoma; (k and l) Mild immunoexpression of AnxA2 demonstrated in the tumor cells of oncocytoma. HE staining: (a, d, g and j) ×100 – Scale bar = 200 µm. Anti-AnxA2 antibody immunostaining: (b, e, h and k) ×100 – Scale bar = 200 µm; (c, f, i and l) ×400 – Scale bar = 50 µm. AnxA2: Annexin A2; RCC: Renal cell carcinoma; HE: Hematoxylin–Eosin.***

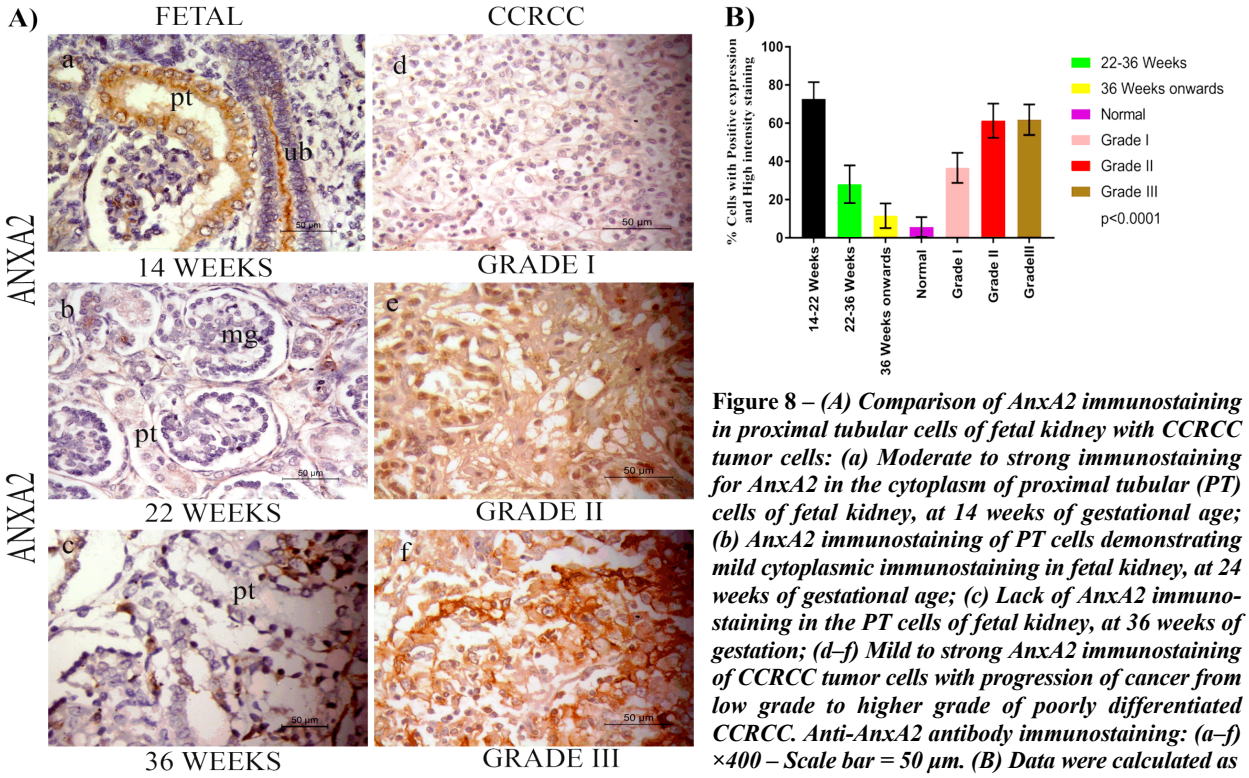

Figure 8 – ***(A) Comparison of AnxA2 immunostaining in proximal tubular cells of fetal kidney with CCRCC tumor cells: (a) Moderate to strong immunostaining for AnxA2 in the cytoplasm of proximal tubular (PT) cells of fetal kidney, at 14 weeks of gestational age; (b) AnxA2 immunostaining of PT cells demonstrating mild cytoplasmic immunostaining in fetal kidney, at 24 weeks of gestational age; (c) Lack of AnxA2 immunostaining in the PT cells of fetal kidney, at 36 weeks of gestation; (d–f) Mild to strong AnxA2 immunostaining of CCRCC tumor cells with progression of cancer from low grade to higher grade of poorly differentiated CCRCC. Anti-AnxA2 antibody immunostaining: (a–f) ×400 – Scale bar = 50 µm. (B) Data were calculated as average percentage of AnxA2 expressing cells in the PT cells of fetal, non-neoplastic adult tissues against CCRCC tumor cells, at different tumor grades. The percentage of AnxA2 immunopositive tumor cells increased with the increasing of tumor grade (p<0.0001). AnxA2: Annexin A2; CCRCC: Clear cell renal cell carcinoma.***

Discussions

Cancer cell orchestrates early embryonic cells. Both kinds of cells undergo deprogramming to become immortal and invasive [23]. Currently, developmental biologists are focused on the genes involved in the biological process of cell differentiation, as tumorigenesis is mainly thought to be due to the disruption of normal cell differentiation process controlled by various genes; mutations of such genes have a major role in tumor initiation and progression [24].

AnxA2 (36-kDa, lipocortin II) is a multifunctional Ca^{2+}-regulated phospholipid-binding protein present abundantly in endothelial, smooth muscle, trophoblast and few tumor cells [12]. AnxA2 functions as either a monomer or heterotetramer. The AnxA2–S100A10 heterotetramer interacts with cytoskeletal, membrane and extracellular matrix (ECM) regulating a wide variety of biological processes that includes tissue remodeling, ECM degradation, angiogenesis, actin cytoskeletal dynamics, endocytosis, exocytosis, cell–cell adhesion and cell polarity, thereby mediating regulatory effects on cell behavior [7, 25]. Kidney development is closely related to cell differentiation, proliferation and apoptosis, and in addition, reorganization of cytoskeleton of metanephric mesenchyme by the formation of cell-to-cell contacts and ECM interactions is leading to the completion of developmental program [26].

