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MASTER'S PROGRAM

DETERMINATION OF *ACTR2* GENE EXPRESSION IN KIDNEY CANCER

MASTER'S THESIS

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Supervisor: Prof. Dr. Haci Omer ATES

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ETHICS CONTRACT

T.C.

TOKAT GAZIOSMANPASA UNIVERSITY

TO THE INSTITUTE OF GRADUATE STUDIES DIRECTORATE

According to the thesis writing instructions of Tokat Gaziosmanpasa University College of Medicine, my Master's thesis named “Determination of *ACTR2* gene expression in kidney cancer” was prepared under the guidance and consultation of “Prof. Dr. H. Omer ATES” is based on scientific ethical values and rules. Furthermore, I declare that I obtained and presented all of the information in accordance with academic guidelines and ethical values. To comply with these standards and principles, I have cited any data, ideas, and discoveries that are not my own.

Ethical Committee for Clinical Research at Tokat Gaziosmanpasa University decision number: 22-KAEK-045.

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JURY ACCEPTANCE AND APPROVAL

The defending examination for the thesis study titled “Determination of *ACTR2* Gene Expression in Kidney Cancer’’ is prepared by Milat Hussein MUSTAFA and was held on 26/09/2024. It has been accepted by jury members as a Master’s Thesis at Tokat Gaziosmanpasa University, Graduate Education Institute, Department of Medical Biology.

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/ /2024

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ABSTRACT

DETERMINATION OF *ACTR2* GENE EXPRESSION IN KIDNEY CANCER

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Kidney cancer, particularly renal cell carcinoma (RCC), poses a significant health challenge globally, with its incidence steadily rising. Understanding the molecular mechanisms underlying its pathogenesis is crucial for developing targeted therapies. Among the multitude of genes implicated in kidney cancer, the Actin-Related Protein 2 (*ACTR2*) gene has lately received attention for its potential role in tumor development and disease furtherance. *ACTR2* is an important member of the actin-related protein (ARP) group that participates in several cellular activities like cytoskeletal dynamics, intracellular transport, and gene expression control. The study aim to explore the functional implications of *ACTR2* dysregulation in kidney cancer, highlighting its role in tumor cell proliferation, invasion, and resistance to conventional therapies. The study used quantitative Real-time polymerase chain reaction (qRT-PCR) to analyse gene expression rates in 11 cancer and 11 normal tissue samples from the kidney of the same patient. The research indicated a 1.206-fold elevation in *ACTR2* gene expression in kidney cancer tissue samples relative to normal kidney tissues; however, this increase did not reach statistical significance ($p = 0.234$). Accordingly, we believe that further studies with large samples are needed to analyze and understand the *ACTR2* expression level relationship to kidney cancer.

Keywords: Actin-related Protein, *ACTR2* Gene, Kidney Cancer, RCC.

ÖZET

BÖBREK KANSERİNDE *ACTR2* GEN EKSPRESYONUNUN BELİRLENMESİ

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Böbrek kanseri, özellikle de renal hücreli karsinom (RCC), görülme sıklığının giderek artmasıyla birlikte küresel olarak önemli bir sağlık sorunu teşkil etmektedir. Patogenezinin altında yatan moleküler mekanizmaların anlaşılması, hedefe yönelik tedavilerin geliştirilmesi için çok önemlidir. Böbrek kanserinde rol oynayan çok sayıda gen arasında Aktin İlişkili Protein 2 (*ACTR2*) geni, tümörigenez ve hastalığın ilerlemesindeki potansiyel rolü nedeniyle son zamanlarda dikkatleri üzerine çekmiştir. ACTR2, hücre iskeleti dinamikleri, hücre içi taşıma ve gen ekspresyonu kontrolü gibi çeşitli hücresel aktivitelere katılan aktin ilişkili protein (ARP) ailesinin önemli bir üyesidir. Bu çalışma, *ACTR2* disregülasyonunun böbrek kanserindeki fonksiyonel etkilerini araştırmakta ve tümör hücresi proliferasyonu, invazyonu ve geleneksel tedavilere dirençteki rolünü ortaya koymayı amaçlamıştır. Çalışmada, aynı hastanın böbreğinden alınan 11 kanser ve 11 sağlıklı böbrek dokusunda gen ekspresyon düzeyini belirlemek için kantitatif gerçek zamanlı polimeraz zincir reaksiyonu (qRT-PCR) kullanılmıştır. Çalışma, normal böbrek dokusuna kıyasla kanserli böbrek dokusu örneklerinde *ACTR2* gen ifadesinde 1.206 kat artış olduğunu ortaya koymuştur, ancak bu artış istatistiksel anlamlılığa ulaşmamıştır ($p = 0.234$). Bu doğrultuda, *ACTR2* ekspresyon düzeyinin böbrek kanseri ile ilişkisini analiz etmek ve anlamak için büyük örneklemli daha fazla çalışmaya ihtiyaç olduğuna inanıyoruz

Anahtar Kelimeler: Aktin ilişkili Protein, *ACTR2* Geni, Böbrek Kanseri, RCC.

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LIST OF ABBREVIATIONS

ABPs	:	Actin Binding Proteins
ACTB	:	Actin-B
***ACTR2* (*Arp* 2)**	:	Actin-Related Protein 2
***ACTR3* (*Arp* 3)**	:	Actin-Related Protein 3
ALKP	:	Alkaline Phosphatase
ALL	:	Acute lymphocytic leukemia
AML	:	Acute Myelocytic Leukemia
Arp2/3 complex	:	Actin-Related Proteins 2 and 3 Complex
ARPC 1-5	:	Actin-Related Protein 2/3 Complex (Subunit 1-5)
ARPC1	:	Actin-Related Protein 2/3 Complex (Subunit 1)
ARPC1A	:	Actin-Related Protein 2/3 complex (Subunit 1A)
ARPC1B	:	Actin-Related Protein 2/3 complex (Subunit 1B)
ARPC-2	:	Actin-Related Protein 2/3 Complex (Subunit 2)
ARPC3	:	Actin-Related Protein 2/3 Complex (Subunit 3)
ARPC5	:	Actin-Related Protein 2/3 Complex (Subunit 5)
BAP1	:	BRCA1-associated protein-1
BMI	:	Body Mass Index
CBC	:	Complete blood cell count

ccRCC	:	clear cell Renal Cell Carcinomas
CDKN2A	:	Cyclin-dependent kinase inhibitor 2A
cDNA	:	Complementary DNA
Chr 2	:	Chromosome 2
CKD	:	Chronic kidney Disease
CLL	:	Chronic lymphocytic leukemia
cm	:	Centimeter
CML	:	Chronic Myelocytic Leukemia
CRC	:	Colorectal cancer
CRCLM	:	Colorectal cancer liver metastasis
crRCC	:	chromophobe Renal Cell Carcinomas
CT	:	Computed Tomography
Ct	:	Cycle Threshold
DCT	:	Distal Connecting Tubules
DKD		Diabetic kidney disease
DNA	:	Deoxyribonucleic Acid
DSBs	:	Double-Strand Breaks
dsDNA	:	Double-stranded DNA
DTT	:	Dithiothreitol
ECM	:	Extracellular Matrix

ESRD	:	End-Stage Renal Disease
F-actin	:	Filamentous actin
G-actin	:	Globular actin
GLUT1	:	Glucose Transporter 1
HCC	:	Hepatocellular Carcinoma
HNSCC	:	Head and Neck Squamous Cell Carcinoma
HR	:	Homologous Recombination
HTN	:	Hypertension
ISUP	:	International Society of Urological Pathology
IVP	:	Intravenous pyelogram
JMY	:	Junction-mediating and regulatory protein
kg/m2	:	kilograms per square meter
LDH	:	Lactate Dehydrogenase
LFT	:	Liver function tests
mL	:	Milliliter
MRI	:	Magnetic Resonance Imaging
mRNA	:	messenger RNA
NPF	:	Neuropeptide F
N-WASP	:	Neuronal Wiskott-Aldrich Syndrome protein
OS	:	Overall Survival

PBRM1	:	polybromo l
PCR	:	Polymerase Chain Reaction
PDGF		Platelet-Derived Growth Factor
PET	:	Positron Emission Tomography
pRCC	:	papillary Renal Cell Carcinomas
qPCR	:	quantitative PCR
qRT-PCR	:	Quantitative real-time polymerase chain reaction
RB1	:	Retinoblastoma 1
RCC	:	Renal Cell Carcinoma
RNA	:	Ribonucleic Acid
ROX	:	Carboxy rhodamine
RUNX1	:	Runt-related transcription factor 1
SEM	:	Standard error mean
SH3	:	Src homology 3
ssDNA	:	single-stranded DNA
TCC	:	Transitional cell carcinoma
TFTs	:	Thyroid function Tests
TKIs	:	Tyrosine kinase inhibitors
TP53	:	Tumor protein p53
TSG	:	Tumor Suppressor Gene

UV	:	Ultraviolet Radiation
VEGF	:	Vascular Endothelial Growth Factor
VHL	:	Von Hippel-Lindau
WASH	:	Wiskott-Aldrich syndrome protein and SCAR homolog
WASP	:	Wiskott-Aldrich Syndrome Protein
WAVE	:	WASP Family Verprolin-homologous protein
WAVE2	:	WASP Family Verprolin-homologous Protein-2
WCA	:	W: WASP-homology 2 domain, Connector, and Acid
WCR	:	World Cancer Report
WH2	:	Wiskott-Aldrich syndrome protein homology 2
WHAMM	:	WASP homolog associated with actin, membranes, and microtubules
WHO	:	World Health Organization
WT	:	Wilms tumor
°C	:	degree Celsius
µL	:	Microliter

1. INTRODUCTION

Cancer is considered the second main cause of mortality worldwide. Therefore, cancer is a severe disease that affects the health of all human communities (Siegel et al., 2013). Cancer is a complicated and widespread collection of illnesses characterized by the uncontrollable development and spread of abnormal cells in the body. It has a significant effect on people, families, and communities as a whole and is regarded as one of the main causes of death globally. It is essential to comprehend cancer's underlying nature, causes, and mechanisms to effectively prevent and cure the disease (Hanahan and Weinberg, 2011).

Cancer can impact virtually all parts of the human body, from vital organs like the lungs, liver, and breast to blood, skin, kidneys, and bones. Its development is often marked by genetic mutations or alterations that disrupt the normal regulatory processes that control cell growth and division. These altered cells avoid the body's natural defensive mechanisms, especially the immune system, allowing tumors to proliferate and form. The risk factors for cancer are diverse and include genetic predisposition, exposure to carcinogens (such as tobacco, radiation, and certain chemicals), dietary choices, and lifestyle factors like obesity and physical inactivity. These factors can interact in complex ways, making it challenging to pinpoint the precise cause of cancer in any given case (Siegel et al., 2020).

Renal cell carcinoma (RCC), commonly referred to as kidney cancer, is a dangerous occasionally harmful condition that develops in the kidneys. In addition to controlling blood pressure and generating hormones that regulate several body processes, the kidneys are essential organs that filter waste and excess chemicals from the blood. Kidney cancer can arise from tumors formed by malignant cells that have spread throughout the renal tissue. (Bray et al., 2018). RCC of the kidney parenchyma causes more than 80% of renal malignancies; these tumors are primarily adenocarcinoma originating from the kidney parenchyma (Gago-Dominguez et al., 1999). RCC comprises diverse histological subtypes, the most prevalent types of which are clear cell RCC (ccRCC) (80%-85%) and papillary RCC (pRCC) (10%). Oncocytoma and chromophobe tumors are the less common subtypes of kidney cancer (Moore et al., 2005; Chow et al., 2010). An additional histological subtype of kidney cancer is transitional cell carcinoma

(TCC) which frequently appears in the kidney pelvis. According to histology, these cancers resemble the bladder TCC (Chow and Devesa, 2008).

Obesity, diabetes, smoking, and high blood pressure are the primary risk factors for RCC. These factors of risk are believed to make up for almost half of the instances (Benichou et al., 1998; Sasco et al., 2004; Chow and Devesa, 2008). Temporal variations in prevalence rates of cancers of the kidney by Nation/locality and within certain subpopulations are explained by the rising prevalence of various risk factors. Certain risk factors involve the use of analgesics and long-term hemodialysis, even though the causes of the other 50% of kidney cancer patients are mostly unknown (Hurst et al., 2011). The heterogeneous RCC is composed of many histology cellular types that are genetically, biologically, and behaviorally different. Much of our understanding of the molecular underpinnings of early sporadic RCC has come from discovering genes that predispose to genetic conditions with RCC. About the several cancer suppressor genes and oncogenes that undergo mutations that cause channel dysfunction in RCC remain to be recognized. This objective will be attained by international research on gene sequencing and expressions, expression of miRNA, and gene methylation in the main RCC. The behavior of this illness may be understood by looking at the historical context of RCC, which is characterized with potential preclinical tumors, multilayer or bilateral illness, and the growing rate of tiny renal lesions under monitoring. A greater focus on the application of genetic markers for prognosis and preliminary diagnosis is warranted given the continuous advancements in omics technology (Cairns, 2011).

The primary cause of cancer-related mortality in patients is the metastasis of cancer cells from their initial site of origin to other regions of the body (Guan, 2015). Kidney cancer or RCCs, eventually evolve into metastatic tumors in around 35% of cases (Lin et al., 2021). The lung, liver, bone, lymph nodes, adrenal gland, and brain make up the bulk of sites (Umer et al., 2018). Metastasis is a highly coordinated, multi-stage process involving the stroma, blood vessels, and cytoskeleton. During this process, the cytoskeleton undergoes deformation, and there are changes in the expression of genes and proteins associated with the cytoskeleton (Strube et al., 2020).

The actin-related protein 2/3 (Arp2/3) complex is an important actin nuclear structure mechanism that has remained in eukaryotic, this is the only molecular machine that can create a branching actin network. The Arp2/3 complex was initially found as a complex of multiple proteins comprising Arp (Machesky,L et al., 1994). However, it was

later found as an actin-nucleating machine (Welch et al., 1997). From that time, the Arp2/3 complex has remained linked to several functions. The Arp2/3 complex offers an exclusive set of actions. It initiates the development of filaments of actin, caps the slowly developing end of the filament, and joins fibers to create complementary networks. These actions enable trigger the creation of a filament of actin while also connecting it to a stiff system able to produce and transfer mechanic stresses (Welch et al., 1997). Furthermore, through facilitating nuclear actin polymerization, the Arp2/3 complex contributes to the cytoplasmic cytoskeleton (Yoo et al., 2007).

ARP2 protein, which is a crucial part of the Arp2/3 complex, is synthesized by the actin-related protein 2 (*ACTR2*) gene (Rotty et al., 2013). The complex's name comes from the fact that two of its members, Arp 2 and Arp 3, are actin-related proteins. These two Arp subunits can be placed at the pointy end of the smaller filament using the framework provided by the remaining five subunits (ARPC1–5) (Rottner et al., 2010). The Arp2/3 complex, which is a necessary part of platelet activation, causes actin cytoskeletal rearrangements through the ARP2 protein (Schneider et al., 2023).

