Age-Related Variations in Gene Expression Patterns of Renal Cell Carcinoma – Biological and Translational Implications

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DEDICATION

This thesis is dedicated to my parents and brother, for their unfailing support throughout my life. Thank you so much.

I'd also like to thank my colleagues from Yasser's lab for making it a pleasant place to work the last two years.

ABSTRACT

Renal cell carcinoma (RCC) is known to occur across a wide age spectrum traversing age-related organismal changes, however little is known as to how the aging process may affect the course of RCC and the repertoire of genes involved. I therefore examined associations between patient age and the gene expression profiles in RCC tumors and normal kidney tissues. Datasets from The Cancer Genome Atlas (TCGA, n=436) and the International Cancer Genome Consortium (ICGC) Cancer Genomics of the Kidney (CAGEKID, n=89) were analyzed for pathways and cellular processes that are affected by aging in RCC.

My analysis revealed different age dependent gene expression spectra in RCC tumors and normal kidney tissues. These findings were significant and reproducible in both datasets examined ($p < 2.2 \times 10^{-16}$). Age-upregulated genes, that is genes that show higher expression in older patients, in normal cells were significantly enriched (FDR<0.05) for pathways associated with immune response, collagen formation and semaphorin signaling, whereas age-upregulated genes in tumors were enriched for metabolism and oxidation pathways. Strikingly, age-downregulated genes in normal cells were also enriched for metabolism and oxidation, while those in tumors were enriched for extracellular matrix organization. Further *in silico* analysis of potential drug targets using connectivity mapping tools predicted preferential efficacy of Phosphoinositide 3-kinase (PI3K) inhibitors or immunotherapy in association with age.

Conclusion: I report on hitherto unrecognized interrelations between human life cycle and RCC, suggesting possible effects of age on response to drug treatments.

RÉSUMÉ

Le carcinome à cellules rénales (CCR) est connu pour se manifester à travers un large spectre d'âges traversant les changements organiques liés à l'âge, mais on en connait peu sur la façon dont le processus de vieillissement peut affecter l'évolution du CCR et le répertoire des gènes impliqués. J'ai donc examiné les associations entre l'âge des patients et les profils d'expression génique dans les tumeurs CCR et les tissus rénaux normaux. Les données de The Cancer Genome Atlas (TCGA, n = 436) et du International Cancer Genome Consortium (ICGC) Cancer Genomics of the Kidney (CAGEKID, n = 89) ont été analysées afin d'identifier les voies et les processus cellulaires affectés par le vieillissement dans le contexte du CCR.

Mon analyse a révélé différents spectres d'expression génique dépendant de l'âge dans les tumeurs CCR et les tissus rénaux normaux. Ces résultats étaient significatifs et reproductibles dans les deux ensembles de données examinés ($p < 2.2 \times 10^{-16}$). Dans les cellules normales, les gènes ayant une expression plus élevée chez les patients âgés étaient significativement enrichis (FDR <0.05) pour les voies associées à la réponse immunitaire, à la formation de collagène et à la signalisation de la sémaphorine, tandis que dans les cellules cancéreuses ces gènes étaient enrichis pour les voies de métabolisme et d'oxydation. De manière frappante, les gènes ayant une expression moins élevée chez les patients âgés dans les cellules normales étaient également enrichis pour le métabolisme et l'oxydation, tandis que ceux des tumeurs étaient enrichis pour l'organisation de la matrice extracellulaire. Une analyse *in silico* des cibles médicamenteuses potentielles utilisant des outils de cartographie de la connectivité a prédit l'efficacité

préférentielle des inhibiteurs de la phosphoinositide 3-kinase (PI3K) ou de l'immunothérapie en association avec l'âge.

Conclusion: Je rapporte des interrelations jusqu'alors méconnues entre le cycle de vie humain et le CCR, suggérant des effets possibles de l'âge sur la réponse aux traitements médicamenteux.

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LIST OF ABBREVIATIONS

Akt: Protein kinase B
AMPK: AMP activated protein kinase
CAGEKID: Cancer Genomics of the Kidney
ccRCC: Clear cell renal cell carcinoma
CGH: Comparative genomic hybridization
cmap: Connectivity map
COL: Collagen
CSF: Colony stimulating factor
DNA: Deoxyribonucleic acid
E: Eigengene
ECM: Extracellular matrix
EF: Elongation factor
EMT: Epithelial-mesenchymal transition
EV: Extracellular vesicle
FDR: False discovery rate
GH: Growth hormone
GLM: General linearized model
GSEA: Gene set enrichment analysis
HDAC: Histone deacetylase
HIF: Hypoxia inducible factor
HSP: Heat shock protein
ICI: Immune checkpoint inhibitor
IFN: Interferon
IGF: Insulin-like growth factor
IIS: Insulin and insulin-like growth factor signaling
IL: Interleukin
MAPK: Mitogen activated protein kinase
MCH: Major histocompatibility complex
MMP: Matrix metalloprotein
mTOR: Mammalian target of rapamycin
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
PD1: Programed cell death 1
PI3K: Phosphatidylinositol-3-kinase
RCC: Renal cell carcinoma
ROS: Reactive oxygen species
RPKM: Reads per Kb per million mapped reads
RSEM: RNA-Seq by Expectation Maximization

TCGA: The Cancer Genome Atlas
TF: Tissue factor
TGF: Transforming growth factor
TKI: Tyrosine kinase inhibitor
TMN: Tumor, Nodes, Metastasis
TNF: Tumor necrosis factor
VEGF: Vascular endothelial growth factor
VHL: von Hippel Lindau
WCGNA: Weighted Gene Co-Expression Network Analysis

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CONTRIBUTION OF AUTHORS

This work was the result of a collaboration between the labs of Yasser Riazalhosseini and Janusz Rak, with significant assistance from Hamed Najafabadi. Detailed contributions are as follows:

- Lara Feulner: Wrote the thesis manuscript; performed all statistical analysis and developed figures.
- Yasser Riazalhosseini and Janusz Rak: Supervision and advising as to direction of study; assistance with data interpretation; editing of manuscript.
- Hamed Najafabadi: Statistical/methodology advising and R code assistance.
- Simon Tanguay: Clinical advising.

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Renal cell Carcinoma (RCC), particularly clear cell RCC (ccRCC), is the most common type of kidney cancer in adults. ccRCC is molecularly defined by loss-of-function mutation of the von Hippel Lindau (VHL) tumor suppressor gene, an event associated with dysregulation of pathogenetically important processes including metabolism, hypoxia response and vascular endothelial growth factor (VEGF)-driven angiogenesis. However, the molecular evolution of ccRCC is complex and ultimately results in altered expression of multiple genes involved in epigenetic regulation, growth factor response, extracellular matrix (ECM) formation and immunoregulation.[1] Consequently, targeted agents directed at tumor stroma, such as VEGF pathway of angiogenesis and immune checkpoint inhibitors (ICIs), have revolutionized treatment for RCC and extended lives of patients with advanced disease.[2] However these gains are restricted by the variability and transiency of therapeutic responses, the mechanisms for which remain poorly defined.

Several factors could contribute to inter-individual diversity among cancer patients. In addition to cell-intrinsic factors, cancer incidence, type, course, metastatic dissemination and therapeutic responses could also be affected by variables associated with individual life cycle,[3] during which various cell compartments, such as the vasculature, immune system and stroma undergo profound age-related changes.[4] Little is known in this regard about ccRCC, a disease which is known to affect adults across a remarkably wide age spectrum spanning several decades. It is

therefore unknown whether and how physiological aging and prevalent age-associated co-morbidities such as atherosclerosis, cardiovascular disease, metabolic conditions, or chronic inflammation may affect the biology and therapy of ccRCC.