To understand the role of AnxA2 in human renal development and cancer, it is necessary to establish its localization in various developing renal structures. Our study on the AnxA2 ontogeny during renal development reveals that this molecule is expressed in the ureteric bud and its derivatives, such as the collecting tubules and collecting ducts at all-time points (as early as 12th week of gestation). AnxA2 immunoexpression often appeared within the cells or on the plasma membrane. In addition, moderate cytosolic AnxA2 immunoexpression was detected in the early developing PT. Similar immunoexpression

pattern was observed in one of the fetal kidney (not included in the study group) at 12th week of gestation, speculating a much earlier AnxA2 immunoexpression. Our study confirmed that AnxA2 immunoexpression in the PT, declined (1+) with increasing gestational age. Earlier works on the AnxA2 immunoexpression in animal models have emphasized its role in Ca^{2+} homeostasis. Ca^{2+} pulses, waves and gradients are involved in coordination of cell movements, axis specification during early vertebrate development. Recent recordings of Ca^{2+} wave in meta-nephric blastemal cells of explanted rat embryonic kidney suggested the requirement of Ca^{2+} for kidney development. Gilbert *et al.* showed a restricted AnxA2 immunoexpression in the ureteric bud and collecting ducts of embryonic mouse kidney, indicating its participation in Ca^{2+} homeostasis *via* membrane traffic or regulation of ion channel activities in the developing permanent kidneys [27]. A study conducted to examine the relationship between dentin phosphoprotein and AnxA2 showed their membranous and cytosolic colocalization and physiological Ca^{2+}-dependent binding in rat kidney ureteric bud cells, this likely indicating the participation of the molecule in Ca^{2+} transport [28]. Another member of annexin family, annexin IV, is also acknowledged to play important role in pronephros morphogenesis [27]. The cytosolic and membranous AnxA2 immunoexpression is often involved in the regulation of actin cytoskeleton dynamics, endocytosis and exocytosis, cell–cell adhesion, cell polarity and endosome formation [7].