The actin gene family includes the protein-coding *ACTR2* gene. It is recognized as a significant component of the Arp2/3 complex, which stimulates the development of branching actin filament networks to regulate a variety of cellular functions. It is yet unknown what exactly this gene does. Research has shown that, like other members of the cytoplasmic Arp subfamily, this gene may have a role in the structure of the cytoskeleton. Centronuclear myopathy and Wiskott-Aldrich syndrome are among the illnesses linked to *ACTR2* (He et al., 2017**).** Our main goal is to express the *ACTR2* gene in our patients between cancer tissue and normal kidney cell tissue in the same person, to provide a global overview, to help researchers emphasize the potential of targeting the *ACTR2* gene as a therapeutic approach for prevention, and as a prognostic marker for kidney cancer.

2. LITERATURE REVIEW

2.1. Cancer

2.1.1. Background

Cancer is a group of diseases that are complex and diverse. It is characterized by the expansion and dispersion of abnormal cells that can impact any organ of the body. Cancer can infect tissues surrounding it or migrate to another organ by a process called metastasis. Changes or mutations in the cell DNA cause cancer, leading to alterations in their normal behavior, including uncontrolled cell division and evasion of the body's natural defense mechanisms (DeVita et al., 2018). The uncontrollably growing cells in a collection called a tumor or neoplasm produce an abnormal lump or mass that may be scattered extensively (Sitki-Copur, 2019).

Uncontrolled cell growth is referred to as a tumor, and there are two kinds of tumors: benign and malignant. Benign tumors are well-differentiated, slow-growing, expansile, encapsulating, and confined to their original location without inflicting widespread damage or infiltrating nearby healthy tissues. On the other hand, malignant tumors often exhibit poor differentiation, proliferate rapidly through several mitoses, lack a capsule, and possess the ability to spread to nearby healthy tissue as well as move (metastasize) to distant bodily tissue via lymphatic and vascular circulation (Alzahrani et al., 2021). As a result, malignant tumors spread to other parts of the body through the up-regulation of receptors that facilitate cell motility and the down-regulation of receptors necessary for cell adherence in certain tissues (Sarkar et al., 2013). Merely malignant tumors are considered to be cancers, and since cancer has the ability to spread, it can be fatal and frequently leads to treatment resistance. Benign tumors often pose no threat to life. On the other hand, benign cell development might become dangerous if it changes widely or, occasionally, develop into cancerous (Alzahrani et al., 2021; Valet and Narbonne, 2022).

2.1.2. Cancer classification

Cancers are categorized in two manners: based on the type of tissue they originate from (histological classification) and by the initial location, or their primary region in the body where cancer emerged (Kumar et al., 2017; Carbone, 2020).

2.1.2.1. Cancer classification by site of origin

Cancers are classified into particular categories based on their primary place of origin, such as breast cancer, lung cancer, prostate cancer, hepatic cancer kidney cancer, oral cancer, brain cancer, etc. (Carbone, 2020).

2.1.2.2 Classification of cancer by tissue types

By histological approach, hundreds of distinct tumors are classified to six primary groups:

2.1.2.2.1. Carcinoma

Carcinomas, or malignancy of the epithelial layer of tissue, constitute 80-90% of the cases of cancer since they are the most prevalent cancers in the human body. They typically attack secretory organs or glands, such as the bladder, lungs, breast, prostate, and colon, forms of carcinoma consisting of adenocarcinoma and squamous cell carcinoma. Adenocarcinoma arises in an organ or gland, whereas carcinoma of squamous cells starts in the squamous epithelial cells. Adenocarcinomas, diagnosed as a thicker plaque-like white mucosa, can spread quickly and cause mucus membrane damage. They are most commonly found in secretory organs or glands (Siegel et al., 2023; Merck Manual Professional Edition, 2007).

2.1.2.2.2. Sarcoma

Sarcomas are cancers that develop in supportive and connective tissue such as bones, muscles, fat, and cartilage. Other types include chondrosarcoma, leiomyosarcoma, rhabdomyosarcoma, mesothelial sarcoma, fibrosarcoma, angiosarcoma, liposarcoma, glioma, myosarcoma, and mixed mesodermal tumors. These cancers resemble the tissue

they develop in and can affect various organs, including blood vessels, adipose tissue, and neural tissue. (Horn et al., 2015).

2.1.2.2.3. Myeloma

These originate from bone marrow plasma cells. Plasma cells can produce a variety of antibody in response to certain infections. Myeloma is a form of cancer of the blood (Bartl et al., 1982). To combat infection, normal plasma cells create a variety of antibodies. Myeloma is a condition where normal plasma cells become malignant and produce a single type of antibody called paraprotein. Paraprotein measurements are used to diagnose and monitor myeloma, which affects multiple parts of the body, including the spine, pelvis, rib cage, and areas surrounding the shoulders and hips (Fitzpatrick et al., 2022).

2.1.2.2.4. Leukemia

Blood cancers are a group of diseases that attack the bone marrow, which causes irregular blood cell production. After the bone marrow becomes malignant, it produces an excess of undeveloped leukocytes, causing the patient to become predisposed to infection. Acute myelocytic leukemia (AML) is a pediatric cancer involving myeloid and granulocytic white blood cells, while Chronic Myelocytic Leukemia (CML) primarily affects adults. Lymphoblastic Leukemia (ALL), acute lymphatic, or lymphocytic, affects children and adults of younger age, while chronic lymphatic, lymphocytic, and lymphoblastic leukemia (CLL) primarily affects individuals of senior age. Polycythemia vera, also called erythema, affects many components of blood cells, most commonly erythrocytes (Volinia et al., 2010).

2.1.2.2.5. Lymphoma

Lymphomas are solid-tumor-causing cancer specifically affecting lymph nodes in areas such as the stomach, brain, and intestines, unlike Leukemia, liquid-tumor cancer, which affects the blood. They are also known as extranodal lymphomas (Mugnaini and Ghosh, 2016). Lymphomas are cancers arising from abnormal white blood cell growth in the lymphatic system, making them one of the most prevalent types of cancer, not including non-melanoma skin cancer. (Matasar and Zelenetz, 2008).

2.1.2.2.6. Mixed types

These contain at least two cancer components, including mixed Mesodermal tumors, Carcinosarcoma, Adenosquamous carcinoma, and Teratocarcinoma. As well as Blastomas which affect embryonic tissues (Bang et al., 2020)

2.2. Kidney cancer

2.2.1. Definition

Kidneys are organs that are shaped like beans, roughly the size of a fist, weigh 130–150 grams each. About 10–12 cm in length, 5–7 cm in width, and 3–4 cm in thickness make up each kidney. A thin, glossy fibromuscular capsule enclosing the kidney is called peri-renal fat, and it serves as protection. They are two solid organs that are highly vascular, accepting 20% of the heart's output. They are situated in the retroperitoneal area, which is between the transverse processes of the T12–L3 vertebrae and the posterior abdominal wall. Because of the liver, the left kidney is positioned more superiorly than the right (Figure 2.1). Multiple functions are carried out by the kidneys, including filtering and expulsing of waste materials from metabolism, the control and upkeep of electrolytes, fluids, and acid-base equilibrium, and calcium is controlled through the synthesis of calcitriol and the activation of vitamin D. They also assist in controlling blood pressure and create hormones such as renin and erythropoietin. (Moinuddin and Dhanda., 2015).

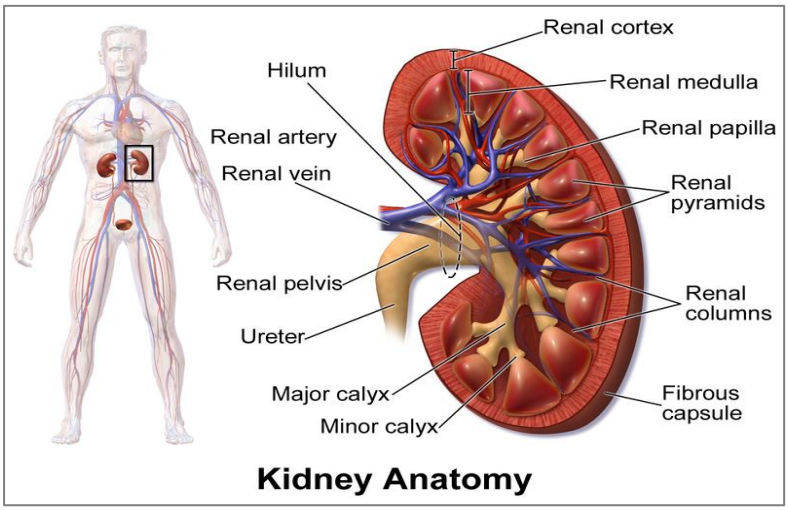

Figure 2.1**.** Kidney Anatomy

(Venkatakrishna et al., 2023)

Kidney cancer develops when normally functioning cells in one or both kidneys undergo mutation and expand beyond normal control, resulting in a malignant growth known as a renal cortical tumor. A tumor might be metastatic, indolent, or innocuous. Metastatic tumors are malignant, which means they may develop and metastasize across the body. An indolent tumor is similarly malignant; however, it seldom progresses across the body. A noncancerous tumor can develop but doesn't expand. Kidney cancer is not a single illness, referring to a group of tumors that arise in the kidney, each with distinctive histology and medical history, respond variably to therapy, and are caused by alterations in particular genes (Figure 2.2) (Linehan et al., 2003).

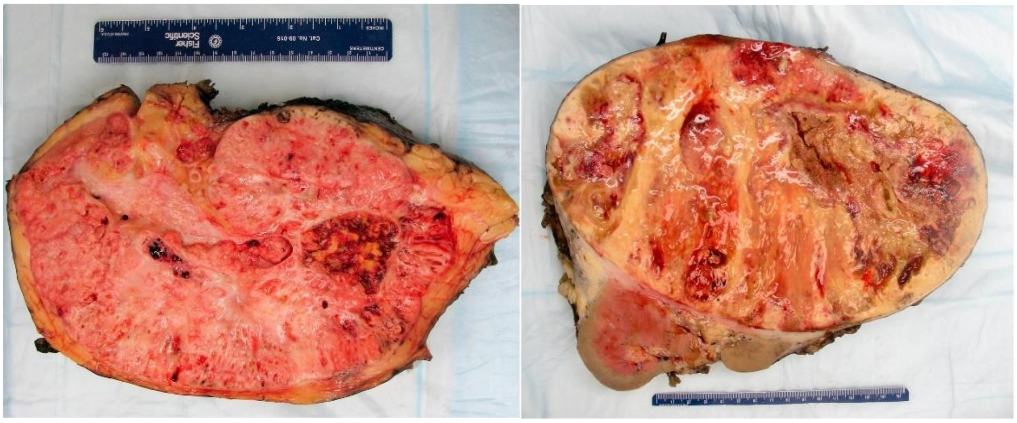

Figure 2.2. Large Tumor of the RCC

(Williamson et al., 2019)

2.2.2. Types of kidney cancer

In 2013, the International Society of Urological Pathology (ISUP) put forth a revised classification of RCC, aligning with WHO guidelines and introducing five newly defined renal neoplasms, along with three emerging categories (Delahunt et al., 2014). Kidney cancer is classified into several types: (Moore et al., 2005; Chow et al., 2010).

2.2.2.1. RCC

RCC is the more frequent kind of mature kidney cancer, accounting for around 85% of cases. This cancer grows in the adjacent renal tubules, which comprise the kidney's purification mechanism. Each kidney has hundreds of tiny filtration units. The three most common histologic subgroups are ccRCC, chromophobe RCC (crRCC), and

pRCC (Muglia and Prando, 2015). The combined prevalence of these three subtypes constitutes over 90% of all RCCs (Lopez-Beltran et al., 2009) ccRCC represents approximately 80% of cases, these cells have an abundance of lipid-rich cytoplasm. Followed by pRCC, which accounts for 10-15%, Type 1 pRCC are spindle-shaped, basophilic cells with minimal cytoplasm, while Type 2 pRCC have spindle-shaped cells with eosinophilic granular cytoplasm and noticeable nucleoli. crRCC, which makes up 5%, has noticeable perinuclear halos and big, pale cells with reticulated cytoplasm. However, recent data indicate that the proportion of pRCC is higher among Black individuals compared to White individuals (Sankin et al., 2011; Tsivian et al., 2014).

2.2.2.2. Urothelial carcinoma

Upper urinary tract TCC initiates malignant changes in the transitional epithelial cells that line the urinary tract from the renal calyces to the ureter. These neoplasms make up <5% of all renal tumors and <1% of genitourinary neoplasms (Jemal et al., 2008). TCC occurs for 6–7% of all kidney cancers. This cancer generally starts where the ureter joins to the major section of the kidney. This region is known as the renal pelvis. TCC can also arise in the ureters and bladder (Melamed and Reuter, 1993).

2.2.2.3. Renal sarcoma

Renal sarcoma is the least frequent kind of kidney cancer, this represents about 1% of kidney cancer cases. It originates in the kidney's connective tissues and, if left untreated, can spread to neighboring organs and bones. The second most prevalent pediatric kidney cancer is renal sarcoma, which accounts for 3-5% of all initial kidney malignancies in children (Gooskens et al., 2012; Köhle et al., 2015).

2.2.2.4. Wilms tumor

Wilms tumor (WT), also known as nephroblastoma, is a kidney embryonal malignant tumor that is typically detected in young children. Roughly 1 in 8000–10,000 children in North America suffer with WT (Al-Hussain et al., 2014). It is closely related to early nephrogenesis, which it mimics morphologically (Treger et al., 2019). And it is handled differently than adult kidney cancer. When paired with surgery, this form of

tumor is more likely to respond favorably to radiation treatment and chemotherapy compared to other kinds of cancer of the kidney (Coorens et al., 2019).

2.2.3. Epidemiology of kidney cancer

Kidney cancer ranks as the fourteenth most prevalent malignancy globally, according to the Global Cancer Observatory (Bukavina et al., 2022). RCC is a complicated and diverse illness with a variety of clinical characteristics. RCC accounts for about 2–3% of various malignancies. It is one of the most dangerous urogenital malignancies, with a death rate of 30-40%, compared to bladder and prostate cancers, which have a death rate of approximately 20%. In incident, which is steadily increasing, vary across the globe, and occur more often in developed countries than in developing countries. Furthermore, males are more likely to develop RCC than women (the ratio of males to females is 1.5:1), and men have a greater death rate than women. Kidney cancer falls in the fifth percentage of all cancers affecting males and is regarded as the sixth prevalent malignancy affecting men (Miller et al., 2018). Furthermore, RCC most commonly arises between the ages of 60 and 70, and it reduces beyond that age (Capitanio et al., 2019). Also, the mortality rate for this disease increased (Chow et al., 1999). According to recent statistics, 400,000 new cases of kidney cancer are reported worldwide annually and about 175,000 people die from the disease (Cirillo et al., 2024).

The development of screening methods and general awareness of the significance of regular health assessments are the main factors contributing to this increase in the prevalence of RCC. As a result, an increasing percentage of individuals are being diagnosed in the beginning stages of the disease. Regardless the rise in overall incidence over the last three decades, particularly in developed countries, the death rate from RCC has fallen dramatically due to earlier identification and therapy. Despite advances in disease management and survival, many patients continue to develop locally advanced illnesses and distant metastases. At the period of screening, around 20-30% of these individuals have cancer that is metastatic and 30-50% develop cancer metastasis with local illness and around forty percent of these individuals with confined RCC enhanced distant carcinoma following the surgery (Pavlakis et al., 2004). The widely distributed site of distant carcinoma includes bones, lungs, and brain; however, the liver, the opposite kidney, and the adrenal gland furthermore be implicated (Ather et al., 2010).