Prior studies have suggested that vascular structures, density and molecular make-up of blood vessels in ccRCC may exhibit age-related alterations.[5] Moreover, transplantable mouse tumors were shown to grow at lower rates in old atherosclerotic mice - a pattern coupled with prominent vascular alterations, diminished infiltration of bone marrow-derived myeloid cells, reduced levels of circulating endothelial progenitor-like cells and favorable responses to VEGF antagonists, all markedly different than in younger and healthier animals harboring the same tumor type.[6,7] These observations suggest that while the core pathways responsible for cellular transformation in ccRCC (e.g. VHL loss) dictate global stromal and vascular responses, the nature and magnitude of these events and their effects on cancer cells may be modulated by age-related pathophysiological processes. The hypothesis and objectives of this study are therefore as follows:

Hypothesis*:* Aging changes the molecular composition of RCC tumors as well as the tumor microenvironment, impacting angiogenesis, metastasis, response to therapies and genetic evolution of the disease.

Initial aim*:* Better understand the influence of aging on the genetic evolution of metastatic RCC cells.

Ultimate aim*:* Improve therapeutic strategies based on age and potentially decrease incidence of resistance.

In order to glean insights as to the relationship between age and RCC, I employed two large independent gene expression data sets to examine the association between age of ccRCC patients and gene expression profiles of tumors and corresponding normal tissues. Before presenting this research, I would like to provide a literature review on what is currently known about aging, cancer and RCC in particular.

1.2 Aging

Human aging is characterized by loss of physiological stability, leading to impaired function and increased risk of pathologies including cancer and cardiovascular disease. The molecular mechanisms behind aging have long been of interest, but some of the genetic pathways and biochemical processes have only recently begun to be understood. López-Otin *et al*[8] enumerated nine hallmarks of aging (Figure 1):

1) Genomic instability
2) Telomere attrition
3) Epigenetic alteration
4) Loss of proteostasis
5) Deregulated nutrient sensing
6) Mitochondrial dysfunction
7) Cellular senescence
8) Stem cell exhaustion
9) Altered intercellular communication

Figure 1: Hallmarks of Aging

Figure 1: The nine hallmarks of aging, from López-Otin *et al*.[8]

1.2.1 Genomic Instability

A significant accumulation of DNA damage and somatic mutations, coinciding with a decrease in total DNA repair capacity, is known to be associated with aging.[9] Furthermore, mutations in DNA repair genes have consistently been found in human progeroid (premature aging) syndromes, and inactivation of those genes have resulted in mutation accumulation and the development of progeria in model organisms.[10] Conversely, activation of DNA repair pathways has been associated with longevity.[11,12]

The impact of DNA damage on stem cells, affecting regenerative capability and contributing to stem cell exhaustion, may be particularly important. In one study progeroid mice transplanted with stem cells from young wild-type mice showed increased lifespan, supporting this notion.[13]

1.2.2 Telomere Attrition

When DNA polymerase was discovered and its mechanism understood in the 1960's, a resulting question was how the complete replication of DNA ends was ensured.[14] As DNA polymerase could only extend a preformed primer, it would be unable to copy the very end of a linear DNA. The subsequent discovery of telomerase provided an answer. Telomerase is a specialized DNA polymerase allowing for replication of the terminal ends of DNA molecules, known as telomeres. However, it is not normally expressed in human somatic cells and so these cells will experience telomere depletion as the cell undergoes multiple replications. This depletion is believed to be associated with human aging, and there is evidence that aging can be reverted by telomerase activation.[15] In particular, expression of the telomerase catalytic subunit TERT following transfection has been found to prevent telomerase shortening and extend the lifespan of human somatic cells[16,17], whereas inhibition of telomerase reduced lifespan.[18]

Because telomeres are bound by a complex preventing access from DNA repair proteins (which would otherwise lead to improper "fixing" and chromosomal fusion), they are also especially vulnerable to DNA damage. Such damage will often trigger cell senescence or apoptosis.[19]

1.2.3 Epigenetic Alterations

Epigenetic alterations including histone methylation, DNA methylation and chromatin remodeling have also been associated with age. Deletion of genes encoding for the components of H3K4 and H3K27 has extended longevity in nematodes and flies,[20,21] and flies with loss of function mutations of the heterochromatin protein 1 alpha (HP1α) have shortened lifespans whereas overexpression promotes longevity.[22]

Of recent interest is sirtuins, stress-responsive histone deacetylases (HDACs) that modulate both transcription and post-translational modifications. Supporting their relevance, premature aging was observed in mice with impaired SIRT6 expression, whereas an increased lifespan was found in mice overexpressing SIRT6.[23,24] Of note, sirtuins are also linked to cancer, with some functioning solely as tumor suppressors and others, notably SIRT1 having both oncogenic and tumor suppressor properties.[25,26]

1.2.4 Proteostasis

Cellular protein homeostasis, or proteostasis, is the regulation and control of protein synthesis, protein folding, conformational maintenance and protein degradation. This is achieved through a proteostasis network, whose role is to ensure cells have the correct amount of protein present while minimizing errors such as misfolding. Of particular importance is the coordination of the proteostasis network with molecular chaperones, particularly heat shock proteins (HSPs), that modulate protein folding. It has been found that the maintenance of proteostasis in cells declines

with age, which may increase the risk of human pathologies such as Alzheimer's and Parkinson's Disease.[27] Notably, mice deficient in a HSP co-chaperone have been found to have premature aging,[28] whereas increased expression of HSPs has been associated with longevity.[29]

1.2.5 Deregulated Nutrient Sensing

Decreased nutrient signaling (ex. from caloric restriction) is known to promote longevity. Conversely, current evidence suggests that anabolic signaling promotes aging, with particular attention given to the insulin and insulin-like growth factor signaling (IIS) pathway. Mutations affecting GH, IGF1 receptor or downstream effectors of the IIS pathway (in particular the transcription factor FOXO) have been associated with longevity.[30,31] Other nutrient sensing systems that have been implicated in aging include mammalian target of rapamycin (mTOR), AMP-activated protein kinase (AMPK) and sirtuins. Downregulation of mTORC1 has been found to increase longevity in mice,[32] whereas upregulation of AMPK and sirtuins promotes longevity.[23,33]

1.2.6 Mitochondrial Dysfunction

Progressive mitochondrial dysfunction has also been implicated in aging, primarily through resulting increased production of reactive oxygen species (ROS). However, recently there has been a reconsideration of the role of ROS,[34,35] and it may be that dysfunctional mitochondria can affect aging through other mechanisms. It has been postulated that ROS serves a positive

homeostatic purpose up to a certain threshold, beyond which they increase rather than alleviate age-associated damage.[34]

1.2.7 Cellular Senescence

Cellular senescence, or irreversible cell cycle arrest, is due in part to telomere shortening, but there are other age-related processes (including non-telomeric DNA damage) that may trigger it. Its primary purpose is to trigger recognition of damaged cells by the immune system. Senescence is regulated by two main pathways, p53 and Rb. Two distinct proteins known to be tumor suppressors, $P16^{INK4a}$ (p16) and $p19^{ARF}$ (p19), play significant roles in inducing cell senescence via activation of those pathways. During cell division, the protein Elongation Factor 2 (EF2) induces G1-S cell cycle progression. In senescent cells, however, dephosphorylated Rb binds to and inactivates EF2, resulting in G1 cell cycle arrest. Both p16 and the protein $p21^{CIP1/WAF1}$ (p21) inhibit cyclin D-dependent kinases, preventing phosphorylation of Rb and promoting senescence. In turn, p21 is activated by p53. p19 stabilizes p53 by sequestering Mdm2, a ubiquitin ligase targeting p53 for degradation.[36,37]

The INK4a/ARF locus encoding p16 and p19 has been among the best documented genes controlling human aging and age-related pathologies including cardiovascular disease, diabetes, Alzheimer's and glaucoma.[38] It has been proposed that activation of INK4a/ARF can be a beneficial compensatory response to damage incurred by aging; but this response becomes deleterious once tissue regeneration is eventually exhausted.[8]

1.2.8 Stem Cell Exhaustion

A decline in tissue regeneration is very strongly associated with aging and is linked to a notable attrition of stem cells termed stem cell exhaustion. This is consistent with a decline in hematopoiesis in older people.[39] Interestingly, transplantation of muscle-derived stem cells from young to progeroid mice has reduced tissue degeneration even in tissues where donor cells are not seen, suggesting there may be beneficial secretory factors at work.[13]

1.2.9 Altered Cellular Communication

Alteration of cellular pathways promoting inflammation is also consistently found with age and play a role in the pathogenesis of obesity, type 2 diabetes and atherosclerosis. Over-activation of the immune response/inflammation-promoting NF-κB pathway is a strong transcriptional signature of aging, and its inhibition in mouse skin cells has been found to promote tissue rejuvenation.[40] Furthermore, mice deficient in the mRNA decay factor AUF1, which inhibits inflammatory response, have been found to have increased cell senescence and a premature aging phenotype.[41] Age has also been linked to decreased immune function, and thus reduced ability to clear infectious agents/cells and cells undergoing malignant transformation.