AnxA2 has specific roles in the kidney. In the collecting duct, it mediates cyclic adenosine monophosphate (cAMP)-induced trafficking of aquaporin 2, while in the thick ascending limb (TAL) of Henle, it participates in the recruitment and activation of Na^+–K^+–$2Cl^-$ (NKCC2) cotransporter. NKCC2, responsible for urine concentration and systemic salt homeostasis interacts with AnxA2 in phosphorylation-dependent manner. The latter promotes its apical translocation in response to vasopressin signaling [29].

Several studies provide evidence of colocalization of AnxA2–S100A10 complex with transient receptor potential cation channel subfamily V member 5 (TRPV5) and TRPV6 Ca^{2+} channel in renal tubules, suggesting its role in apical ion transport in Madin–Darby canine kidney (MDCK) cells (an epithelial line with features of collecting ducts) and active Ca^{2+} reabsorption [13, 30]. Further, an association of the heterotetramer complex with neuroblast differentiation-associated protein (AHNAK) in the formation of cell-to-cell contacts and cell polarity in MDCK cells was observed [13]. Domoto *et al.* demonstrated the immunoexpression of both antigens (AnxA2 and S100A10) in the renal medulla of normal human kidney, primarily in the LOH, distal convoluted tubules and collecting duct cells [30]. Current study also demonstrated a similar immunoexpression pattern, AnxA2 being largely expressed in the endothelial cells, collecting tubules and collecting duct cells of the normal adult kidney, and occasional in the LOH and distal tubules cells, this suggesting a possible role of the protein in Ca^{2+} uptake from these nephron' components. The lack of AnxA2 immuno-expression in the PT of adult human kidney suggests that in these cells probably AnxA2 fails to play a role in structural organization of the membrane, housekeeping functions or regulation of the membrane intracellular traffic [31].

AnxA2 is expressed in a wide spectrum of cancer that includes: cervical, breast, hepatic, non-small cell lung carcinoma and multiple myeloma [32]. RCCs are the most common, amongst the genitourinary cancers, and AnxA2 has been found in a subset of renal neoplasms, such as CCRCC, papillary and chromophobe RCC [32]. AnxA2 monomer exists in the cell cytoplasm and nucleus and AnxA2 heterotetramer is present on the cell membrane. Nuclear AnxA2 is thought to promote deoxyribonucleic acid (DNA) replication and chromosomal instability, explaining the rapid tumor growth and resistance to chemotherapy [12]. When it is located on the extracellular surface, AnxA2, a natural ligand of S100A10, also promotes the formation of plasmin and active forms of matrix metalloproteinases, resulting in ECM degradation, with further membrane mediate cytoskeletal remodeling, thus permitting tumor invasion and metastases [7, 25, 30, 32].

Our findings corroborate earlier reports regarding the strong AnxA2 immunoexpression in cancer cell membranes of CCRCC [32]. While the tumor cells of chromophobe, collecting duct carcinoma and oncocytoma were AnxA2 immunopositive, the tumor cells of papillary RCC were immunonegative. CCRCC and papillary RCC are thought to be derived from the PT epithelium, while chromophobe, collecting duct carcinoma and oncocytomas are derived from the ureteric bud [33]. This probably explains the variability of AnxA2 immunoexpression in different types of renal tumors. Accordingly, we observed the presence of AnxA2 immunoexpression in the normal renal epithelia of the distal tubules, LOH, collecting duct system, endothelial cells, but not in the PT (thought to be the origin of the most CCRCC), this possibly indicating a malignant transformation of the normal cells.

Conclusions

Our study points towards AnxA2 immunoexpression in embryonic and neoplastic kidney that could be of clinical relevance in designing new standard markers against CCRCC and diseases attaining ureteric bud and embryonic collecting ducts. The membranous and cytosolic AnxA2 immunoexpression in the fetal and adult renal tubules may presumably play an essential regulatory role in the maintenance of cell–cell contacts, membrane cytoskeletal dynamics, Ca^{2+} homeostasis and other cell signaling events. If AnxA2 is not often expressed in the PT of normal adult kidney, fetal kidneys show moderate immunoexpression in the PT and a strong immunoexpression in the CCRCC, suggesting a deregulation of the gene during tumorigenesis. AnxA2 is also expressed in the ureteric bud and its derivatives, such as collecting tubules, collecting ducts and chromophobe, collecting duct RCC. The analysis of AnxA2 immunoexpression in the nascent, adult and neoplastic human kidney could be of interest to the developmental and cancer biologists in designating of molecular networks that could bridge the gap between the involvement of this molecule in the developing kidney and renal tumorigenesis.