Although RCC metastasis into the pancreas and small intestine are relatively unique, gastrointestinal bleeding in individuals probably be the initial sign of implication in these parts of the body. Furthermore, around 40% of individuals with RCC pass away (Cairns, 2011). Incidence rates of kidney cancer vary greatly throughout humanity, with the greatest rates the province degree recorded in the Republic of Czech (21.9/100,000 in men) and Lithuania (18.7/100,000 in males). Minimal exposure countries, including, Africa, Thailand, and China have incidence rates of less than 2/100,000. In the United States, black males had an elevated exposure rate (15.6 per 10^5) than white men (14.0 per 10^5). Hispanics and non-Hispanics exhibit comparable levels. Native Americans in the US have median levels ($10.9/10^5$ in men) (Li P et al., 2015).

2.2.4. Histopathology

RCC is a diverse malignancy that can develop from many cells across the kidney. Numerous studies have revealed that prognosis and available treatments are significantly impacted by the RCC's histological subtype classification. RCC is classified into different subtypes by the World Health Organization (WHO), utilizing morphological, genetic, and molecular characteristics. According to histological features, clear cell, pRCC (types I and II), and chromophore are the most prevalent subtypes of solid RCC, accounting for 70–90%, 10-15%, and 3-5% of kidney cancers, respectively. Whereas crRCC arises from the collecting duct system and distal connecting tubules (DCT), particularly intercalated cells, clear cell and pRCC develop from proximal tubule characteristics (Warren and Harrison, 2018). Only 5% of clear cell carcinomas are linked to genetic conditions such as von Hippel-Lindau (VHL) disease or tuberous sclerosis, with the remainder (95%) occurring randomly. This type of cancer grows quickly and can spread to the bones, lungs, liver, and, most importantly, lymph nodes (15–25% of cases). Compared to crRCC and pRCC, clear cell carcinoma has a worse prognosis. pRCCs can be either sporadic or inherited. Papillary type I has a better prognosis than type II because it may be identified sooner. In one's sixth decade of life, crRCC is more common. Of all the RCC types, this one is less invasive than cc-RCC (Capitanio et al., 2019).

The WHO reports that in 2004 there were additional less common histological types of RCC, including Xp11.2 translocations-TFE3 carcinoma, unclassified lesion, medullary carcinoma, collecting duct carcinoma, mucinous tubular and spindle cell

carcinoma, multilocular cystic RCC, and neuroblastoma-associated RCC. (Table 2.1) Based on a consensus meeting held in Vancouver by the (ISUP), the WHO released a new categorization for RCCs in 2016. Advances in electron microscopy, immunohistochemistry, cytogenetics, and molecular diagnostics provide the basis for this new classification. (Moch et al., 2016), This RCC categorization is based on various criteria, including pathognomonic, cytoplasmic, and cancer anatomical sites, and different aspects, Also, RCC is divided into sixteen variants (Ulbright et al., 2016).

Table 2.1: The Classification of RCC as Per the WHO Guidelines from 2016 (Moch et al., 2016)

NO.	Renal cell tumors
1	ccRCC
2	pRCC
3	crRCC
4	Renal medullary carcinoma
5	Collecting duct carcinoma
6	Multilocular cystic renal neoplasm of low malignant potential
7	Hereditary leiomyomatosis and RCC—associated RCC
8	Papillary adenoma
9	Tubulocystic RCC
10	RCC, unclassified
11	Clear cell papillary RCC
12	MiT family translocation RCC
13	Succinate dehydrogenase—deficient renal carcinoma
14	Mucinous tubular and spindle cell carcinoma
15	Acquired cystic disease-associated RCC
16	Oncocytoma

2.2.5. Risk factors and causes

Gender and age are the primary hazards for RCC. Geography, ethnic origin, obesity, hypertension (HTN), and excessive smoking or usage of tobacco products are additional hazards (Johansson et al., 2019). End-stage renal disease (ESRD), acquired kidney cystic illness, chronic kidney disease (CKD), extended palliative care utilization, exposure to trichloroethylene and cadmium, intake of processed and red meat, viral liver infections, vitamin D deficiency, type two diabetes, elevated cholesterol, reduced physical activity, and hereditary conditions are some minor risk factors for RCC (Choueiri et al., 2014).

2.2.5.1. Tobacco smoking

Tobacco contains several carcinogenic chemicals that have been linked to various cancers. A significant prevalence of RCC among smokers has been demonstrated. (Vogelzang and Stadler 1998). Cigarette smoking regularly remained identified as a moderate hazard factor for kidney cancer. There was a 1.2 to 2.3 increase in hazard when compared to nonsmokers. Dose-response is associated with increased cigarette use with smokers up 30% as compared to nonsmokers (Kreiger et al., 1993). There is an interaction between hazard and daily tobacco intake in epidemiologic data that supports a causative role for tobacco smoking. Additionally, the hazards decrease with the number of years that one has quit smoking. Tobacco usage is to be the cause of 6% of kidney cancer deaths in wealthy countries (Yu et la., 1986; Dy et al., 2017).

2.2.5.2. Obesity

Many large-scale studies have analyzed the correlation between the risk of kidney cancer and being overweight. An ongoing and stronger connection has been examined between obesity and kidney cancer, particularly in women. Perhaps the cause is a hormonal shift in fat people, such as elevated amounts of endogenous estrogens. There are few epidemiological evidence connecting hormone-related factors to RCC, despite the fact that estrogens can cause kidney cancer in some experimental animals. Additionally, obesity can elevate the risk of arterionephrosclerosis, which elevates the risk of carcinogenesis in the renal tubules. Diuretics are also occasionally used to treat obesity, and this may be a risk factor (Janout and Janoutová 2004).

Extra body weightiness is most commonly measured by an increase in body mass index (BMI) (Wang and Xu, 2014). An increased BMI is thought to account for 26% of kidney cancer worldwide (Arnold et al., 2015).

The worldwide obesity epidemic is suspected to have played a part in raising the amount of RCC. Considering the lengthy latent phase of cancer development, it seems unlikely that the recent stabilization of obesity incidence in high-resource nations has had a significant influence on contemporary RCC trends. Obese individuals are more likely to acquire RCC due to obesity-induced inflammatory response, lipid peroxidation, oxidative stress, chronic tissue hypoxia, insulin resistance, altered endocrine milieu, and adipokine production (Klinghoffer et al., 2009).

2.2.5.3. Physical activity

Regular physical exercise has remained connected to a lower incidence of colon and breast cancers, among other malignancies. Although there is currently a dearth of information relating physical action to the hazard of RCC, the majority of research on this topic has found that increased physical activity lowers the risk. Numerous cohort studies (Van Dijk et al., 2004; Choi et al., 2005; Setiawan et al., 2007; Moore et al., 2008) have demonstrated a dosage response ratio whereby the probability of RCC decreases with increased activity levels, as determined by daily energy expenditure, regular exercise, leisure activities, or daily physical activity. Physical exercise has been demonstrated to lower blood pressure and body weight, enhance insulin sensitivity, and lessen oxidative stress and chronic inflammation. (McTiernan 2008; Richardson et al., 2008; Solomon et al., 2009). These modifications may all help lower the risk of RCC.

2.2.5.4. Environmental and occupational exposure

Another possible risk factor is environmental and occupational exposure. One of the most studied organic solvents is trichloroethylene which is commonly used as a cleaning and degreasing metal. Based on a significant amount of evidence indicating trichloroethylene causes kidney cancer, the International Agency for Research on Cancer categorized occupational exposure to this substance as carcinogenic to humans. According to the most recent meta-analysis, there is an increased risk of kidney cancer of 30% to 40% while working with trichloroethylene (Karami et al., 2012).

Another environmental exposure is aristolochic acid. Both chronic and acute exposures to aristolochic acid have historically been associated with nephropathy and carcinomas of the upper urinary tract (Turesky et al., 2016).

2.2.5.5. Hypertension

Hypertension influences kidney cancer, according to the available data (McLaughlin et al., 2006). Furthermore, numerous studies indicate a correlation between antihypertensive medications and an elevated risk of kidney cancer; however, distinguishing the effects of the underlying condition from those of the treatment remains challenging (Yoon et al., 2011). According to estimates, having a history of hypertension increases a patient's chance of kidney cancer by twice for white patients and three times for black patients in the US (Colt et al., 2011). The incidence of kidney cancer is increased by hypertension and decreases with lowering, according to Swedish cohort research. This suggests that efficient blood pressure control might reduce the risk of kidney cancer (Sun et al., 2015).

2.2.5.6. Kidney diseases

One risk factor for RCC has been linked to a history of kidney illness, including renal infections, cysts, stones, and end-stage kidney disease, kidney cancer risk is increased two to three times (Hofmann et al., 2015). Empirical data indicates that African Americans are more likely than White Americans to experience an increase in risk. This might perhaps account for the greater incidence rates of kidney illness among Black patients, given their higher prevalence of chronic kidney disease (Lipworth et al., 2012). Some case-control studies have indicated a link between kidney stone history and a later risk of kidney cancer (Cheungpasitporn et al., 2015).

2.2.5.7. Diabetes mellitus

Diabetes and cancer are related, however, their effects on organs differ (Figure 2.3) (Hartmann et al., 2012). Cohort studies have evaluated the relationship between diabetes mellitus and the risk of kidney cancer. Some of these studies have shown intriguing evidence of an independent biological influence from diabetes comorbidities, such as obesity and hypertension. There is a 42% higher incidence of kidney cancer in

individuals with diabetes. In women compared to males, there was a greater positive correlation (Larsson and Wolk, 2011).

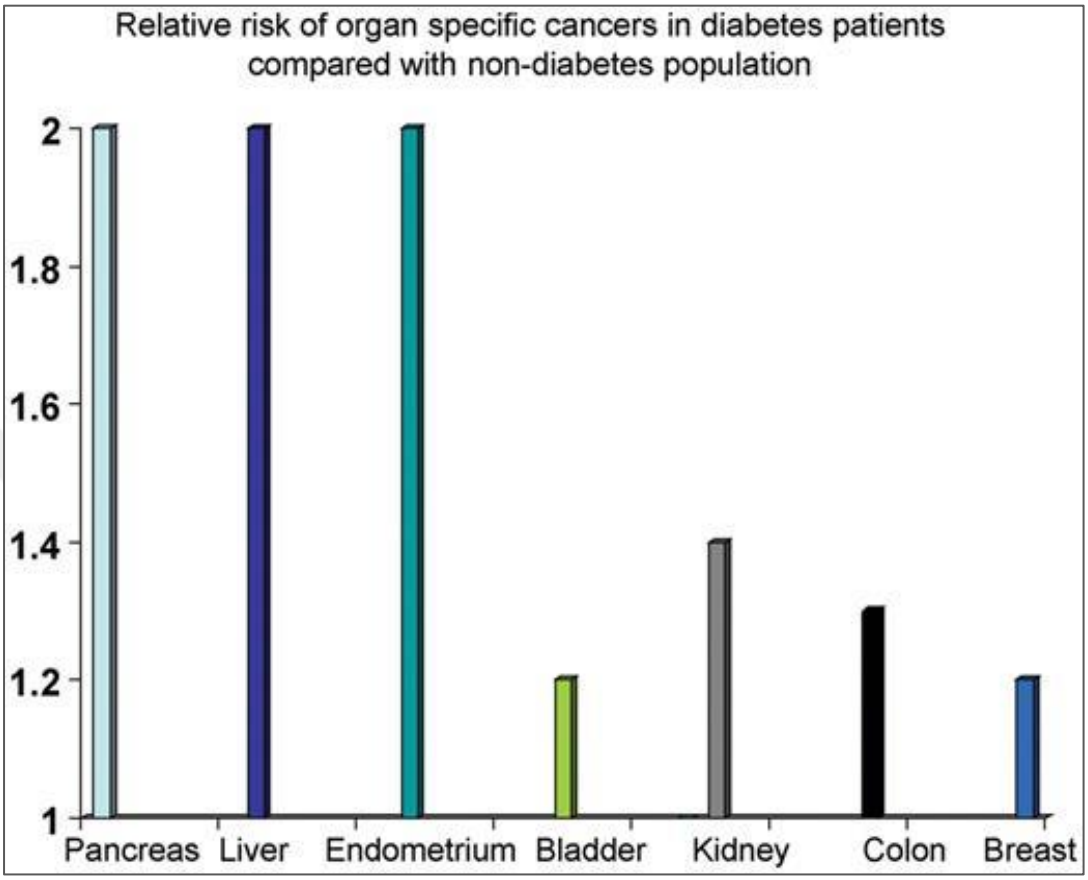

Figure 2.3. Comparative Risk of Organ-Specific Cancer in Individuals with Diabetes vs those without the Disease
(Hartmann et al., 2012)

2.2.5.8. Genetic

Some people having RCC might have inherited several genes that put them at higher risk for the disease. It is unclear what these genes do and how they induce carcinoma of the renal cells. RCC has been found to have hereditary variants, encompassing both clear cell and papillary cell types; however, the particular mutations responsible for these tumors vary. This hereditary form of carcinoma of the renal cells typically affects renal systems and results in the development of multiple tumors. These data suggest that many tumor suppressor genes on the short arm of the 3rd chromosome may be involved in the early stages of carcinoma of the renal cells (Cohen et al., 1979).

Recently, researchers discovered gene mutations that cause certain unusual disorders, including tuberous sclerosis (Choyke et al., 2003) and Von Hippel Lindau (VHL) (Pavlovich and Schmidt 2004). People who carry these mutations have a higher chance of getting kidney cancers. VHL syndrome is marked by the presence of multiple tumors in various organs, such as the kidney, pancreas, adrenal glands, brain, spine, eyes, inner ear, and epididymis. About 1 in 36,000 newborns are affected with VHL illness, which often develops in clumps across generations. People with VHL are significantly more likely to develop ccRCC, which often develops at a young age. Approximately forty percent of persons having the condition have tumors or cysts in both kidneys. The *VHL* gene has been recognized as the cause of VHL disease. It's situated on 3rd chro1mosome. Testing for mutations in the *VHL* gene is growing. The importance of the *VHL* gene in the etiology of ccRCC has also been highlighted by the discovery that a significant fraction of sporadic ccRCC includes genetic alterations related to this gene. (Pavlovich and Schmidt 2004). In several tumor classifications, VHL syndrome has also been observed in renal carcinoma (Latif et al., 1993).

Tuberous sclerosis, characterized by skin bumps, convulsions, mental impairment, and kidney, liver, and pancreatic cysts, increases the hazard of RCC and is connected to blood type A, according to research (Choyke et al., 2003). Hereditary pRCC is the third kind of inherited kidney cancer and is brought on by germinal mutations in the MET proto-oncogene on chromosome 7. Rare occasional instances of renal clear cell and papillary carcinomas have also been linked to somatic mutations in this gene (Gnarra et al., 1995).