1.3 Cancer Development

As will be seen, many of the hallmarks that were just discussed for aging are also applicable to cancer. Hanahan and Weinberg's "The Hallmarks of Cancer"[42] can be considered a landmark

review article in the field of cancer research. It initially listed six hallmarks, subsequently extended to ten[43] (Figure 2):

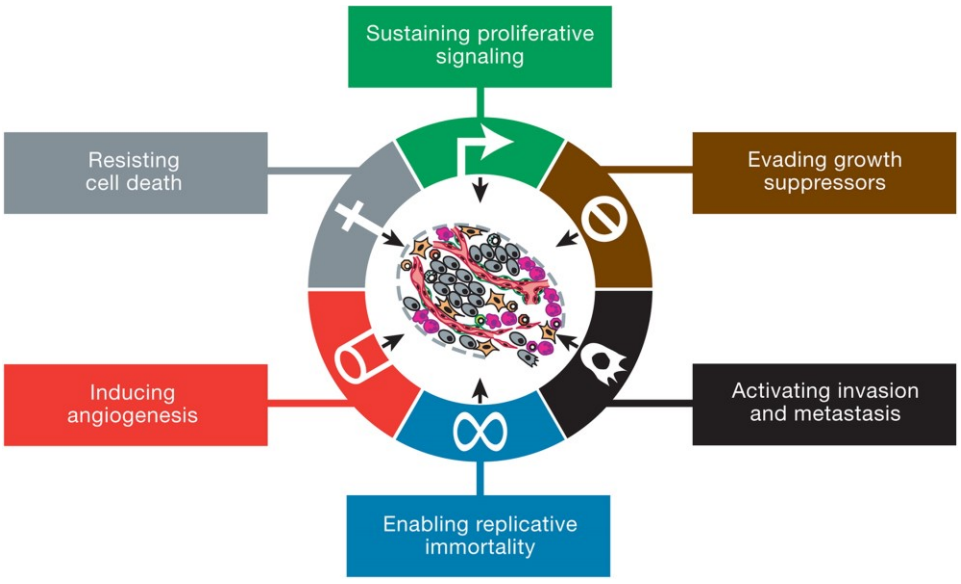
Original Hallmarks:

1) Sustaining proliferative signaling
2) Evading growth suppressors
3) Resisting cell death
4) Enabling replicative immortality
5) Inducing angiogenesis
6) Activating invasion and metastasis

Emerging Hallmarks

7) Genomic instability
8) Tumor promoting inflammation
9) Reprogramming energy metabolism
10) Evading immune destruction

Figure 2: Hallmarks of Cancer



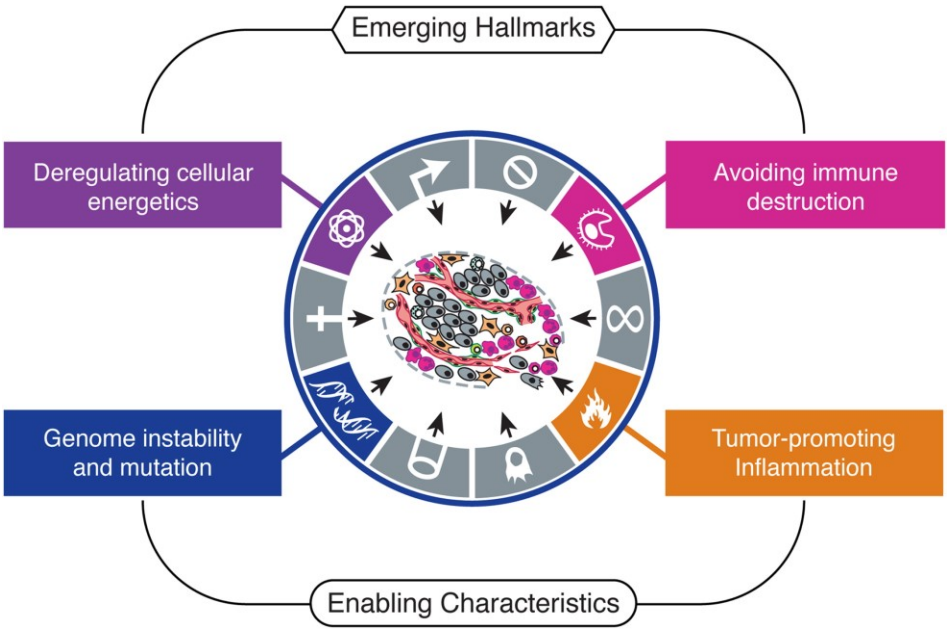


Figure 2: The hallmarks of cancer, from Hanahan *et al*[42,43]

1.3.1 Sustaining Proliferative Signaling

A number of signaling pathways promoting cell growth and proliferation are frequently exploited by cancer cells. Chief among them is the PI3K-Akt-mTOR signaling pathway.

Phosphatidylinositol-3-kinases, or PI3Ks, are serine/threonine kinases that, when activated, act on phosphatidylinositol-4,5-bisphosphate (PIP_2) to produce the second messenger phosphatidylinositol-34,5-trisphosphate (PIP_3). PIP_3 in turn activates protein kinase B (Akt), an enzyme with multiple downstream targets chief among which is the mammalian target of rapamycin (mTOR). Activated mTOR then regulates translation by phosphorylating components of the protein synthesis machinery, including ribosomal protein S6 kinases ($p70^{S6K}$) and 4E-binding protein (4E-BP), necessary for cell growth and proliferation. The PI3K-Akt-mTOR

pathway is regulated by PTEN, a phosphatase and known tumor suppressor that dephosphorylates PIP_3 reverting it to PIP_2 (Figure 3).

Figure 3: PI3K Pathway

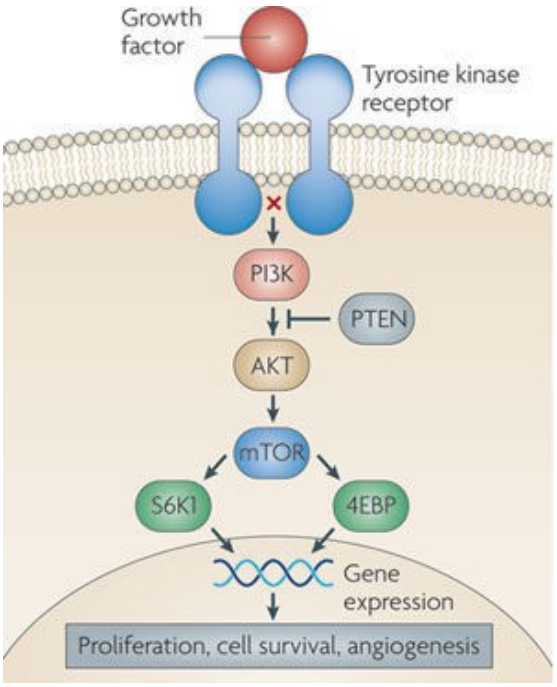


Figure 3: A simplified overview of the PI3K-Akt-mTOR pathway, from Holmes D.[44]

The Ras-Raf-MEK-ERK pathway (also called the MAPK/ERK pathway) is also significant for cell growth. Extracellular mitogen binding activates the G protein Ras via exchange of GDP for GTP. This sets a chain of activation of members of the mitogen-activated protein kinase (MAPK) family. Ras phosphorylates and activates Raf (MAP3K), which in turn activates MEK (MAP2K). MEK then activates ERK (MAPK), which regulates transcription factors including C-myc and CREB (Figure 4).