Conflict of interests

The authors declare that they have no conflict of interests.

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References

[1] Čukuranović R, Vlajković S. Age related anatomical and functional characteristics of human kidney. Facta Univ Med Biol, 2005, 12(2):61–69.
[2] Song R, Yosypiv IV. Development of the kidney medulla. Organogenesis, 2012, 8(1):10–17.
[3] Lacson A, Bernstein J, Risdon RA, Gilbert-Barness E. Chapter 27: Renal system. 1. Kidneys and urinary tract. In: Gilbert-Barness E (ed). Potter's pathology of the fetus, infant and child. 2nd edition, vol. 2, Mosby–Elsevier, China, 2007, 1281–1344.
[4] Leroy X, Devisme L, Buisine MP, Copin MC, Aubert S, Gosselin B, Aubert JP, Porchet N. Expression of human mucin genes during normal and abnormal renal development. Am J Clin Pathol, 2003, 120(4):544–550.
[5] Sulemanji M, Vakili K. Neonatal renal physiology. Semin Pediatr Surg, 2013, 22(4):195–198.
[6] Bharadwaj A, Bydoun M, Holloway R, Waisman D. Annexin A2 heterotetramer: structure and function. Int J Mol Sci, 2013, 14(3):6259–6305.
[7] Xu XH, Pan W, Kang LH, Feng H, Song YQ. Association of annexin A2 with cancer development (review). Oncol Rep, 2015, 33(5):2121–2128.
[8] El-Shorbagy SH, Alshenawy HA. Diagnostic utility of vimentin, CD117, cytokeratin-7 and caveolin-1 in differentiation between clear cell renal cell carcinoma, chromophobe renal cell carcinoma and oncocytoma. J Microsc Ultrastruct, 2017, 5(2):90–96.
[9] Ozcan A, Zhai J, Hamilton C, Shen SS, Ro JY, Krishnan B, Truong LD. PAX-2 in the diagnosis of primary renal tumors: immunohistochemical comparison with renal cell carcinoma marker antigen and kidney-specific cadherin. Am J Clin Pathol, 2009, 131(3):393–404.
[10] Farber NJ, Kim CJ, Modi PK, Hon JD, Sadimin ET, Singer EA. Renal cell carcinoma: the search for a reliable biomarker. Transl Cancer Res, 2017, 6(3):620–632.
[11] Dormoy V, Jacqmin D, Lang H, Massfelder T. From development to cancer: lessons from the kidney to uncover new therapeutic targets. Anticancer Res, 2012, 32(9):3609–3617.
[12] Cheng CW, Rifai A, Ka SM, Shui HA, Lin YF, Lee WH, Chen A. Calcium-binding proteins annexin A2 and S100A6 are sensors of tubular injury and recovery in acute renal failure. Kidney Int, 2005, 68(6):2694–2703.
[13] Markoff A, Gerke V. Expression and functions of annexins in the kidney. Am J Physiol Renal Physiol, 2005, 289(5): F949–F956.
[14] Eble JN, Grignon DJ, Young RH. Chapter 12: Tumors of the urinary tract. Part A: Tumors of the kidney. In: Fletcher CDM (ed). Diagnostic histopathology of tumors. 4th edition, vol. 1, Elsevier–Saunders, Philadelphia, 2013, 559–601.
[15] Shetty P, Patil VS, Mohan R, D'Souza LC, Bargale A, Patil BR, Dinesh US, Haridas V, Kulkarni SP. Annexin A2 and its downstream IL-6 and HB-EGF as secretory biomarkers in the differential diagnosis of Her-2 negative breast cancer. Ann Clin Biochem, 2017, 54(4):463–471.
[16] Shetty PK, Thamake SI, Biswas S, Johansson SL, Vishwanatha JK. Reciprocal regulation of annexin A2 and EGFR with Her-2 in Her-2 negative and herceptin-resistant breast cancer. PLoS One, 2012, 7(9):e44299.