2.2.6. Clinical diagnosis and staging

2.2.6.1. Stages of kidney cancer

Kidney cancer is categorized into four distinct stages according to the size of the tumor and its spread within the body, which can aid in determining treatment options (Thakur and Jain 2011). See (Figure 2.4) for a breakdown of these stages.

First stage: The tumor is relatively small, measuring less than 7 centimeters, and remains confined to the kidney without any spread to surrounding tissues.

Second stage: In cases where the tumor exceeds 7 centimeters, patients diagnosed with stage I or II RCC typically undergo surgical resection, which can involve either a radical or partial nephrectomy. Postoperative adjuvant treatments, involving immunotherapy, radiation therapy, or chemotherapy, are generally unadvised for stage I or II RCC (Thakur and Jain 2011).

Third stage: The tumor has either started to develop further in the kidney, into the adjoining adipose tissue, spread to a nearby lymph node, or migrated to the kidney's main blood veins. Radical nephrectomy is the most common therapy for stage III RCC (Thakur and Jain 2011).

Fourth stage: The cancer has migrated to other body parts or several lymph nodes, such as the lungs, brain, or bones. Stage four RCC has spread too far from the kidney to be treated surgically (Thakur and Jain 2011).

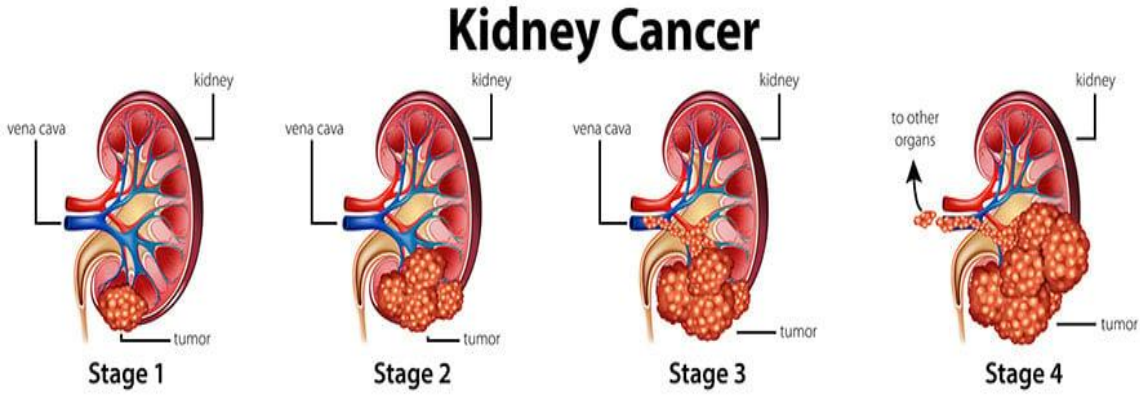

Figure 2.4. Stages of Tumor Development in the Kidney

(Thakur and Jain 2011)

2.2.6.2. Signs and symptoms of kidney cancer

Kidney cancer patients may encounter symptoms such as 1) blood in their urine. 2) Side pain or back pressure. 3) A lump in the back or side. 4) Swelling in the ankle and leg. 5) Having high blood pressure. 6) Anaemia (lack of red blood cells). 7) Feeling weak and tired. 8) Decreased appetite. 9) Loss of weight. (Ikuerowo et al., 2019) (Figure 2.5). Despite the limited utility of physical examinations in diagnosing RCC, certain symptoms

can be clinically significant. These include the presence of peripheral lymphadenopathy (LAP), lower limb edema, abdominal tumor, and varicocele which may result from renal thrombosis or inferior vena cava (IVC) involvement. (Mota et al., 2018).

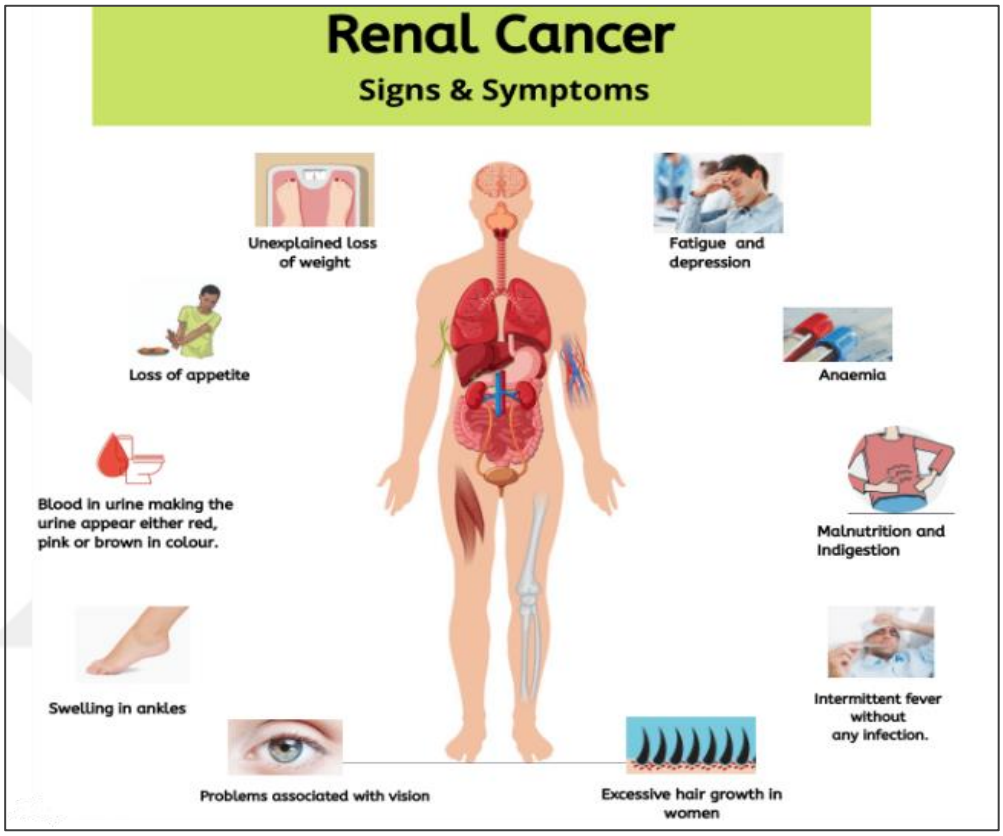

Figure 2.5. Signs and Symptoms of Kidney Cancer

(Thakur and Jain 2011)

2.2.6.3 Diagnosis of kidney cancer

Imaging modalities can effectively detect kidney masses with high precision. RCC, for instance, is frequently identified using gastrointestinal ultrasonography, while diagnostic mild cystic renal lesions can be readily identified through ultrasound alone, eliminating the necessity for additional imaging modalities (Hélénon et al., 2018). Recently, contrast-enhanced ultrasonography (CEUS) has emerged as a dependable and economically feasible imaging technique for estimating uncertain kidney lesions. It avoids

ionizing radiation and nephrotoxicity, enabling swift assessment of the improvement patterns of kidney lesions. Nevertheless, many investigations indicate that CEUS lacks the reliable ability to distinguish between benign and malignant solid renal tumors. When malignancy is suspected, it is advisable to process Computed Tomography (CT) scanning or Magnetic Resonance Imaging (MRI) (Agnello et al., 2020). Whenever feasible, contrast-enhanced (MRI) and (CT) intend to be employed, however, uptake plays a pivotal role in identifying cancerous tumors. Additional indicators of malignancy comprise of rate of growth, size, contrast uptake pattern, fat composition, and hyper attenuation of the mass in CT scans (Benjaminov et al. 2006).

To minimize radiation exposure, MRI is recommended in patients who are young, pregnant, with kidney irregularities, or iodine-based contrast material allergies. Moreover, MRI is recommended for evaluating small tumors with diameters of 1-2 centimeters, as measurements of attenuation can be unreliable in these cases (Kim et al., 2016). Presently, as per the Bosniak classification, MRI provides more detailed information compared to CT scans due to its ability to detect greater enhancement in unequivocal cysts (Israel et al., 2004). The principal objective of imaging is to analyze the features of the lesion, detect suspected abdominal metastases, assess tumor size, and determine venous involvement for staging purposes. Further imaging modalities, such as thoracic and brain CT scans, whole-body bone scans, or comprehensive MRI scans, can be recommended in patients who are symptomatic, with extensive abdominal disease, or in metastatic RCCs. While positron emission tomography (PET) scans can be valuable for RCC diagnosis and monitoring, they are not currently considered routine in clinical practice. Consequently, regular utilization of PET/CT is recommended (Farolfi et al., 2020).

Emerging imaging techniques, such as advanced MRI methodologies or the integration of iodine PET and CT for detecting carbonic anhydrase IX-binding antibodies, may be employed for comprehensive assessment and identification of renal masses. While imaging is capable of detecting benign lesions, histopathological analysis remains crucial for possible malignant tumors. Because of the standard approach of promptly treating and surgically removing solid kidney masses, biopsy minimally influences treatment decisions. As nephrectomy serves as a therapeutic choice for both primary and metastatic tumors, performing nephrectomy, excluding percutaneous needle biopsy, can provide histopathological confirmation (Ljungberg et al., 2017).

Histopathological confirmation through percutaneous biopsy is indicated for patients managed conservatively, those undergoing percutaneous ablation, and those with locally advanced masses or metastases ineligible for nephrectomy (Marconi et al., 2016). Renal biopsy exhibits a low morbidity rate, with an overall complication rate (primarily bleeding, infection, and arteriovenous fistula) estimated to be around 3.5-3.5%. Importantly, advancements in biopsy methodologies have minimized the risk of tumor seeding via the biopsy tract (Lubomirova et al., 2015). Renal biopsy has a crucial role in the assessment and management of renal tumors, yet its widespread adoption is limited by concerns regarding safety and diagnostic precision (Tøndel et al., 2012).

Based on symptoms, the expert may do one or more of the procedures:

- Physical exam:

The doctor verifies typical health symptoms and conducts testing for fever and high blood pressure. The doctor also examines the abdomen and looks for tumors.

- Urine testing:

Urine is tested for blood and other indicators of illness.

- Blood testing:

This is done to monitor the kidney's function. An increased amount of numerous chemicals, including creatinine, indicates that the kidneys are not performing properly.

- The Intravenous Pyelogram (IVP):

This includes intravenous injection of a dye into the vein of the arm. The dye moves throughout the body and accumulates in the kidneys. The dye is then tracked via the kidneys to the ureters and bladder using a series of X-rays. The x-rays confirm the presence of a kidney tumor and any other issues.

- CT scan:

A succession of full kidney images might be acquired using a computer-connected X-ray device. Injecting dye into a patient may produce clear images of the kidney, which can be used to forecast kidney cancer. Abdominal and chest CT can reveal the

original tumor extension, the shape of the contralateral kidney, and the presence of metastases (Israel and Bosniak, 2008).

- Ultrasound test:

  An ultrasound is a test that employs high-frequency sound waves to create a picture of the bodily portion being examined. The waves bounce off the kidneys, and a computer utilizes the echoes to generate a sonogram. An ultrasonography reveals the existence of a solid tumor or cyst.

- Biopsy:

  A biopsy is the procedure of extracting tissue from a suspicious area of the body to identify cancerous cells. A small needle is inserted into the skin to reach the kidney in order extract a small sample of tissue. A pathologist uses a microscope to search for cancer cells in tissue. Renal tumor biopsy is increasingly being employed in diagnosis and is continually designated prior to ablative and systemic therapy without prior histology, and in surveillance schemes to stratify follow-up (Shannon et al., 2008).

2.2.7. Pathophysiology of kidney cancer

2.2.7.1. Loss of the VHL tumor suppressor gene

One important event in the pathophysiology of RCC is the loss of the *VHL* tumor suppressor gene. According to studies, the primary cause of ccRCC is believed to be the inactivation of the *VHL* gene, which is thought to be the main causative agent of the disease. *VHL* gene inactivation is associated with certain genetic mutations or epigenetic changes (Majo et al., 2020). The *VHL* gene a tumor suppressor, and stabilizes hypoxia-inducible factors (HIF-α), is located on chromosome 3p25 and plays a significant role in ccRCC development (Mazumder et al., 2023). The VHL protein ubiquitinates and degrades HIF-α when oxygen levels are normal. But, in the absence of functioning VHL, HIF-α builds up and moves to the nucleus, where it triggers the transcription of many genes related to angiogenesis, metabolism, and cell survival (Gossage et al., 2015). This involves the overexpression of factors that promote the growth and evolution of tumors, such as platelet-derived growth factor (PDGF), glucose transporter 1 (GLUT1), and

vascular endothelial growth factor (VEGF) (Kaelin, 2008). Targeting the dysfunction of the VHL-HIF-α pathway is crucial for understanding the pathogenesis of RCC and should be included in treatment methods (Rini et al., 2009). Furthermore, knowing the molecular pathways of VHL loss and HIF-α stabilization has made it easier to create targeted treatments like VEGF inhibitors, which have demonstrated notable therapeutic benefits in the treatment of RCC (Linehan et al., 2010).

Furthermore, in addition to the *VHL* gene, the etiology of ccRCC involves additional genes, including the polybromo l (*PBRM1*) gene, the BRCA1-associated protein-1 (*BAP1*) gene, the histone-modifying enzyme (*SETD2*) gene, and the lysine-specific demethylase 6A (*KDM6A*) gene (Petejova and Martinek, 2016).

2.2.7.2. Growth factors

The progression and metastasis of kidney cancer, particularly RCC, are heavily influenced by several growth factors. VEGF is essential for angiogenesis in RCC, promoting new blood vessel formation that supplies the tumor with nutrients and oxygen, aiding its growth (Ferrara, 2004). This overexpression of VEGF is frequently due to the inactivation of the *VHL* gene, which results in the accumulation of HIF and subsequent upregulation of VEGF (Rini and Small, 2005). PDGF is another critical factor that supports angiogenesis and recruits stromal cells to the tumor microenvironment (Gerber & Ferrara, 2003). Additionally, insulin-like growth factor (IGF) is involved in RCC (Mennitto et al., 2020). Furthermore, fibroblast growth factors (FGFs) and their receptors (FGFRs) contribute to tumor growth, angiogenesis, and resistance to targeted therapies (Turner and Grose, 2010). These growth factors and their associated signaling pathways have become crucial targets for therapeutic intervention in RCC, with many drugs developed to inhibit these pathways, leading to improved patient outcomes (Escudier et al., 2012).

2.2.7.3. Degradation of extracellular matrix (ECM) and matrix metalloproteinases (MMPs) in kidney cancer

The ECM is made up of a highly dynamic matrix of protein filaments that envelops cells, provides structural support and interaction between malignant cells and the stromal environment. The degradation of the ECM and the activity of MMPs are critical processes

in the progression and metastasis of kidney cancer, particularly RCC. MMPs are a group of zinc-dependent proteolytic enzymes that break down different ECM constituents, which makes tumor invasion and metastasis easier (Kessenbrock et al., 2010; Hashmi et al., 2021). Because these enzymes degrade ECM components including collagen and laminin, cancer cells can spread across surrounding tissues, enter the bloodstream, and form distant metastases (Björklund & Koivunen, 2005). Elevated levels of MMPs, including MMP-2 and MMP-9, have been seen in RCC; these levels are correlated with poor prognosis and enhanced tumor aggressiveness (Gong et al., 2012). The regulation of MMP activity is intricate, including both transcriptional control and the action of endogenous inhibitors referred to as tissue inhibitors of metalloproteinases (TIMPs) (Visse & Nagase, 2003). In RCC, an imbalance between MMPs and TIMPs shifts the equation in favor of ECM breakdown and faster tumor growth (Bergers & Coussens, 2000). It has been demonstrated that most MMP family members are overexpressed in some subtypes of kidney cancer (Gobin, 2019).