Figure 4: MAPK Pathway

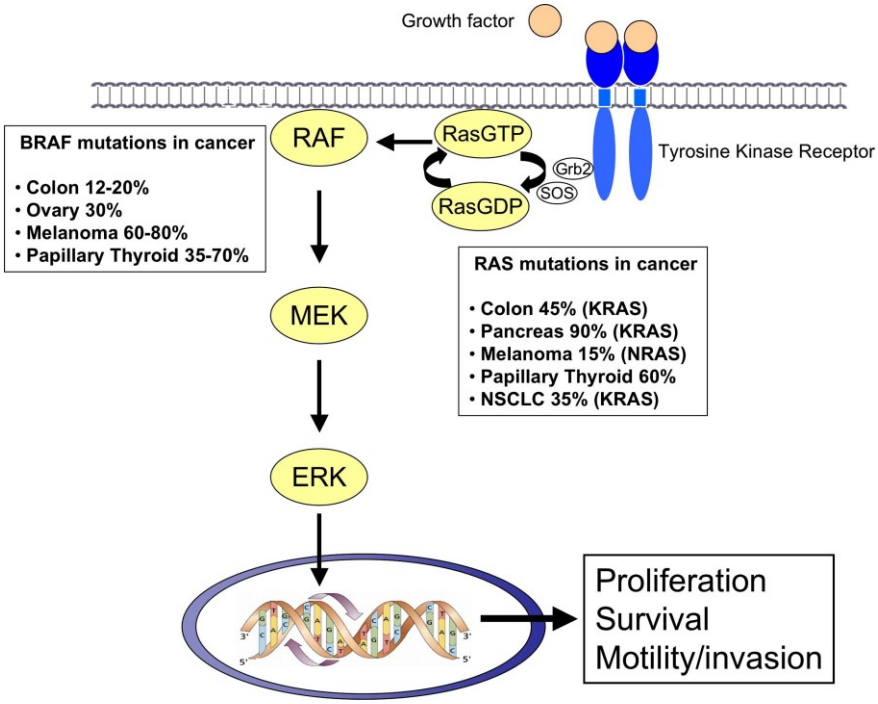


Figure 4: A linear simplified version of the MAPK pathway, from Montagut and Settleman.[45] Mutations of Ras and Raf are prevalent in a number of cancers.

In order to sustain proliferation cancer cells may produce growth factor ligands themselves, or induce stromal cells to supply growth factors. Somatic mutations can also allow for constitutive activation of signaling pathways promoting cell growth. For example, melanoma tumors are frequently associated with mutations that interfere with B-Raf protein structure, resulting in activation of MAPK-ERK.[46]

Many of the growth signaling pathways involve negative feedback mechanisms that must be taken into consideration. For example, loss of function mutations in PTEN may actually amplify PI3K signaling.[47] mTOR activation is also known to inhibit PI3K via negative feedback, an

unfortunate consequence being that pharmacological inhibition of mTOR may actually increase PI3K activity, counteracting the effect of the mTOR inhibitor.[48]

A third signaling pathway of note is the Notch signaling pathway, which promotes cell proliferation and differentiation (stemness) in particular. Binding of ligands including Jagged-1 (JAG1) and delta-like ligand 1 (DLL1) to the Notch receptor promotes translocation of the Notch Intracellular Domain into the nucleus which subsequently interacts with the transcription factor CSL (*C*BF-1, *S*uppressor of Hairless, *L*ag-2, aka RBPJ). This promotes transcription of target genes including cyclinD1, cMyc and p21. Dysregulation of the Notch signaling pathway has been found in various cancers including breast, prostate, colorectal and lung.

1.3.2. Evading Growth Suppressors

Many genes that limit cell growth and proliferation have proven to be tumor suppressors, prime examples being those coding for RB (retinoblastoma-associated) and tumor protein 53 (TP53 or p53) proteins. These proteins act as gatekeepers of cell cycle progression by inducing cell cycle arrest when activated, which may be triggered upon DNA damage or other stress. Mutations in their coding genes therefore significantly increase the risk of cancer development.

1.3.3. Resisting Cell Death

One important mechanism of cancer prevention is programmed cell death by apoptosis. Apoptosis may be triggered by detection of DNA breaks and chromosomal abnormalities. One

such damage detector functions via p53, which plays a critical role in inducing apopotosis in addition to cell cycle regulation. Tumors may evade apoptosis via the loss of p53 or increased expression of anti-apoptotic regulators. Autophagy, an intracellular degradation system, is another mechanism used to induce cell death. Inhibition of Beclin-1, a component of autophagy machinery, is associated with increased risk of cancer in mice.[49]

1.3.4. Enabling Replicative Immortality

Cells typically have a finite number of replications that can be undergone before either senescence or cell death occur. Evasion of these endpoints and the enablement of unlimited replicative potential is strongly associated with cancer development. As previously mentioned, the enzyme telomerase is not expressed in adult somatic cells. It is however expressed in the vast majority of immortalized cells including cancers, a certain key component to cancer development.

1.3.5. Inducing Angiogenesis

One of the most significant advances in cancer research over the past decades was the recognition of what is termed the "angiogenic switch".[50] Tumors promote proliferation and reduce apoptosis, thus resulting in a period of hyperplastic growth. However, without a supply of oxygen and nutrients this growth will eventually lead to cell death via apoptosis or necrosis. Tumors thus induce angiogenesis, the formation of new blood vessels from pre-existing blood vessels, in order to maintain survival. This is done via induction of VEGF (particularly VEGF-

A). Fibroblast-derived growth factor (FGF), platelet-derived growth factor (PDGF), matrix metalloproteinase (particularly MMP9), cathepsins and angiopoetins also play important roles in the induction of angiogenesis.

One function of p53 as a tumor suppressor is to regulate the expression of thrombospondin 1 (TSP-1), a glycoprotein that inhibits angiogenesis.[51] It has also been discovered that a class of angiogenic inhibitors is contained within proteins that are not themselves inhibitors, for example angiostatin (a component of plasminogen)[52]. The overall balance of angiogenic inducers and inhibitors is highly significant in governing the angiogenic switch.

1.3.6. Activating Invasion and Metastasis

Carcinoma cells are known to transform from an epithelial to a mesenchymal cell phenotype, a process known as the epithelial-mesenchymal transition (EMT). This process can facilitate tumor cell migration and invasion. Overexpression of transcription factors including ZEB1 and Snail and repression of the cell adhesion molecule e-cadherin are associated with EMT. A gene set analysis by Chen *et al* strongly suggested that EMT does indeed occur in RCC.[53]

Induction of EMT in cancer cells has also been found associated with the release of extracellular vesicles (EVs), of which tissue factor (TF) is a major component. The EVs may then interact with endothelial cells, contributing to systemic coagulopathy in cancer.[54]

Degradation of the extracellular matrix is an important component of cancer dissemination. This is accomplished with the help of matrix metalloproteinases (MMPs) activated outside the cell by other active MMPs or serine proteases. MMPs also participate in tumor proliferation, angiogenesis, EMT transformation and invasion.