[17] Lacunza E, Ferretti V, Barbeito C, Segal-Eiras A, Croce MV. Immunohistochemical evidence of Muc1 expression during rat embryonic development. Eur J Histochem, 2010, 54(4): e49.
[18] Kang WY, Chen WT, Huang YC, Su YC, Chai CY. Over-expression of annexin 1 in the development and differentiation of urothelial carcinoma. Kaohsiung J Med Sci, 2012, 28(3): 145–150.
[19] Bertelli E, Regoli M, Fonzi L, Occhini R, Mannucci S, Ermini L, Toti P. Nestin expression in adult and developing human kidney. J Histochem Cytochem, 2007, 55(4):411–421.
[20] Young B, Lowe JS, Stevens A, Heath JW. Wheater's functional histology: a text and colour atlas. 5th edition, Churchill Livingstone–Elsevier, Edinburgh, 2006, 302–327.
[21] Truong LD, Shen SS. Immunohistochemical diagnosis of renal neoplasms. Arch Pathol Lab Med, 2011, 135(1):92–109.
[22] Kuroda N, Tanaka A, Ohe C, Nagashima Y. Recent advances of immunohistochemistry for diagnosis of renal tumors. Pathol Int, 2013, 63(8):381–390.
[23] Monk M, Holding C. Human embryonic genes re-expressed in cancer cells. Oncogene, 2001, 20(56):8085–8091.
[24] Ma Y, Zhang P, Wang F, Yang J, Yang Z, Qin H. The relationship between early embryo development and tumourigenesis. J Cell Mol Med, 2010, 14(12):2697–2701.
[25] Hitchcock JK, Katz AA, Schäfer G. Dynamic reciprocity: the role of annexin A2 in tissue integrity. J Cell Commun Signal, 2014, 8(2):125–133.
[26] Jeon YJ, Kim J, Chae JI. Comparative proteomic analysis of kidney development-related proteins in the pig. *In Vitro* Cell Dev Biol Anim, 2013, 49(5):315–323.
[27] Gilbert T, Leclerc C, Moreau M. Control of kidney development by calcium ions. Biochimie, 2011, 93(12):2126–2131.
[28] Alvares K, Stern PH, Veis A. Dentin phosphoprotein binds annexin 2 and is involved in calcium transport in rat kidney ureteric bud cells. J Biol Chem, 2013, 288(18):13036–13045.
[29] Dathe C, Daigeler AL, Seifert W, Jankowski V, Mrowka R, Kalis R, Wanker E, Mutig K, Bachmann S, Paliege A. Annexin A2 mediates apical trafficking of renal Na^+–K^+–$2Cl^-$ cotransporter. J Biol Chem, 2014, 289(14):9983–9997.
[30] Domoto T, Miyama Y, Suzuki H, Teratani T, Arai K, Sugiyama T, Takayama T, Mugiya S, Ozono S, Nozawa R. Evaluation of S100A10, annexin II and B-FABP expression as markers for renal cell carcinoma. Cancer Sci, 2007, 98(1):77–82.
[31] Tribolo S, Maroux S, Massey-Harroche D. Kidney proximal tubule cells: epithelial cells without EGTA-extractable annexins? Biochem Cell Biol, 2000, 78(4):495–502.
[32] Yang SF, Hsu HL, Chao TK, Hsiao CJ, Lin YF, Cheng CW. Annexin A2 in renal cell carcinoma: expression, function, and prognostic significance. Urol Oncol Semin Orig Investig, 2015, 33(1):22e.11–22.e21.
[33] Terpe HJ, Störkel S, Zimmer U, Anquez V, Fischer C, Pantel K, Günthert U. Expression of CD44 isoforms in renal cell tumors. Positive correlation to tumor differentiation. Am J Pathol, 1996, 148(2):453–463.

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