2.3. The actin cytoskeleton

The cytoskeleton of eukaryotic cells comprises three primary elements, intermediate filaments, microfilaments, and microtubules (Zhao et al., 2022). The filaments offer structure, form, and internal transport pathways to the cell, allowing it to move and divide. The microfilament system includes actin, an immensely conserved and copious protein that constitutes 5-10% of the total cellular protein. Actin was first extracted from frog muscle (Halliburton, 1887), then purified, identified, and characterized by Straub (Straub, 1942). Actin is a 42 kDa protein known for its unique structure, which includes four subdomains and an ATP-binding site situated between its primary domains 1 and 2. Actin exists in two forms: monomeric, globular actin (G-actin) and polymeric, filamentous actin (F-actin). The filaments are composed of two helical strands intertwined with monomeric actin subunits, creating a right-handed helix with a diameter of approximately 7-9 nm. Actin filaments begin with an actin "nucleus," usually consisting of an actin trimer. The development of this nucleus is energetically unfavorable, but once established, additional monomers spontaneously elongate the filament (Figure 2.6) (Straub, 1942).

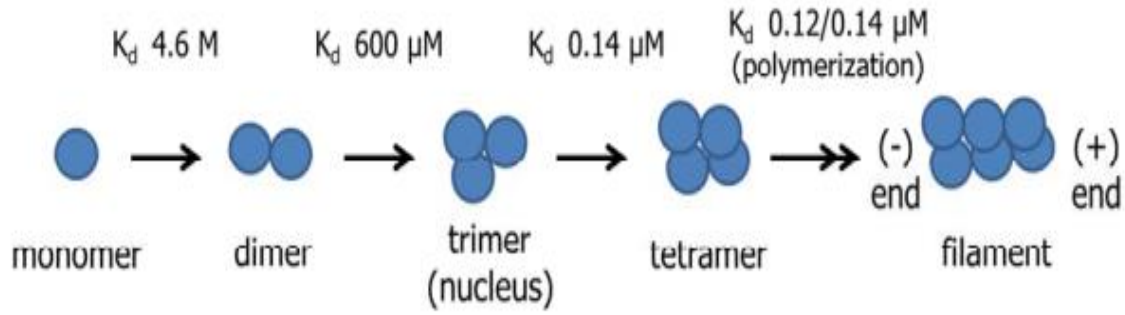

Figure 2.6. The Process of Spontaneous Actin Polymerization

(Straub, 1942)

In cellular environments, actin exists in two states: as a single, isolated monomer and as part of elongated filaments. The dynamics of the actin cytoskeleton are controlled by maintaining a delicate balance between these two states in response to external stimuli (Ridley and Hall, 1992; Ridley et al., 1992). Throughout evolutionary development, eukaryotic actin has maintained a high degree of conservation, ranging from yeast to humans. Its atomic structure is characterized by two main domains, each containing two smaller subdomains. The actin filament comprises two primary domains referred to as the outer domains (subdomains 1 and 2) and inner domains (subdomains 3 and 4). These main domains share structural similarities and are believed to have arisen from a gene duplication event early in evolutionary history (Graceffa and Dominguez, 2003).

Therefore, regulation and remodeling of the actin filament are critical to numerous physiological systems in cells, like membrane traffic pathways, cell movement, and cytokinesis (Lee and Dominguez, 2010). Nevertheless, alteration or dysfunction of proteins that regulate the actin cell skeleton may induce various diseases, including cancer progression (Sossey-Alaoui, 2013). Cell migration and invasion commence through interaction with diverse chemoattractants. When binding to receptors on the surface of the cell, these chemoattractants initiate intracellular signaling pathways that control the rearrangement of the actin cytoskeleton. Many essential proteins implicated in signaling pathways have been found to be upregulated in multiple malignancies (Sahai, 2005). Extensive research has focused on the Wiskott-Aldrich syndrome protein (WASP) family proteins/Arp2/3 complex, LIM-kinase/cofilin, and cortactin pathways, highlighting their crucial roles in promoting cell migration and invasion. (Millard and Machesky 2001).

2.3.1. Arp2/3 complex

2.3.1.1. Structure of Arp2/3 complex

Arp2/3 is a flattened spheroid complex measuring approximately 15 nm in length, 14 nm in width, and with a thickness varying from 7 to 10 nm. It regulates actin polymerization and nucleation (Molinie and Gautreau, 2018). Figure 2.7 shows the structure of the Arp2/3 complex. The Arp2/3 complex consists of seven subunits, which include actin-related proteins Arp2 and Arp3, demonstrating structural resemblances to actin. The other five subunits are named *ARPC1* through *ARPC5*, arranged in order of their molecular weights (Pizarro et al., 2017). The primary components of the daughter filament include *Arp2* and *Arp3*, which both exhibit a structural fold similar to actin. *ARPC1*, known for its 7-bladed β-helix structure, is believed to have an embedding site. It makes minimal contact with actin filaments and binds to nucleation promoting factors (NPFs) lightly (Kelly et al., 2006). The *ARPC2* and *ARPC4* dimers create a "C"-shaped clamp structure that surrounds the *Arp2* and *Arp3* subunits, establishing the fundamental architecture of the Arp2/3 complex. This arrangement forms the primary interface through which the complex interacts with the parent filament (Gournier et al., 2001). ARPC3 is a globular alpha-helical component that acts as a bridge between *Arp3* and the parent actin filament, thereby enhancing nucleation efficiency (Pizarro et al., 2017). Furthermore, *ARPC5* and the architecturally comparable *ARPC3* are located near the complex's periphery and play an important role in its structural integrity (Pollard and Beltzner, 2002). Several subunits of the Arp2/3 complex (*Arp3*, *ARPC1*, and *ARPC5*) have been identified in multiple isoforms, although the functional implications of these variations have yet to be fully understood (Pizarro et al., 2017).

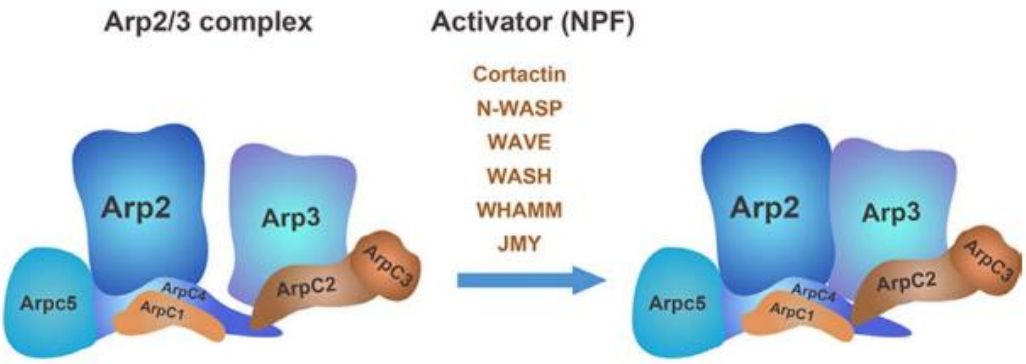

Figure 2.7. Structure of the Arp2/3 Complex

(Zheng et al., 2023)

2.3.1.2. Activation of the Arp2/3 complex

To successfully stimulate actin nucleation, the Arp2/3 complex must be coupled with a nucleation-promoting factor (NPF), as it is inefficient on its own. The human genome has several NPF families, including WASP Family Verprolin-homologous protein (WAVE), WASP, WASP homolog associated with actin, membranes, and microtubules (WHAMM), Wiskott-Aldrich syndrome protein and SCAR homolog (WASH), and junction-mediating and regulatory protein (JMY). With the exception of cortactin, all NPFs contain a WCA domain (WH2, C, A), which activates the Arp2/3 complex, facilitating actin polymerization and nucleation (Zheng et al., 2023).

2.3.1.3. The role of the actin-related proteins 2/3 complex in carcinoma

The Arp2/3 complex shows limited tissue specificity and is observed to be overexpressed in diverse malignancies. Its involvement in modulating the actin cytoskeleton is thought to profoundly influence tumor cell movement, invasion, and metastasis, thus tightly associating it with tumor prognosis (Yamazaki et al., 2005) The role of the Arp2/3 complex in numerous malignancies underscores its associations as detailed in Table 2.2 (Yamazaki et al., 2005). Immunohistochemical studies have demonstrated elevated expression of Arp2/3 subunits in several malignancies, including lung cancer (Semba et al., 2006), breast (Iwaya et al., 2007), gliomas (Liu et al., 2013), gastric (Zheng et al., 2008), and colorectal tumors (Otsubo et al., 2004). As the Arp2/3 subunits assemble into a complex, the presence of one subunit typically indicates the presence of the entire complex. Consistent staining patterns were observed in consecutive

tissue sections using antibodies against *Arp2* and *Arp3* (Otsubo et al., 2004). Nevertheless, Molli et al. (2010) have also identified a distinct functional role for a free pool of *ARPC1B* in maintaining acentrosomal homeostasis. In three independent investigations, elevated levels of *Arp2* along with its NPF *WAVE2* were detected in specific cell types of lung carcinomas (Semba et al., 2006), breast carcinomas (Iwaya et al., 2007), and colorectal carcinomas (Oikawa et al., 2007). This indicates a coordinated increase in both the Arp2/3 complex and *WAVE2* within tumor cells, which frequently display heterogeneous expression patterns. Co-localization of these markers was commonly observed at the invasive fronts of breast and colorectal carcinomas (Iwaya et al., 2007; Oikawa et al., 2007).

Table 2.2. Some Cancers Exhibit Elevated Expression of Arp2/3 Complex Subunits (Yamazaki et al., 2005)

Cancer name	Significantly high expression of subunits	Inhibition or silencing of the Arp2/3 complex or highly expressed subunits affects cancer invasion and migration
Head and neck squamous cell carcinoma	*ARPC5*	Inhibition
Colorectal cancer	*Arp2, Arp3*	Inhibition
Pancreatic cancer	*ARPC3, ARPC4*	Significant inhibition
Gastric cancer	*Arp2, Arp3, ARPC2*	Inhibition
Breast cancer	*Arp2* (Arp2 is co-expressed with *WAVE2*)	Significant inhibition
Hepatocellular carcinoma	*ACTR2, ACTR3, ARPC1A, ARPC1B, ARPC2*	Inhibition
Lung squamous cell carcinoma	*ARPC5l*	Significant inhibition

- *Arp2*: Actin-related protein 2 - *Arp3*: Actin-related protein 3 , *ACTR2*: Actin-Related Protein 2 , *ARPC2*: Actin-Related Protein 2/3 Complex (Subunit 2) , *ARPC3*: Actin-Related Protein 2/3 Complex (Subunit 3) , *ARPC4*: Actin-Related Protein 2/3 Complex (Subunit 4) , *ARPC5*: Actin-Related Protein 2/3 Complex (Subunit 5) , *ARPC1A*: Actin-Related Protein 2/3 Complex (Subunit 1A) , *ARPC1B*: Actin-Related Protein 2/3 Complex (Subunit 1B) , *ARPC5l*: Actin-Related Protein 2/3 Complex (Subunit 5-like) , *WAVE2*: WASP Family Verprolin-homologous Protein-2.

2.3.2. ACTR2 gene

2.3.2.1. Gene expression, regulation, and its importance

ACTR2, also known as *ARP2*, encodes a protein that is integral to the Arp2/3 complex, which is pivotal for inducing actin polymerization within the nucleus. This complex regulates essential cellular processes including gene transcription and DNA damage repair (Yoo et al., 2007). *ACTR2* shows increased expression in hepatocellular carcinoma and diffuse large B-cell lymphoma, which correlates with poor clinical prognosis (Huang et al., 2021; Chen and Jiang, 2022).

ACTR2 has also been recognized as a diagnostic indicator for primary thrombocythemia. While the precise function of *ACTR2* (*Arp2*) remains incompletely understood, it is a key component of the Arp2/3 complex. This complex is pivotal in regulating cell shape, motility, and the assembly of actin filaments. It resides on the cellular membrane and facilitates the assembly of actin in lamellipodia, leading to protrusion. Actin polymerization mediated by the Arp2/3 complex generates the driving force for cellular motility. Intriguingly, *Arp2* additionally facilitates actin polymerization within the nucleus, thereby regulating processes such as gene transcription and DNA repair. In particular, *ARP2* facilitates the movement of double-strand breaks (DSBs) during homologous recombination (HR) repair in response to DNA damage (Yoo et al., 2007). The *ACTR2* gene has been found to encode two transcript variants that produce distinct isoforms. To summarize, *ARP2* plays diverse roles ranging from molding the cell's external structure to impacting gene expression and DNA repair mechanisms.

Recent research has revealed the aberrant expression and functions of *ACTR2* in several human diseases and cancers. For example, *ACTR2/3* was identified as downregulated in serum-derived exosomes from patients diagnosed with Moyamoya disease (Wang, X et al., 2021). *ACTR2* exhibited decreased expression in essential thrombocytosis and carries significant prognostic implications (Wang, J et al., 2021). In contrast, *ARPC2* was found to be markedly expressed in hepatocellular carcinoma (HCC) and correlated with unfavorable overall survival (OS) and progression-free survival in HCC (Huang et al., 2021). The Arp2/3 complex comprises seven proteins, among them Arp2 and Arp3, which exhibit substantial sequence homology with actin (Frankel and

Mooseker, 1996; Mullins et al., 1997). Additional proteins interact with the Arp2/3 complex to augment its ability to initiate actin polymerization (Pollard et al., 2001).

The organization of the actin cytoskeleton is essential for cellular functions such as movement, division, and phagocytosis. Three-dimensional actin networks serve as barriers that influence the autonomous movement of cellular organelles and contribute to the regulation of signal transduction and other intracellular processes (Stossel et al., 2001). Since actin polymers are naturally linear, their effective organization into networks requires crosslinking, a process greatly facilitated by two critical entities: the Arp2/3 complex and filamins (Stossel et al., 2001; Janmey, 2001). The Arp2/3 complex promotes the creation of branched actin filaments, usually at angles near 70 degrees (Mullins et al., 1998; Blanchoin et al., 2000).

2.3.2.2. Structure of the ACTR2 gene

The *ACTR2* gene, also known as *Arp2* (Actin-Related Protein 2 Homolog), is located on chromosome 2 in humans (Figure 2.8). The *ACTR2* length is 43423 base pairs and this gene's mRNA includes 3798 base pairs (NM_001005386) (Figure 2.9). It encodes a protein that is integral to the Arp2/3 complex, a crucial regulator of actin cytoskeleton dynamics. The Arp2/3 complex is essential for fundamental cellular processes such as cell motility, endocytosis, and cytokinesis (Welch et al., 1997).