Integrins are cell surface receptors that interact with ECM proteins. In doing so, they activate signaling pathways involved in cell proliferation and motility. They can promote tumor growth and survival by supporting PI3K-Akt or MAPK pathway activity, upregulating expression of NF-κB or attenuating p53 mediated apoptosis. Integrins are also known to induce and enhance angiogenesis via upregulation of VEGF and the PI3K/Akt/NO signaling pathway. They additionally promote cell adhesion and basement membrane degradation, facilitating cell migration and metastasis. Finally, they can promote cell adhesion-mediated drug resistance.[55]

1.3.6.1 Tumor Microenvironment

In conjunction with this hallmark, the role of interaction between cancer cells and the tumor microenvironment in cancer progression has also been examined.[56] The transplantation of mammary carcinoma cells into cleared fat pads containing normal mammary stroma has been found to reverse malignant properties.[57] Conversely, vehicle-exposed epithelial cells became cancerous when recombined with carcinogen-exposed mammary stroma.[58] It is believed that tumor-associated stroma is activated by malignant epithelial cells to promote growth via secretion of growth factors and promotion of angiogenesis. Interestingly, breast cancer studies found 3 epithelial genes differentially regulated at the transition of DCIS to invasive cancer,

whereas 305 stromal genes were dysregulated. Those stromal genes contained both cell cycle and ECM components.[59] Studies also showed that patients with stromal caveolin-1 (cav1) expression had a 2.5 higher 10-year mortality risk.[60]

1.3.7. Genomic Instability

As with aging, defects in DNA repair machinery are a hallmark of cancer.[61] Such defects can impact genes encoding proteins that detect DNA damage, actively repair DNA, or intercept mutagenic molecules before they have damaged DNA. Paradoxically, while telomerase allows unlimited replication favorable for tumor development, loss of telomeric DNA generates karyotypic instability frequently found in tumors.[62] Comparative genomic hybridization (CGH) studies have found pervasive genomic aberrations in gene copy number in tumors, evidence that control of genomic integrity has been lost.[63]

1.3.8. Tumor Promoting Inflammation

Also in parallel to aging, immune infiltration - ranging from subtle infiltrations to gross inflammation - has been recognized as a characteristic of tumors. The infiltrate may consist of lymphocytes, mast cells, neutrophils and what are known as tumor-associated macrophages. Initially, this was thought to reflect an attempt by the immune system to eradicate tumors, indeed a necessary process. However, it has been found that inflammation itself actually contributes to tumor growth by supplying growth factors, angiogenic mediators and ECM degrading enzymes.

It can also in itself promote genetic instability.[64] Inflammation has been evident at the earliest stages of neoplastic progression and has been shown capable of inducing full-blown cancer.[65]

1.3.9. Reprogramming Energy Metabolism

Tumor development requires not only deregulated control of cell proliferation, but also alteration in energy metabolism in order to fuel growth. It has been found that even in the presence of oxygen (which normally favors glucose production over glycolysis), tumor cells can reprogram their glucose metabolism to favor glycolysis - a state termed as "aerobic glycolysis". This is done in part by upregulating glucose transporters, increasing expression of glycolytic enzymes and inhibiting mitochondrial metabolism.[66] As many tumors promote a hypoxic environment, this process is even further accentuated by activation of hypoxia-inducible transcription factors promoting glycolysis.[67]

1.3.10. Evading Immune Destruction

An important component of tumor progression is evasion of immune system recognition and destruction. Cells that present tumor antigens are naturally vulnerable to immune destruction. However, genetic instability and continued replication results in cells with reduced immunogenity, a process known as "immunoediting".[68] Initially there may be a balance between immune control and tumor growth, resulting in the appearance of tumor dormancy. Eventually, however, the tumor cells are able to impair the capacity of the immune system to eradicate them.

Exploitation of immune suppression via regulatory T cells is a particularly major mechanism of tumor immune escape. Another mechanism is down-modulation of antigen processing machinery including the major histocompatibility complex (MHC) I pathway. Tumors may also produce immune suppressive cytokines including transforming growth factor (TGF-β),[69] tumor necrosis factor (TNFα),[70] colony stimulating factor (CSF-1),[71] interleukins and interferons.

1.4 Renal Cell Carcinoma

1.4.1 Introduction and Epidemiology

Renal cell carcinoma (RCC) is the most common type of kidney cancer, comprising over 90% of cases. For 2017, the American Cancer Society estimated 63,990 new cases and 14,400 deaths within the United States[72] while the Canadian Cancer Society estimated 6,600 cases and 1,900 deaths.[73] It is approximately twice as common in males than in females. It most commonly occurs in the 6th to 8th decade of life with a median age diagnosis of 64, but has a wide age spectrum. Among countries the highest rates of RCC are observed in the US and Czech Republic, and Americans of Asian descent have been noted to have a lower incidence of RCC compared to other racial groups in the US.[74]

1.4.2 Presentation

RCC is often only symptomatic once the disease is advanced, and approximately 25% of patients have distant metastasis upon diagnosis. Those diagnosed due to an incidental procedure therefore

tend to have improved prognosis. Patients most frequently present with hematuria, flank pain and a palpable abdominal renal mass, but only approximately 10% exhibit all three of these symptoms.[75] Weight loss, scrotal varioceles in males and symptoms associated with inferior vena cava involvement such as hepatic dysfunction and ascites may also occur.[76] Diagnosis is generally confirmed by the presence of a renal mass on abdominal computer tomography (CT).

1.4.3 Subtypes, Grade and Stage

In 2016, the World Health Organization updated its classification of renal cell tumors to the following subtypes, taking into account cytoplasmic and architectural features, anatomic background, association with pre-existing renal disease and molecular alterations (Table 1).[77] Of those, clear cell renal cell carcinoma (ccRCC) is by far the most prevalent subtype, occurring in approximately 85% of cases.

Table 1: Renal Cell Carcinoma Subtypes

Renal Cell Carcinoma Subtypes
• Clear cell renal cell carcinoma • Multilocular cystic renal neoplasm of low malignant potential • Papillary renal cell carcinoma • Hereditary leiomyomatosis and renal cell carcinoma (HLRCC) -associated • Chromophobe renal cell carcinoma • Collecting duct carcinoma • Renal medullary carcinoma • MiT family translocation renal cell carcinomas • Succinate dehydrogenase-deficient renal carcinoma • Mucinous tubular and spindle cell carcinoma • Tubulocystic renal cell carcinoma • Acquired cystic disease-associated renal cell carcinoma • Clear cell papillary renal cell carcinoma • Renal cell carcinoma, unclassified • Papillary adenoma • Oncocytoma

Histological tumor grading is an accepted prognostic factor of RCC. A four-tiered grading system based on nucleolar prominence is currently recommended by the WHO in lieu of the Furhman system, which was commonly used but known not to be applicable to chromophobe RCC. It has been verified for ccRCC and papillary RCC.

Table 2: WHO Grading System

Grade	Description
I	Nucleoli absent and basophilic at x400 magnification.
II	Nucleoli conspicuous and eosinophilic at x400 magnification and visible but not prominent at x100 magnification.
III	Nucleoli are conspicuous and eosinophilic at x100 magnification.
IV	Extreme nuclear pleomorphism, multinucleate giant cells, and/or rhabdoid and/or sarcomastoid differentiation.

An especially significant prognostic factor of RCC is its stage, based on tumor size, location and spread. The TNM (for Tumor, Nodes, Metastasis) system is used for renal cell carcinoma staging as detailed below:

Table 3: TMN Staging System

Stage	Grouping	Description
I	T1, N0, M0	Tumor 7cm or smaller and only in kidney.
II	T2, N0, M0	Tumor > 7cm large and only in kidney.
III	T3, N0, M0	Tumor is growing into a major vein or in surrounding tissue, but not beyond Gerota's fascia or into adrenal gland.
	T1-T3, N1, M0	Tumor is not beyond Gerota's facia, but has spread to surrounding lymph nodes.
IV	T4, Any N, M0	Tumor is beyond Gerota's facia and may be growing in adrenal gland. It may have also spread to surrounding lymph nodes.
	Any T, Any N, M1	Tumor has spread to distant lymph nodes and/or other organs.

1.4.4 Molecular Characteristics

ccRCC is characterized by loss of the short arm (p) of chromosome 3, occurring in over 90% of cases. This area encompasses the von Hippel Lindau (VHL) gene on chromosome 3p25, which has long been known to be associated with RCC. The VHL protein (pVHL) forms part of an E3 ubiquitin ligase complex that targets the hypoxia-induced factor (HIF).

HIF-1 is a transcription factor consisting of a beta subunit that is constitutively expressed, and an alpha subunit that is regulated by oxygen. When oxygen is present, the VHL-ubiquitin complex binds to the alpha subunit of HIF-1 in the cytosol, targeting it for degradation by proteasomes. Under hypoxic conditions, HIF-1α is stable, re-enters the nucleus and binds with HIF-1β, leading to downstream expression of genes promoting anaerobic metabolism, angiogenesis and cell proliferation (Figure 5).

HIF-regulated genes include glucose transporters (GLUT1), vascular/endothelial growth factors (VEGF, EGF), nitric oxide synthases (ENOS) and matrix metalloproteinases (MMP2). Bilateral inactivation of VHL, resulting in HIF overexpression in normoxic conditions, has been observed in most cases of ccRCC and is a hallmark of this disease. This in turn results in constitutive VEGF expression and is a primary driver of angiogenesis in ccRCC, explaining its high level of vascularization.

Figure 5: HIF Pathway

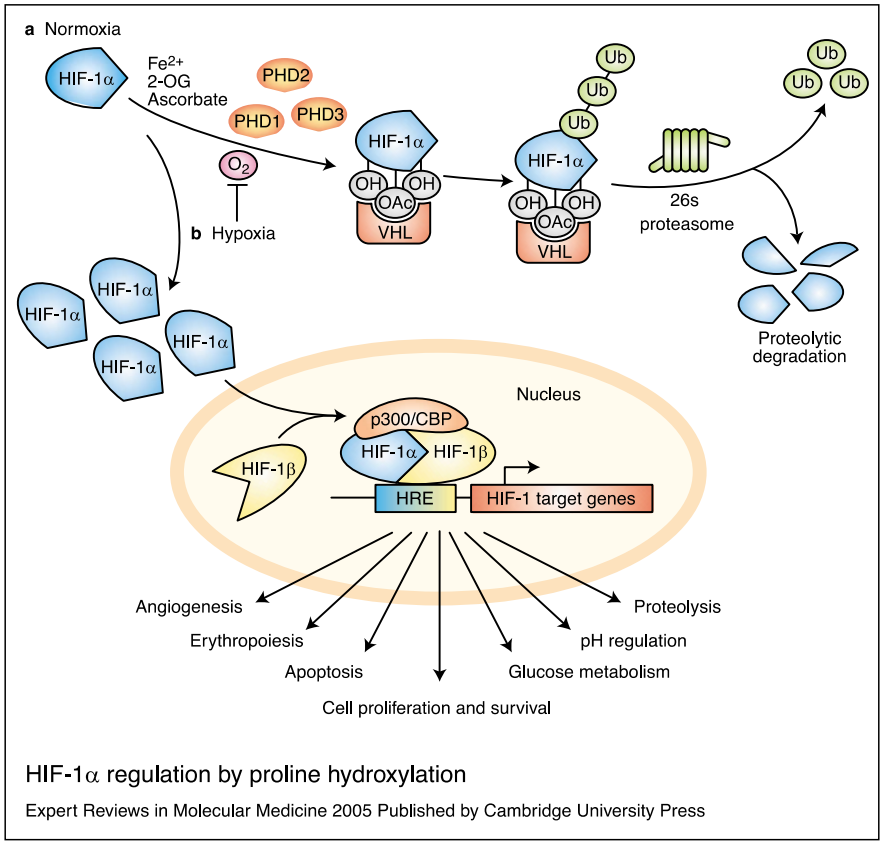


Figure 5: HIF regulation under normoxia (a) and hypoxia (b), from Carroll and Ashcroft.[78] The presence of oxygen triggers proline hydroxylation of HIF-1α through VHL, leading to ubiquitination and subsequent proteolytic degradation. Under hypoxia, HIF is functional and directs expression of cell-proliferation genes.

Mutations in the gene polybromo-1 (PBRM1), located at chromosome 3p21, have also been observed in RCC in association with 3p loss.[79] PBRM1 codes for BAF180, a subunit of the PBAF SWI/SNF chromatin remodeling complex. Approximately 41% of ccRCC cases were found to contain a truncating mutation in PBRM1, second only to VHL in mutation rate and particular to this specific subtype of renal cancer. Inhibition of PBRM1 in ccRCC cell lines was associated with increased cell proliferation,[80] supporting the theory that PBRM1 functions as a tumor suppressor gene in the kidney. Further research by Nargund *et al.* suggests that PBRM1

prevents over-amplification of the HIF pathway upon inactivation of VHL.[81] Loss of PBRM1 in ccRCC has been correlated with higher tumor grades and stages and worse patient outcomes.[82] This is consistent with the finding that an allele of PBRM1 remains functional and expressed in early stage ccRCC, and that subsequent mutation and loss of that allele is a significant driver of cancer progression.

BRCA-associated protein-1 (BAP1), additionally on chromosome 3p21, has also been found significantly mutated in ccRCC.[83] BAP1 protein functions as a deubiquinating enzyme, i.e. a protease that cleaves ubiquitin or ubiquitin-like proteins from subtrates. BAF1 complexes are associated with open chromatin. While the molecular mechanism of tumor suppression of BAP1 is unclear, it is believed that binding to host cell factor 1 (HCF1) plays a role.[84] Interestingly enough, mutations in BAP1 and PBRM1 have been found to be mutually exclusive.[85]

Another gene on chromosome 3p21 significantly impaired in ccRCC is Set domain-containing 2 (SETD2).[86] SETD2 is a histone H3 lysine 36 methytransferase posited to have tumor suppression function through nucleosome stabilization, suppression of replication stress and coordination of DNA repair.[87] While not on chromosome 3p, KDM5C is a histone H3 lysine demethylase also found inactivated along with SETD2, underscoring the role of histone modification in RCC.[86]

Several comprehensive genomic studies over the past decade have allowed for improved understanding of the molecular mechanisms behind subtypes of RCC. One landmark study was by The Cancer Genome Atlas, which performed genetic analysis on ccRCC primary tumors.[88] It further confirmed the significance of the VHL/HIF pathway and gave importance to chromatin

remodeling (particularly involving the SWI/SNF complex and PBRM1) and the PI3K-Akt pathway in ccRCC development as well. The study also found correlation between worsened prognosis in ccRCC patients and a metabolic shift involving increased pentose phosphate cycle activity and decreased Krebs cycle activity.

It is important to reiterate that the molecular characteristics discussed above pertain primarily to the ccRCC subtype, as it has been most extensively studied given its prevalence. TCGA has however created databases for chromophobe RCC and papillary RCC in addition to ccRCC. Per TCGA's analysis, chromophobe RCC was found to originate from the distal nephron and is associated with changes in mitochondrial function as well as genomic rearrangements causing structural breakpoints within the TERT promoter region, leading to increased expression of TERT and subsequent hypermutation.[89] Papillary RCC can be divided in two clinically and biologically distinct types. Type 1 is associated with alterations in the MET pathway. Type 2 is associated with activation of the NRF2-ARE pathway and can be further subdivided in at least three subtypes. CDKN2A loss and CpG island methylator phenotype (CIMP) in type 2 are associated with poor prognosis.[90]

In one significant study, Chen et al analysed a total of 894 TCGA samples using five separate platforms – mRNA expression, DNA methylation, DNA copy, miRNA expression and protein expression.[91] From these analyses, they identified a novel "mixed" RCC subtype and a hypermethylated subset of RCC associated with more aggressive disease. In addition, they found evidence of proximal nephron as the origin of papillary RCC, the association of hereditary

papillary RCC with increased levels of genomic rearrangement, and the association of DNA copy-unstable patterns and CDKN2A loss with more aggressive ccRCC and papillary RCC.

As noted in a review by Manley and Hakami,[92] it is remarkable how little the molecular and genetic characteristics of RCC overlap. It can therefore be inferred that subtype-specific therapy is required to improve outcomes for non ccRCC cases.