The *ACTR2* gene is structured with multiple exons and introns. Exons constitute the coding regions of the gene that are transcribed into messenger RNA (mRNA) and translated into proteins. Introns, on the other hand, are non-coding segments removed during mRNA processing. Specifically, the *ACTR2* gene consists of 10 exons and 9 introns (Figure 2.9), (Welch et al., 1997). Widely, *ACTR2* has expression inside the human body, which includes 27 different tissues (Figure 2.10) (Fagerberg et al., 2014).

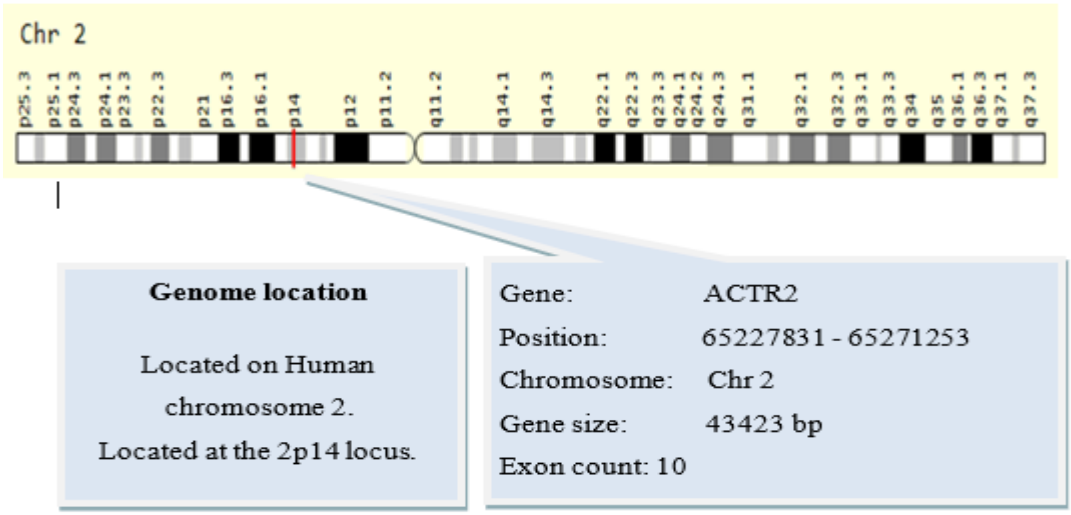

Figure 2.8. *ACTR2* Gene Location on Chromosome 2

(https://www.ncbi.nlm.nih.gov/gene/10097)

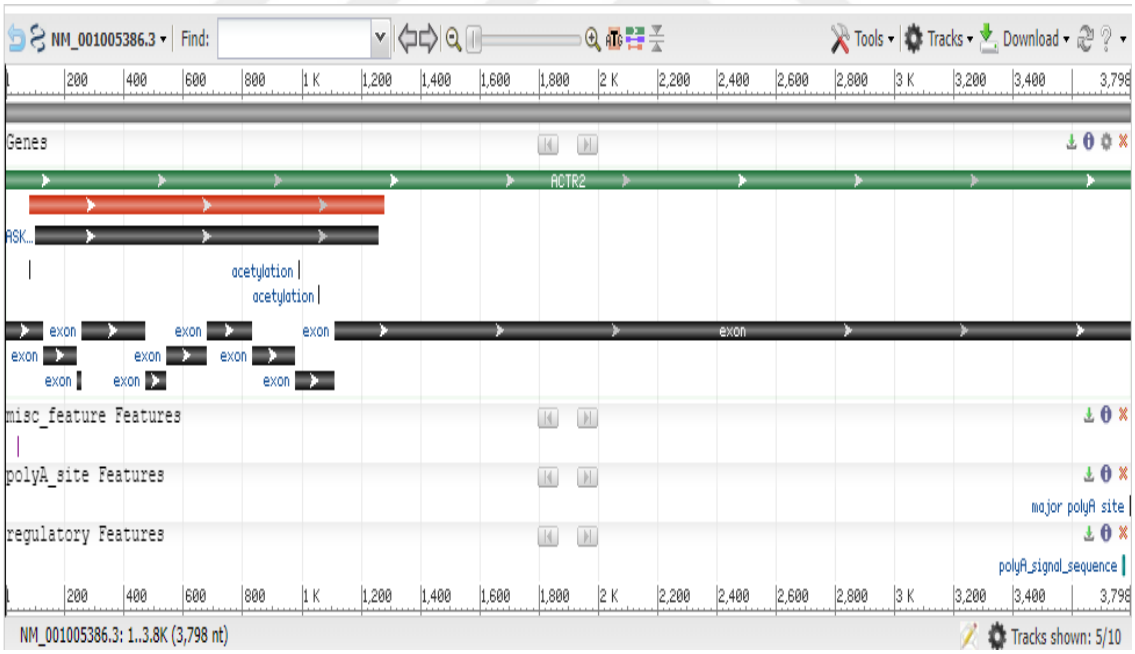

Figure 2.9. Graphic the mRNA of the *ACTR2* Gene

(https://www.ncbi.nlm.nih.gov/nuccore/NM_001005386.3?report=graph)

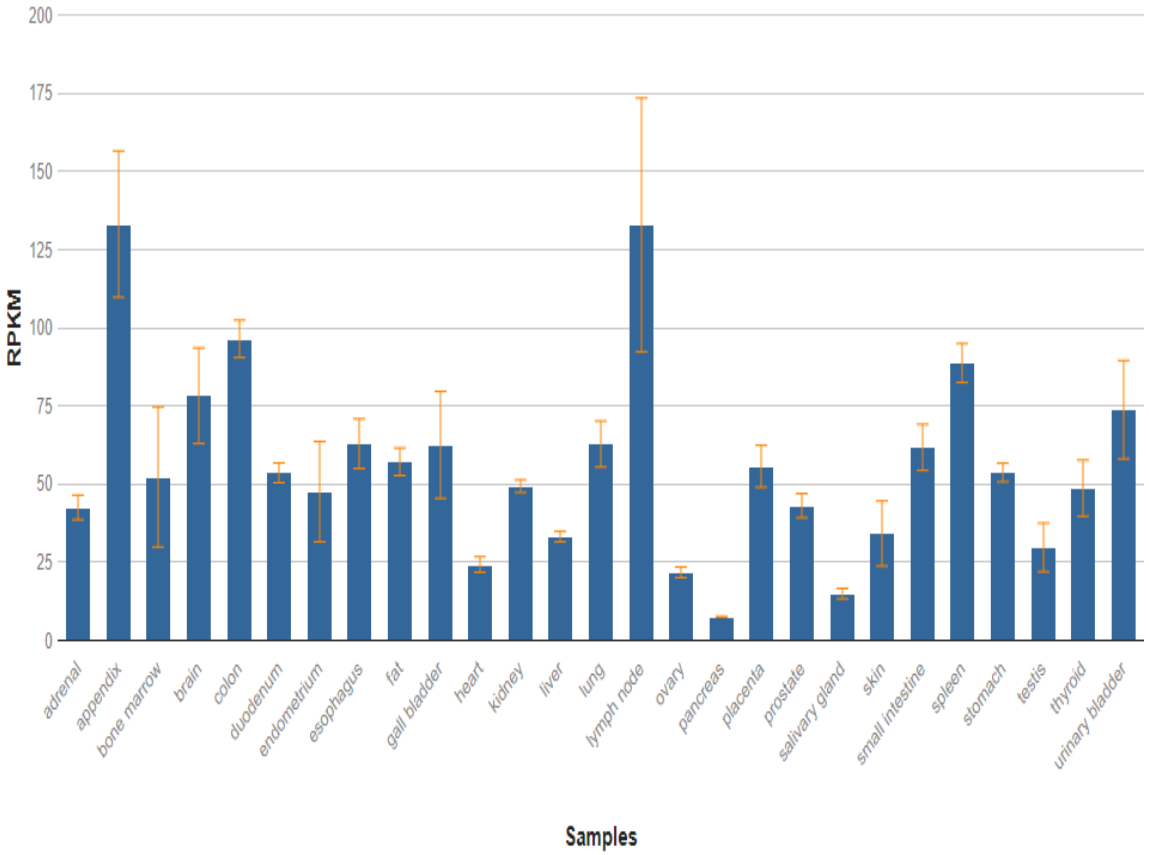

Figure 2.10. *ACTR2* Gene Expressions in Different Tissues

(Fagerberg et al., 2014)

2.3.3. Organs that are affected by alterations in ACTR2 gene and Arp2/3 complex

Specific information about organs affected by alterations in the *ACTR2* gene has not be extensively documented. However, considering the role of the *ACTR2* gene in encoding a component of the Arp2/3 complex, which plays a fundamental role in regulating the organization of the actin cytoskeleton, alterations in this gene could potentially impact various organs and tissues throughout the body. The actin cytoskeleton is essential for maintaining cellular shape, enabling cell mobility, facilitating intracellular transport, and regulating a wide range of cellular processes. Therefore, alterations in the *ACTR2* gene could potentially affect organs and tissues where these processes are critical (Haarer et al., 2023) This could include organs involved in:

- Cellular motility: Alterations in the *ACTR2* gene may affect cell motility processes, which are crucial for tissue development, wound healing, and immune response. Organs involved in these processes, such as the skin, muscles, and immune organs like the spleen and lymph nodes, could be impacted.
- Cell shape maintenance: The actin cytoskeleton is crucial for maintaining the structural integrity and shape of cells. Thus, alterations in the *ACTR2* gene may affect organs where cell shape is critical for function, such as epithelial tissues in the intestines, lungs, and skin. (Siebert, 2017)
- Intracellular transport: Actin cytoskeleton-mediated intracellular transport is essential for the movement of organelles, vesicles, and other cellular components within the cell. Organs with high metabolic activity and extensive intracellular transport, such as the brain, may be particularly sensitive to alterations in *ACTR2*. (Velle and Fritz-Laylin, 2023).
- Cellular adhesion and migration: Actin cytoskeleton dynamics are crucial for cell adhesion and migration processes, which are essential for tissue development, wound healing, and immune response. Organs involved in these processes, such as the cardiovascular system, could be affected. (Rauhala et al., 2013).
- Cancer: Dysregulation of actin dynamics is implicated in cancer metastasis, so alterations in *ACTR2* or Arp2/3 complex could contribute to cancer progression by affecting cell migration and invasion. The way that *ACTR2* gene modifications influence Arp2/3 complex activity, the precise cellular processes that are impacted, and the type of gene mutation determine how the changes affect organs and tissues. (Molinie and Gautreau, 2018).

3. MATERIAL AND METHOD

3.1. Material

3.1.1. Working group

Twenty-two samples were taken from eleven patients. 11 samples with kidney cancer tissues and 11 samples with healthy kidney tissues were taken from the same participants who submitted an application to the outpatient clinic of the Tokat Gaziosmanpasa University Faculty of Medicine, Department of Urology. With the permission and knowledge of the participants, kidney cancer samples taken during surgery were used as the patient group, and the normal kidney tissues belonging to the same kidney of the same patients were used as the control groups. For this study, the requisite approval for the study was secured from the Clinical Research and Ethics Committee of the Faculty of Medicine at Tokat Gaziosmanpasa University, by project number 22-KAEK-045. The research was carried out in Medical Biology and Genetics laboratories.

3.1.2. Tissue storage

The tissues were kept frozen at -80 °C until use. After obtaining cDNA from the tissue samples collected, gene expression analysis was conducted using quantitative Real-time polymerase chain reaction (qRT-PCR).

3.1.3. Tools and equipment used in the study

1. -80 Deep Freezer (Nuaire, NU-9483E, USA).
2. Refrigerator (Arcelik, 570465 MB, Turkey).
3. Centrifuge (Hettich, D'78532, Germany).
4. Refrigerated Centrifuge (Hettich. Germany).
5. Microcentrifuge (Mikro120, Hettich Zentrifugen D- 78 532n).
6. Vortex (Velp Scientifica; F20220176, Italy).
7. Micropipette Set (Gilson, Thermo Scientific, Finnpipette).
8. PCR Device (MiniAmo Plus Thermal Cycle).
9. Qubit 3.0 Fluorometer (Invitrogen, Life Technology, Australia).
10. qRT-PCR machine (Applied Biosystems, UK).

11. Homogenizer (Heildolph Silent Crusher, Finnpipette).
12. Plate (Aplllid Biosystem, 4346907, UK).

3.1.4. Chemical substances and kits used

1. Isolation of RNA kit (Thermo Scientific GeneJet RNA Purification kit, Lithuania, Lot. No 01125929, REF K0731).
2. cDNA synthesis kit (ThermoFisher Scientific High-Capacity cDNA reverse transcription kit, Lithuania, LOT: 01152470).
3. Qubit dsDNA Assay kit Qubit™ 1X dsDNA HS assay kit (Invitrogen catalog No: Q33230, USA).
4. The 2× Master Mix Green with (High ROX™) (Enzo, Cat. No.: ENZ – NUC – 1000, Lot No: 05272212) kit.

3.2. Method

Tissue samples obtained during the operation were stored at −80°C. The stored tissue pieces were used for RNA extraction and the determination of gene expression.

3.2.1. RNA isolation and purification

The GeneJET RNA Purification Kit (REF-K0731), was utilized for RNA separation, the components of the RNA isolation kit were identified in Table 3.1.

Table 3.1. GeneJET RNA Purification Kit.

NO.	GeneJET RNA Purification Kit	50 preps #K0731
1	Proteinase K	600 µL
2	Lysis Buffer	40 mL
3	Wash Buffer 1 (concentrated)	40 mL
4	Wash Buffer 2 (concentrated)	23 mL
5	Water, nuclease-free	30 mL
6	GeneJET RNA Purification Columns pre-assembled with Collection Tubes	50
7	Collection Tubes (2 mL)	50
8	Collection Tubes (1.5 mL)	50

➢ Before starting:

- Proteinase K solution has been prepared using the necessary volume, by diluting 10 µl of the provided Proteinase K with 590 µl of TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA).

3.2.1.1. Procedure (mRNA isolation protocol)

1. Tissue specimens (30 mg) using a surgical scalpel, were cut into tiny pieces and homogenized. The tissues that had disintegrated were transferred directly into a 1.5 µl microcentrifuge (Eppendorf) tube.
2. The 300 µl of Lysis Buffer were added to the samples and vortexed for 10 seconds to mix thoroughly (lysis buffer; with 2-MERCAPTOETHANOL or DTT).
3. We added 600 µl diluted proteinase K. Mixed by vortexing, incubated for 10 minutes at 25°C. (Proteinase K 10µl was diluted in TE buffer 590µl).
4. For 5 minutes, we centrifuged at 12,000 × g and transferred the supernatant to a new microcentrifuge tube free from RNase contamination.
5. 450 µL of 96% ethanol was added, and the mixture was pipetted.
6. 700 µl of the lysate were added to a GeneJET RNA purification column. We centrifuged the column at 12000 × g for 1 min. We threw the bottom containing the liquid and used a purification column.
7. We loaded the GeneJET RNA Purification Column with 700 µL of Wash Buffer 1 containing ethanol, then centrifuged it at 12,000 × g for one minute. After discarding the flow-through, we placed the purification column back into the collection tube.
8. Following the addition of 600 µL of Wash Buffer 2, enriched with ethanol, to the GeneJET RNA Purification Column, centrifugation was performed at 12,000 × g for one minute. Subsequently, the excess liquid was discarded, and the purification column was placed back into the collection tube.
9. The GeneJET RNA purification column's membrane medium was supplemented with 100 µl of nuclease-free water. To extract RNA, centrifuge at 12,000 × g for one minute.
10. We threw away the packing column. We stored RNA at −20°C.