1.4.5 Treatment

Surgical resection remains a first-line treatment for localized RCC. Partial nephrectomy is recommended in tumors < 7cm, and radical nephrectomy for tumors > 7cm. Biopsy is rarely performed beforehand for isolated solid masses, but may be performed for sites of suspected metastasis.[93]

Traditional chemotherapy and radiation have been ineffective in treating advanced RCC, and are only used for managing metastases outside the kidney. The cytokines interferon-alpha (IFN-α) and interleukin 2 (IL-2) were instead among the first therapies to demonstrate efficiency against RCC.[94] High-dose IL-2 was originally approved for advanced RCC in 1992 as a first line treatment, and remains a primary therapeutic agent today.[95] Studies have suggested that prior therapy with other agents decreases response to IL-2, supporting first-line use.[96] Toxicity however remains a significant concern with IL-2,[97] and restricts the amount of institutions capable of administering therapy.

The advent of targeted therapy hailed a revolution in the treatment of RCC. The glycoprotein vascular endothelial growth factor receptor (VEGF) is a key promoter of angiogenesis, which is essential for tumor development and growth. Sunitinib (sunitinib malate; SU11248; SUTENT™) is a small multitargeted tyrosine kinase inhibitor (TKI) of VEGFR-1, VEGFR-2 and other kinases including, fetal liver tyrosine kinase receptor 3 (FLT3), KIT (stem-cell factor receptor), PDGFRα, and PDGFRβ. Sunitinib obliterates VEGFR2 signaling in endothelial cells, a property of particular significance in RCC where high levels of VEGF drive florid tumour angiogenesis. Based on this mechanism sunitinib was approved for use in metastatic RCC in 2006 after studies showed improved overall survival compared to placebo.[98] It was later found to improve disease-free survival of patients who receive nephrectomy and are at high risk of recurrence[99] and was approved as an adjuvant therapy for these patients in 2017.

Other VEGF TKIs currently in use against RCC include sorafenib, axitinib, cabozantinib and pazopanib. Bevacizumab is a monoclonal antibody against circulating VEGF and so also acts to prevent VEGF pathway activation.

In addition to VEGF, the mTOR pathway is also a therapeutic target in RCC. As was previously discussed, mTOR is a serine/threonine kinase functioning as a downstream effector of the PI3K-Akt pathway. It forms the multiprotein complexes mTORC1 and mTORC2, the former of which activates transcription factors S6K1 and 4EBP1, leading to mRNA translation of proteins involved in cell growth, proliferation, metabolism and angiogenesis. The pathways impacting mTOR signaling are dysregulated in RCC, and mTOR additionally regulates production of HIF-1α, making inhibition particularly relevant to RCC. mTORC1 is sensitive to rapamycin, and the

analogues everolimus and tenserolimus function as mTOR inhibitors. However, clinical trials found treatment with everolimus to be inferior compared to other agents including sunitinib,[100] and so mTOR inhibitors are generally only used in patients who have proven refractory to several other therapies.

A recent advance in RCC treatment has come in the form of immunotherapy through immune checkpoint inhibitors (ICIs).[101] One major target is Programmed Cell Death 1 (PD-1), a receptor expressed on B and T lymphocytes, natural killer cells and monocytes belonging to the immunoglobulin (Ig) superfamily and known to attenuate immune response. Binding of ligands PD-L1 and PD-L2 to PD-1 phosphorylates two tyrosine motifs on PD-1, inducing the recruitment of Src homology 2 domain-containing tyrosine phosphatase 2 (SHP2) and in turn modulating downstream effectors necessary for downstream TCR signaling.[102,103] An additional ICI target is cytotoxic leukocyte antigen 4 (CTLA-4).

Nivolumab is a monoclonal antibody targeting PD-1 and the first ICI to demonstrate significant clinical activity against cancer. Phase II clinical trials with RCC patients showed objective response rates in 20-22% of patients and overall survival of 18.2-25.5 months.[104] In the subsequent phase 3 Checkmate 025 clinical trial, nivolumab was found to have improved overall (though not progression-free) survival, objective response rate and quality of life along with decreased toxicity compared to everolimus.[105] It is therefore most currently recommended for patients failing to respond to VEGF TKI therapy.

1.4.6 Mortality

Per the SEER Cancer Statistics Review, the overall five-year survival rate for kidney cancer for all races and sexes was 74.6% in 2013.[106] Of note is that while the annual incidence of RCC rose by 126% in the United States since 1950, the mortality rate only rose by 36.5% over the same period.[107] This lower increase in mortality can be primarily credited to earlier diagnosis and surgical intervention,[74] and recent therapeutic advances can be anticipated to have an effect as well.

1.4.7 Inter-Individual Heterogeneity in RCC

Patient response to both surgical and medical treatment for RCC is notoriously heterogeneous, and so predicting individual prognosis is especially challenging. In one example, a study of 172 patients with unilateral nonmetastatic RCC treated with surgery between 1978 and 1988 found that 30 patients (17%) subsequently developed distant metastases.[108] While targeted and immunological therapy for metastatic RCC has clearly extended duration of progression-free survival (ex. 11.1 months vs. 2.8 months for panopazib vs. placebo),[109,110] many ultimately fail to respond.

Why some patients do respond vs. others is, at least in part, related to molecular differences in the tumors of different patients, including gene expression patterns.[111] A study by Pantuck *et al*. identified 73 differentially expressed genes between responders and nonresponders to IL-2, with CAIX, PTEN and CXCR4 being associated with complete response.[112] Another study found that

12/13 (92%) of patients with high HIF-2α expression responded to sunitinib, as compared to only 4/15 (27%) of patients with low HIF-2α expression.[113]

Changes in gene expression associated with aging ultimately result in alteration of various molecular pathways, and as we have seen these pathways are frequently associated with cancer development as well. Further insight and understanding of age-related gene expression patterns in RCC could therefore ultimately lead to improved therapeutic strategies based on age and a decrease in incidence of resistance – hence the previously outlined aim of this study.

CHAPTER 2: MATERIALS AND METHODS

2.1 Patient Data

Gene expression and clinical data was available from two independent genomic studies of ccRCC; the Cancer Genome Atlas (TCGA)[88] and the Cancer Genomics of the Kidney (CAGEKID)[114] program of the International Cancer Genome Consortium (ICGC).

TCGA data was available in form of RNA-Seq by Expectation Maximization (RSEM),[115] reported as expected counts and consisted of 436 tumor samples and 69 paired normal samples. Of the 436 patients, 285 were males and 151 were females. Patient age ranged from 26 to 90 years, with a mean age of 60.94 and a median age of 61.

CAGEKID data was available in form of Reads per Kb per Million Mapped Reads (RPKM) and consisted of 89 tumor samples and 43 paired normal samples. Of the 89 patients, 50 were males and 39 were females. Patient age ranged from 35 to 83 years, with a mean age of 61.1 and a median age of 61.7. Additional information including number of samples corresponding to each tumor grade and stage are presented in Table 4.

Table 4: Clinical Data for CAGEKID and TCGA Datasets

Characteristic	Parameter	TCGA	CAGEKID
Type	Tumor Paired Normal	436 69	89 43
Age	Range Mean Median	26-90 60.94 61.1	35-83 61.12 61.7
Sex	Male Female	285 151	50 39
Grade	1 2 3 4	8 184 179 65	3 54 15 17
Stage	1 2 3 4	211 43 114 68	49 9 22 9

2.2 Regression Analysis

This study was predicated on the notion that the organismal aging process at the time of ccRCC onset and development may impose different molecular make-ups upon evolving cancer cell populations. To this end, I examined the association between gene expression levels and patient age in tumor and normal kidney tissues via regression analysis involving both TCGA and CAGEKID datasets independently.

A general linearized model (GLM) regression analysis was performed on gene expression data using R.[116] All data was normalized to mean gene expression via the equation log10 ((gene expression + 0.01) / (mean gene expression across all samples + 0.01))). This allowed for

improved functioning of the regression analysis and comparison between TCGA and CAGEKID data.

Genes with a mean expression of 0 for TCGA samples were filtered out for that dataset (leaving a total of 20,229 genes for tumor cells and 19,788 genes for normal cells), while genes with a mean gene expression < 1 for CAGEKID samples were filtered out for that dataset (leaving a total of 14,108 genes for tumor cells and 13,273 genes for normal cells). Y chromosome genes were analyzed separately using only male samples. The results were then added to those from non-Y chromosome genes.