3.2.2. cDNA synthesis from RNA

Reverse transcription was performed using a High-Capacity cDNA Reverse Transcription Kit to convert RNA into cDNA. The components of the reverse transcriptase reaction for each sample are identified in Table 3.2.

First, we prepare the amount of each component needed to synthesize cDNA (Table 3.2).

Table 3.2. The Reverse Transcriptase Reaction Components for Each Sample

No.	The components for the Reverse Transcriptase Reaction	Volume
1	10X RT Buffer	2.0 µL
2	25X dNTP Mix (100 mM)	0.8 µL
3	10X RT Random Primers	2.0 µL
4	MultiScribe™ Reverse Transcriptase (50 U/µL)	1.0 µL
5	Nuclease-Free water	4.2 µL
6	RNA Sample	10.0 µL
Total volume		20.0 µL

- The RT master mix was prepared on ice and gently mixed.
- The total mixing volume was 20µl. We started by combining all the ingredients, excluding the RNA sample, and then we increased the volume of each ingredient by the sample size.
- Then 10µl of the mixture was added into 22 new tubes and then we transferred the RNA samples to other tubes with the same label as tubes of purified RNA.
- For tubes containing a mixture of RNA samples with reverse transcriptase reaction components, we placed them into the Thermocycler, with these conditions (Table 3.3).

Table 3.3. The PCR Program Applied for cDNA Synthesis

RT reaction	Step Name	Temperature	Time
Step I	Annealing	25 °C	10 min
Step II	Elongation	37 °C	120 min
Step III	Termination	85 °C	5 min
Step IV	Hold	4 °C	Hold

Finally, the acquired cDNA can be kept in a freezer at -20°C for later use, or it can be utilized straight away, without purification, for endpoint or qRT-PCR.

3.2.3. cDNA concentration measurements.

cDNA measurements were performed using the Qubit™ 1X dsDNA HS Assay Kits. The necessary procedures for DNA measurement were performed. The steps were done as below:

1. We brought all components to room temperature before use.
2. We prepared the necessary quantity of (0.5 ml) test tubes for standard and specimens. For the Qubit™ 1X dsDNA HS Assay, two standards are needed.
3. We added 10 µL of each Qubit™ standard to the suitable tube.
4. We added 5 µL of each user sample to the suitable tube.
5. The QubitTM 1X dsDNA working solution was added to each tube until the final volume reached 200 µL.
6. For three seconds, we vortexed each sample to thoroughly mix it.
7. We incubated all the tubes for two minutes at room temperature.
8. For ACTR2 as a target gene and ACTB as a reference gene, serial dilution was performed, and cDNA samples were obtained at various concentrations (1/10, 1/100, 1/1000, and 1/10000).

3.2.4. ACTR2 mRNA expression

Using the SYBR-Green qRT-PCR technique, the expression of the *ACTR2* gene in kidney cancer and healthy kidney tissues was measured in the Applied Biosystem StepOnePlus device (Figure 3.1).

Figure 3.1. Applied Biosystem Steponeplus Real-Time PCR Device

3.2.5. qRT- PCR

qRT-PCR is a method employed for quantifying gene expression in real time. This method is highly sensitive and precise for quantifying the quantity of RNA or DNA present in a sample. qRT-PCR is utilized for studying gene expression through the measurement of gene-specific transcript levels. The technique involves several steps, including RNA isolation, reverse transcription, cDNA amplification, and the selected transcript quantification. qRT-PCR is widely used in various fields, including cancer research, neurodegenerative and complex disease research, and biomarker signature studies (Derveaux et al., 2010).

Due to its high sensitivity, qRT-PCR can detect minute variations in gene expression, including rare transcripts. qRT-PCR offers several benefits, such as faster analysis, better process quality control, less chance of contamination, enhanced sensitivity, reproducibility, and accuracy, and simpler quantification (Morillo et al. 2003; Novais et al. 2004).

qPCR requires a thermocycler equipped with an optical system for fluorescence detection, alongside computer software capable of recording data and conducting a final analysis of the reaction. The programs that are offered vary in sensitivity depending on the supplier, excitation mechanism, and sample capacity. Regarding data processing, there are variations as well. The signal produced by the fluorescence emission rises according to the concentration of PCR products. Fluorescence levels, measured at each cycle, indicate the amount of amplified product. SYBR® Green and TaqMan® are commonly utilized fluorescent dyes in this process (Novais et al. 2004; Kubista, M et al. 2006)

SYBR Green I is widely used as a specific dye for dsDNA binding in qRT-PCR. Its fluorescence is only detectable when bound to dsDNA. Compared to ethidium bromide, the most commonly used dsDNA binder in traditional PCR, SYBR Green I exhibits a 100-fold higher affinity for DNA binding (Wittwer et al. 1997; Morrison et al. 1998). One drawback of SYBR Green I is its tendency to bind indiscriminately to all double-stranded DNA, including primer dimers and non-specific amplification products. The effectiveness of amplification of specific items is impacted by amplified non-specific products. As a result, analysis has to be tuned to prevent non-specific amplification.

Controlling the production of dimer primers may be achieved by doing a melting curve examination after the PCR procedure. When the temperature of the amplified product rises, fluorescence is monitored as a function of temperature and steadily decreases (Nygren et al. 1998).

However, the stain splits and the fluorescence quickly decreases when the temperature reaches the point where the double-stranded DNA separates (Ririe et al. 1997). SYBR Green I detection is highly sensitive, capable of identifying a single molecular target within the reaction mixture once optimized. The main benefit is that it's less expensive than a probe since it may be used with various pairings of primers.

Steponeplus Real-Time PCR device (Figure 3.1) utilized the SYBR-Green MasterMix and ROX dye in the kit exclusively for the purpose of amplifying cDNAs made from RNA samples obtained through the reverse transcriptase reaction from the preceding step. The MasterMix was combined with the forward and reverse primers (Table 3.4), of the target gene (*ACTR2*) and reference gene (*ACTB*) along with additional components (Table 3.5).

Table 3.4. Primers for *ACTR2* and *ACTB* Genes used in qRT-PCR

ACTR2- F	5’GGCAGTTCTGAGTTTGTACGC3’
ACTR2- R	5’CCAGTCTCCTGGTAAGATGAGG3’
ACTB- F	5’GCATGGGTCAGAAGGATTCC3’
ACTB- R	5’CACGCAGCTCATTGTAGAAGG3’

Table 3.5. The Components for the qRT-PCR Reaction.

No.	PCR Reaction Components	Volume
1	2x Master Mix Green High ROX™	12.5 µL
2	Forward-Primer	0.5 µL
3	Reverse-Primer	0.5 µL
4	dH2O	8.5 µL
5	cDNA sample (Template)	3.0 µL
	Total amount	25.0 µL

The reaction was performed in a 25 µL volume with the components listed in the ACTR2 gene expression study; B-actin served as the internal control gene. And then we prepared two mixtures, one for the *ACRT2* gene and one for the B-Actin. Before mixing, we multiplied the component volume by 22 and combined the solutions without the cDNA sample.

The mixture was added to the PCR plate (Figure 3.2). It contained two genes target gene (*ACTR2*) and a control gene (*ACT-B*). Next, 3 µL cDNA samples were added to the plate of each gene, along with their previously prepared forward and reverse primers. Subsequently, the plate was placed into the qRT-PCR machine under the required conditions. The qRT-PCR machine system was prepared for the cycling reaction (Table 3.6).

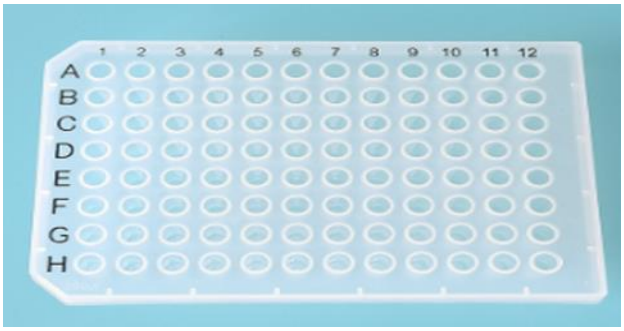

Figure 3.2. qRT-PCR Plate.

Table 3.6. qRT-PCR Protocol

Stages Name	Cycles	Temperature	Time
Initial Denaturation	X 1	95.0 °C	15 minutes
PCR Amplification	X 40	95.0 °C	15 second
		60.0 °C	1 minute
Melt Curve	X 1	95.0 °C	30 second
		60.0 °C	15 second
		95.0 °C	15 second

3.2.6. Statistical evaluation of qRT-PCR data

The qRT-PCR outputs of the ACTR2 gene and the ACTB gene were quantified with the use of step one plus the software version 2.3. The levels of the ACTR2 gene expression have been normalized with the ACTB gene, which is statistical analysis was carried out using the threshold formula data to calculate the fold change amount (Livak & Schmittgen, 2001) (Table 3.7). Absolute quantification, which employs a standard cure to ascertain the target gene's copy number, is employed when a precise number of amplifications is required. Comparative quantification, on the other hand, compares the data obtained from a reference gene (internal control gene) to the data obtained from the target gene. The *ACTB* gene, an internal control gene and a frequently used reference gene to normalize and reduce mistakes while amplifying with the target gene was utilized to normalize the data about the *ACTR2* gene using relative quantification. The $2^{-\Delta\Delta Ct}$ method, which computes the fold change, was applied in conjunction with comparative CT (Table 3.7). The Mean ± SEM was used to show the data, and the 0.9–1.1 range was used to examine the findings. In kidney cancer tissue, the linked gene's expression level was lower than in normal kidney tissue if the values were less than 0.9. If it was in the range of 0.9–1.1, it did not differ from normal kidney tissue. Additionally, if the result exceeded 1.1,

the gene's expression was higher than it was in normal kidney tissue (Livak & Schmittgen, 2001).

The expression data underwent statistical analysis using SPSS 20.0. The correlation of the *ACTR2* gene between normal and abnormal tissues was assessed using the Paired t-test. The acceptable statistical significance threshold was set at 0.05; if p is less than 0.05, then the data was considered significant, indicating a substantial degree of difference. On the other hand, if p>0.05, no significant difference was observed among the samples, and the data value was deemed unimportant.

Table 3.7. The $2^{-\Delta\Delta CT}$ Equation for Kidney Cancer Samples and Control Samples (Livak & Schmittgen, 2001)

- **For kidney cancer sample:**

  ΔC_T (cancer tissue) = Ct (*ACTR2* gene) - Ct (*ACTB* gene)

- **For control samples:**

  ΔC_T (Normal tissue) = Ct (*ACTR2* gene) - Ct (*ACTB* gene)

- **Each sample ΔΔCT value is obtained by subtracting both.**

  $\Delta\Delta C_T = \Delta C_T$ (cancer tissue) - ΔC_T (Normal tissue)

- **Fold change = $2^{-\Delta\Delta CT}$**

4. RESULTS

In this investigation, specimens of kidney cancer were collected from eleven individuals who had been diagnosed with kidney cancer, along with samples of healthy renal tissue obtained from the same participants through collaboration with the Department of Urology at Tokat Gaziosmanpasa University Hospital. *ACTR2* gene expression was investigated using qRT-PCR for normal and abnormal biopsies of study participants.

The ages of patients ranged from 39 to 75 years, with a mean of 62 ± 11.61, As seen in Figure 4.1, there were five male patients and six female patients. Six patients were diagnosed with stage I cancer, one with stage II cancer, and four with stage III cancer. The demographic and clinical characteristics of the kidney cancer patient sample are presented in Table 4.1.

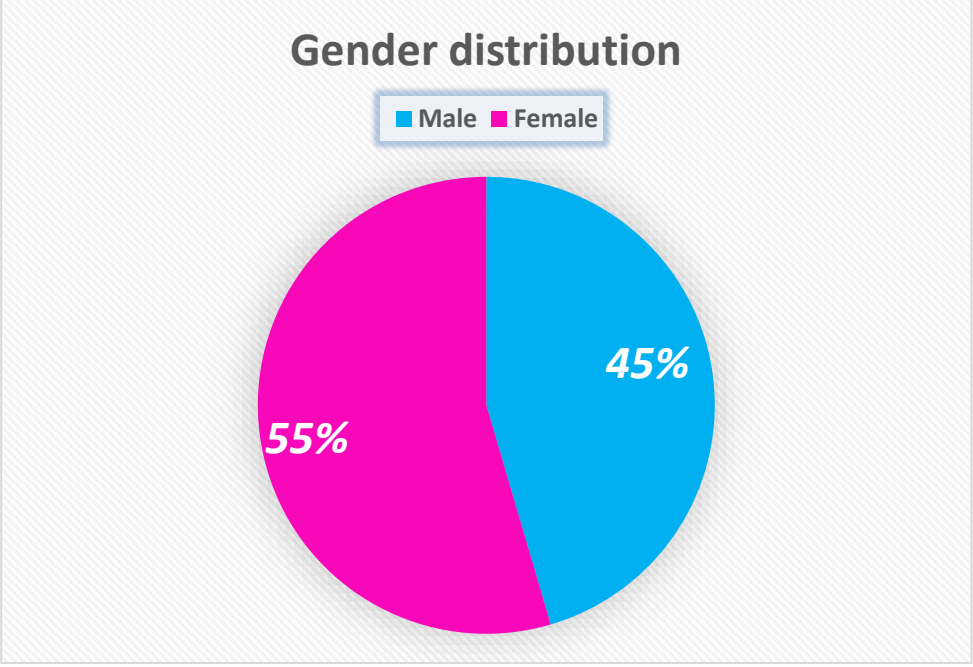

Figure 4.1. Showing Gender Distribution

Table 4.1: Clinical and Demographic Characteristics of Kidney Cancer Patients

(A) Demographic

Number of cancerous kidney samples N=11	
Age, Mean. ± S.D.	62 ± 11.61
Genders	Male = 5 Female = 6
Stage	stage I = 6 stage II = 1 stage III = 4

(B) Details of Kidney Cancer Sample Stages

Sample ID.	Genders	Age	Stage
1	Male	67	1
2	Male	73	3
3	Female	67	1
4	Female	53	2
5	Female	39	1
6	Female	70	3
7	Male	66	1
8	Female	67	3
9	Female	45	1
10	Male	75	3
11	Male	60	1

4.1. The *ACTR2* and *ACTB* Gene Image from qRT-PCR

The evaluation of the levels of the *ACTR2* gene expression within renal cancer and non-cancerous tissues was determined using the SYBR Green-based qRT-PCR method (Applied Biosystems StepOnePlus device). ACTB was selected as the housekeeping gene (a reference gene). The amplification plot and melt curve of *ACTR2* and *ACTB* genes were obtained by qRT-PCR results. A graph of the *ACTR2* gene amplification plot was demonstrated in Figure 4.2; a graph of the *ACTB* gene amplification plot was demonstrated in Figure 4.3; a graph of the melt curve of the *ACTR2* gene was demonstrated in Figure 4.4; a graph of the melt curve of *ACTB* gene was demonstrated in Figure 4.5.

Figure 4.2. Amplification Plot of *ACTR2* Gene

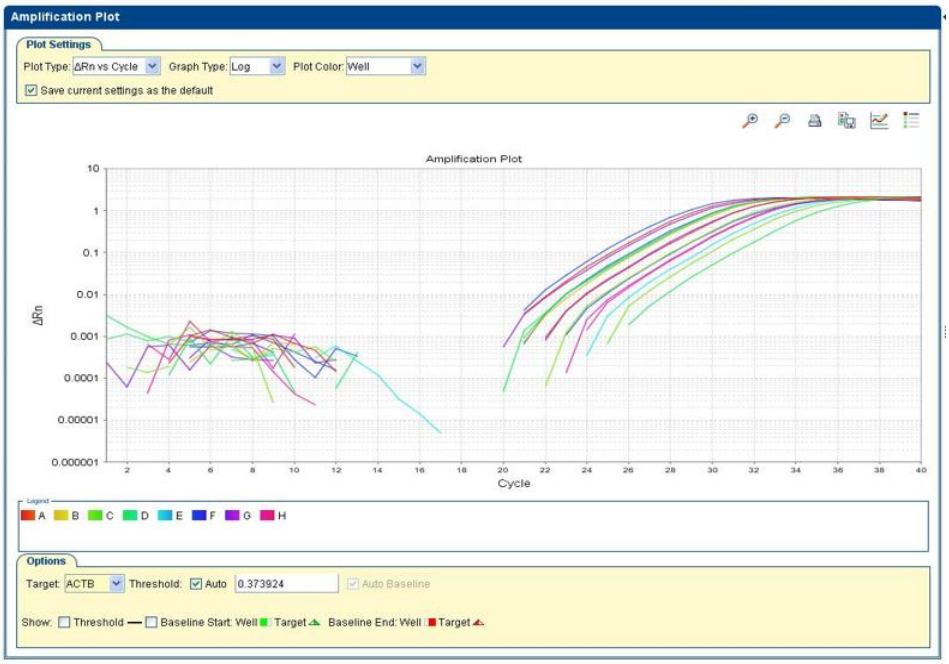

Figure 4.3. Amplification Plot of *ACTB* Gene

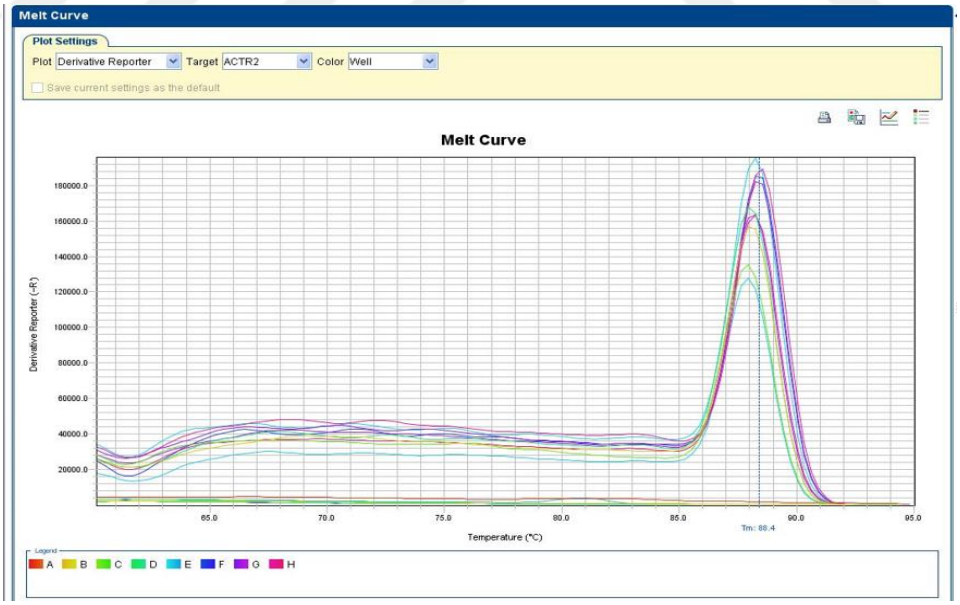

Figure 4.4. Melting Curve of *ACTR2* in qRT-PCR

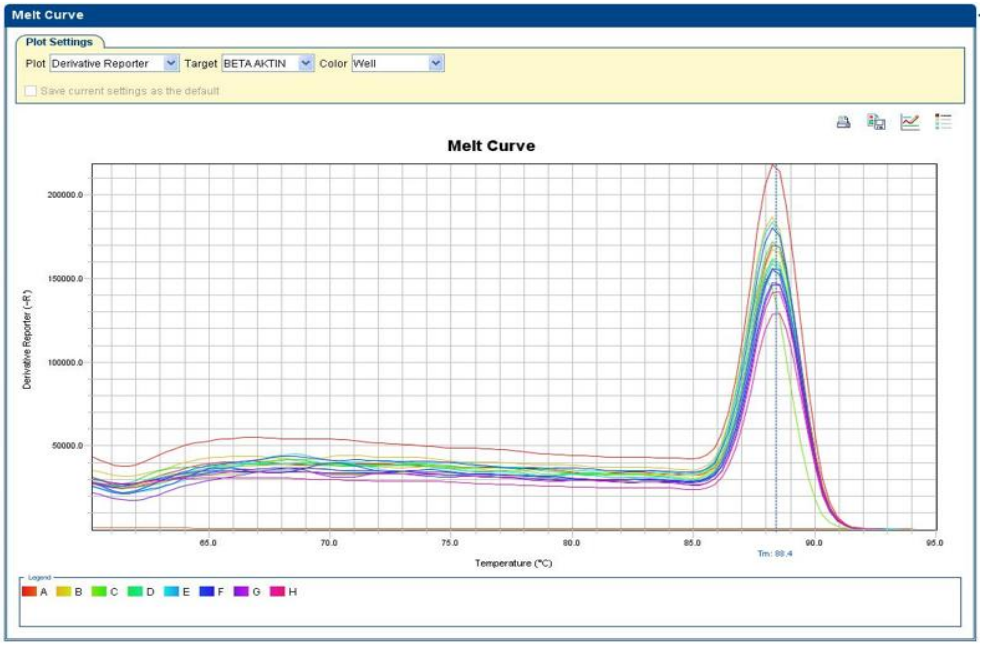

Figure 4.5. Melting Curve of *ACTB* in qRT-PCR

According to the statistical analysis of the results of qRT-PCR, we found the *ACTR2* gene expression increased 1.206-fold in patients' tissues compared to healthy tissues. This result is demonstrated in Figure 4.6. Statistically, no significant relationship was found between tissues ($p = 0.234$). However, the observed increase in *ACTR2* gene mRNA fails to attain statistical significance, as indicated by a p-value exceeding the conventional threshold of 0.05. Consequently, discernible differences between the gene expressions in kidney cancer tissue and healthy kidney tissue samples are not evidenced (Figure 4.6). This suggests that there is no statistically significant difference in *ACTR2* gene expression levels between the two types of tissues examined (Table 4.2).

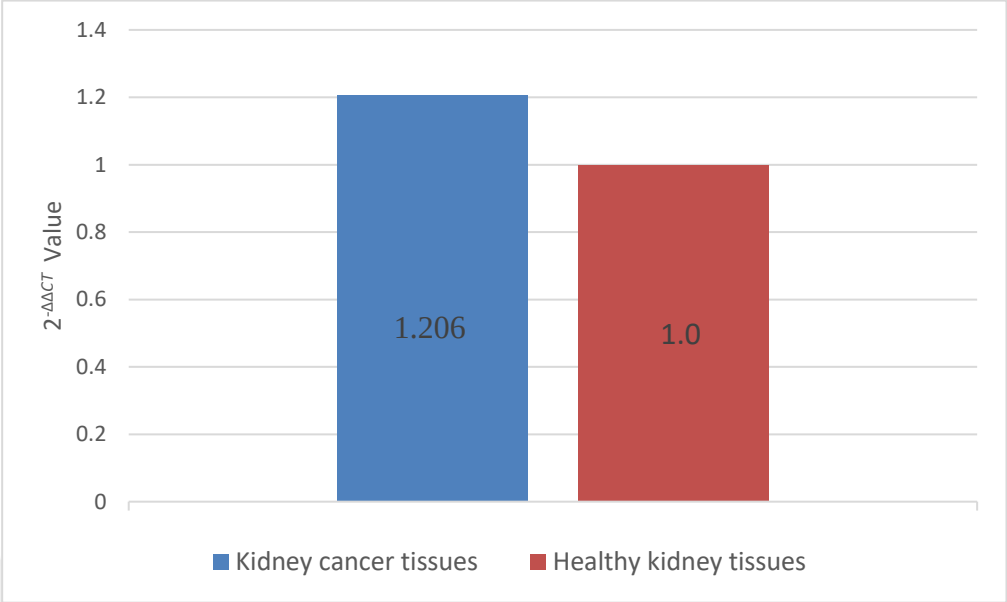

Figure 4.6. Fold Change Graph of the *ACTR2* Gene Expression Between Kidney Cancer Tissues and Healthy Kidney Tissues.

(qRT-PCR was used to determine the relative quantification values of the *ACTR2* gene. *ACTR2* gene quantitative expression data were normalized with that of the *ACTB* gene. The fold change of tissues with kidney cancer was determined to be = 1.206, whereas the fold change of healthy kidney tissues was found to be = 1)

Table 4.2. Statistical Analysis of the *ACTR2* Gene Expression in both Normal and Cancerous Kidney Samples

Samples	*ACTR2* gene (Average±SEM)	P Value
Kidney Cancer Tissues (n=11)	1.206 ± 0.16	*p=0.234
Healthy Kidney Tissues (n=11)	1	

(SEM: Standard Error Mean; *:0.05 significant level)

5. DISCUSSION

Kidney cancer refers to a variety of malignancies that develop in the tissues of the kidneys, which are essential organs responsible for waste filtration and urine production (Hancock and Georgiades, 2016). Advances in genomic research identifying genes like *VHL*, *MET*, *FLCN*, fumarate hydratase, succinate dehydrogenase, TSC1, TSC2, and TFE3 linked to kidney cancer have profoundly altered the therapeutic approach for affected individuals. Despite the availability of seven FDA-approved medications aimed at the VHL pathway for managing advanced kidney cancer, additional genomic investigations, such as whole genome sequencing, analysis of gene expression profiles, and exploration of gene amplification, are imperative for gaining a thorough comprehension of the genetic mechanisms influencing kidney cancer and its related pathways (Linehan et al., 2003).

ACTR2, a pivotal member of the actin-related protein family, is integral to various cellular processes including cytoskeletal dynamics, intracellular transport, and the regulation of gene expression. Positioned on chromosome 2q31.2, the *ACTR2* gene encodes the Arp2 protein, a crucial constituent of the Arp2/3 complex (Rottner and Stradal, 2011).

A research has revealed heightened expression of Arp2/3 subunits in various cancer types, including liver cancer. Utilization of the cancer genome atlas (TCGA) and the University of Alabama at Birmingham cancer data analysis portal (UALCAN) databases confirmed substantial upregulation of all Arp2/3 family components in liver cancer tissues. Moreover, analysis of cancer cell line encyclopedia (CCLE) databases identified widespread expression of Arp2/3 subunits in 23 liver cancer cell lines, notably demonstrating elevated levels of *ACTR2* and *ARPC3* expression. HCC tissues also had greater protein expression of these subunits than normal liver tissues (Huang et al., 2021).

Semba et al. report that their work demonstrated the presence of both Arp2 and WAVE2 in the same lung cell adenocarcinoma, and the clinical data suggested that the expression of these two proteins constituted a separate risk factor for tumor recurrence. The degree of Arp2 and WAVE2 expression would dictate the need for severe adjuvant treatment, particularly at stage IA; Semba et al, imply that expression of WAVE2 and

Arp2 is part of a process that increases the tumor cells' capacity for malignancy. (Semba et al., 2006).

In a study analysing the expression of seven genes in 32 primary gastric cancers, including *p41-Arc*, significant reductions in expression were noted, particularly in the *Arp2, p21-Arc,* and *p34-Arc* genes. Decreases in expression levels were also observed in the *p16-Arc, p20-Arc,* and *p41-Arc* genes (Kaneda et al., 2004).

In a study conducted by Jiang L. in 2022 at Nantong Haimen People's Hospital, the role of Actin-Related Protein 2 (ACTR2) in diffuse large B-cell lymphoma (DLBCL) was explored using well-established experimental methodologies. The research involved both knockdown and overexpression of ACTR2 in DLBCL cell lines to assess its impact on cellular behavior. This functional manipulation was critical in evaluating the oncogenic potential of ACTR2 in DLBCL. The study revealed that ACTR2 expression was upregulated in 12 types of cancer, including DLBCL, and was highly expressed in DLBCL tissues. Elevated ACTR2 expression was significantly associated with poorer overall survival in DLBCL patients. Western blot analysis further confirmed that ACTR2 levels were notably higher in DLBCL cells compared to WIL2S control cells, indicating a potential oncogenic role for ACTR2 in this type of lymphoma. The correlation between high ACTR2 expression and unfavorable survival outcomes suggests that ACTR2 may serve as a prognostic biomarker and a potential therapeutic target in DLBCL (Chen and Jiang, 2022).

Research conducted by Liu et al. highlights the involvement of Arp2/3 in the development and movement of pseudopodia in bladder cancer cells. Additionally, increased Arp2/3 expression is associated with greater malignancy in glioma specimens, where its abnormal function promotes cancer progression and adversely affects patient survival. Therefore, Arp2/3 emerges as a crucial factor influencing tumor invasion and metastasis (Liu et al., 2013; Molinie and Gautreau, 2018).

Beyond its role in cytoskeletal organization, *ACTR2* is involved in transcriptional regulation, bridging the cytoskeleton with the nucleus. *ACTR2* interacts with chromatin remodeling complexes and transcription factors, influencing gene expression patterns and cellular responses to extracellular stimuli. This dual functionality underscores the

versatility of *ACTR2* in orchestrating cellular behaviours in response to diverse environments (Yao et al., 2014). Mutations or dysregulation in the *ACTR2* gene can have profound effects on cellular functions and organismal development. For instance, mutations in *ACTR2* have been associated with certain developmental disorders and diseases. Additionally, alterations in the expression or activity of *ACTR2* may contribute to abnormalities in cell morphology, migration defects, or impaired cellular processes, potentially leading to pathological conditions such as cancer metastasis or neurodevelopmental disorders (Siebert et al., 2017).

In our study, we examined a total of 11 samples of kidney cancer. Our findings revealed a 1.206-fold rise in *ACTR2* gene expression in kidney cancer tissue samples compared to control kidney tissues. However, this increase did not reach statistical significance ($p = 0.234$).

Our results point to *ACTR2's* possible role in kidney cancer, but more research is necessary given the lack of statistical significance. Future studies with larger sample sizes could provide more definitive insights into the role of *ACTR2* in kidney cancer and enhance the reliability of the results. Further exploration of the functions and molecular interactions involving *ACTR2* is crucial for advancing our comprehension of cellular biology. This research could potentially pave the way for the development of targeted therapies aimed at treating cancer and other related disorders.

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