Regression analysis included age, sex, tumor grade and tumor stage as covariates for tumor samples. Grade and stage were not included as covariates for normal samples, and sex was not included as a covariate when analyzing the Y chromosome genes. Results were obtained for TCGA normal samples, TCGA tumor samples, CAGEKID normal samples and CAGEKID tumor samples. I then evaluated the correlation between corresponding TCGA and CAGEKID age beta coefficients results to verify the reproducibility of my findings.

2.3 Identification of Age-Associated Expressed Genes

Results were sorted by age beta coefficients. The 1000 genes with the highest coefficients (indicating progressive upregulation with increased age) and lowest coefficients (indicating progressive downregulation with increased age) were selected. The list of genes corresponding to age-upregulation in TCGA normal samples was then compared with that for age-upregulation in

CAGEKID normal samples. The same was done for age-downregulation in TCGA and CAGEKID normal samples, age-upregulation in TCGA and CAGEKID tumor samples, and age-downregulation in TCGA and CAGEKID tumor samples. From each corresponding set, the number of shared/overlapping genes in each list were obtained. Fisher's exact test was then performed for each of the four sets (age-down normal, age-down tumor, age-up normal, age-up tumor) to obtain significance of the overlap.

2.4 Downstream Pathway Analysis

The overlapping age-associated genes in both TCGA and CAGEKID from each of the four above-mentioned sets were next subjected to downstream pathway analysis using ConsensusPathDB.[117] Results were selected to come from Kyoto Encyclopedia of Genes and Genomes (KEGG),[118] Reactome,[119] Biocarta[120] and PID[121] pathways in addition to Gene Ontology terms. A list of all genes analyzed in both CAGEKID and TCGA datasets was used as background in pathway enrichment analysis (11,358 and 11,953 genes in normal and tumor samples respectively).

These analyses were repeated using a stringent background in ConcensusPathDB of only the top age-associated genes in both TCGA and CAGEKID datasets (797 and 677 genes in normal and tumor samples respectively), using only patients with stage 1 RCC, and with male and females separated. These analyses addressed the stability of our findings in terms of pathway enrichment with regards to the high variations in gene expression, which can be affected by the stage of the tumors as well as sex of patients.

A flow chart of the methodology described is presented in Figure 6.

Figure 6: Methodology

1. Selected genes with 1,000 highest (Up) and lowest (Down) age regression coefficients for each set of results
2. Obtained genes shared between corresponding datasets
3. Performed downstream pathway analysis of genes shared in both datasets

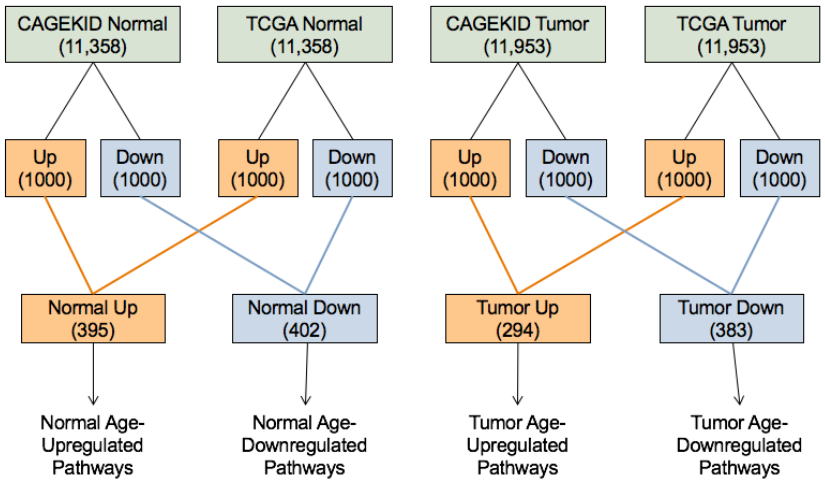


Figure 6: Flow chart of methods used to obtain age-associated pathways for normal and tumor samples.

CHAPTER 3: RESULTS

3.1 Reproducibility of Regression Results

In order to confidently enable a selection of gene subsets for functional pathway analysis, I verified reproducibility between the TCGA and CAGEKID regression results. As the dataset sample size for CAGEKID was too small to provide significant p-values, I focused on the resulting beta coefficients for age – i.e. the amount of change in gene expression predicted from the regression with one year of increase in patient age.

The age-associated gene expression patterns were stable and reproducible as a comparison between regression beta coefficients from TCGA and CAGEKID revealed significant correlations (R= 0.416, $p < 2.2 \times 10^{-16}$ and R= 0.403, $p < 2.2 \times 10^{-16}$ for tumor and normal samples, respectively) between analysis results from these datasets (Figure 7).

Figure 7: CAGEKID vs TCGA Age Beta Coefficients

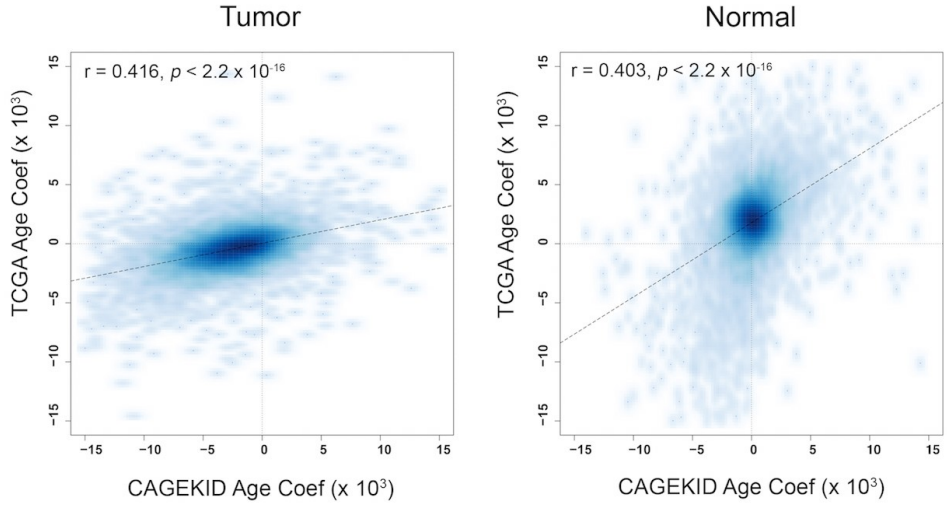


Figure 7: Plot of obtained age beta coefficients from CAGEKID versus TCGA regression analyses for tumor samples and normal samples. There is significant correlation between the results from both datasets.

3.2 Genes with Age-Associated Expression

Following the methods previously outlined in Section 2.3, 294 genes were found to be commonly age-upregulated and 383 genes were found to be commonly age-downregulated among tumor samples in both CAGEKID and TCGA datasets ($p < 2.2\times10^{-16}$, Fisher's exact test; Table 5); indicating a significant overlap between genes identified with the same pattern in independent patient cohorts.

Similar analysis in normal samples from both datasets, revealed that 395 genes were found to be commonly age-upregulated and 402 genes were found to be commonly age-downregulated ($p < 2.2\times10^{-16}$, Fisher's exact test; Table 5). Fold-enrichment of overlap compared to chance ranged from 3.51 to 4.58. These analyses confirmed that my findings were not limited to a specific data or sample set, and age-associated gene expression patterns in ccRCC are stable.

Table 5: Significance of Overlap for Age-Associated Genes Between CAGEKID and TCGA Datasets

Type	Relationship with Increased Age	No. of Genes	Fold-enrichment	*p* value
Normal	Upregulated Downregulated	395 402	4.49 4.57	$< 2.2\text{x}10^{-16}$ $< 2.2\text{x}10^{-16}$
Tumor	Upregulated Downregulated	294 383	3.51 4.58	$< 2.2\text{x}10^{-16}$ $< 2.2\text{x}10^{-16}$

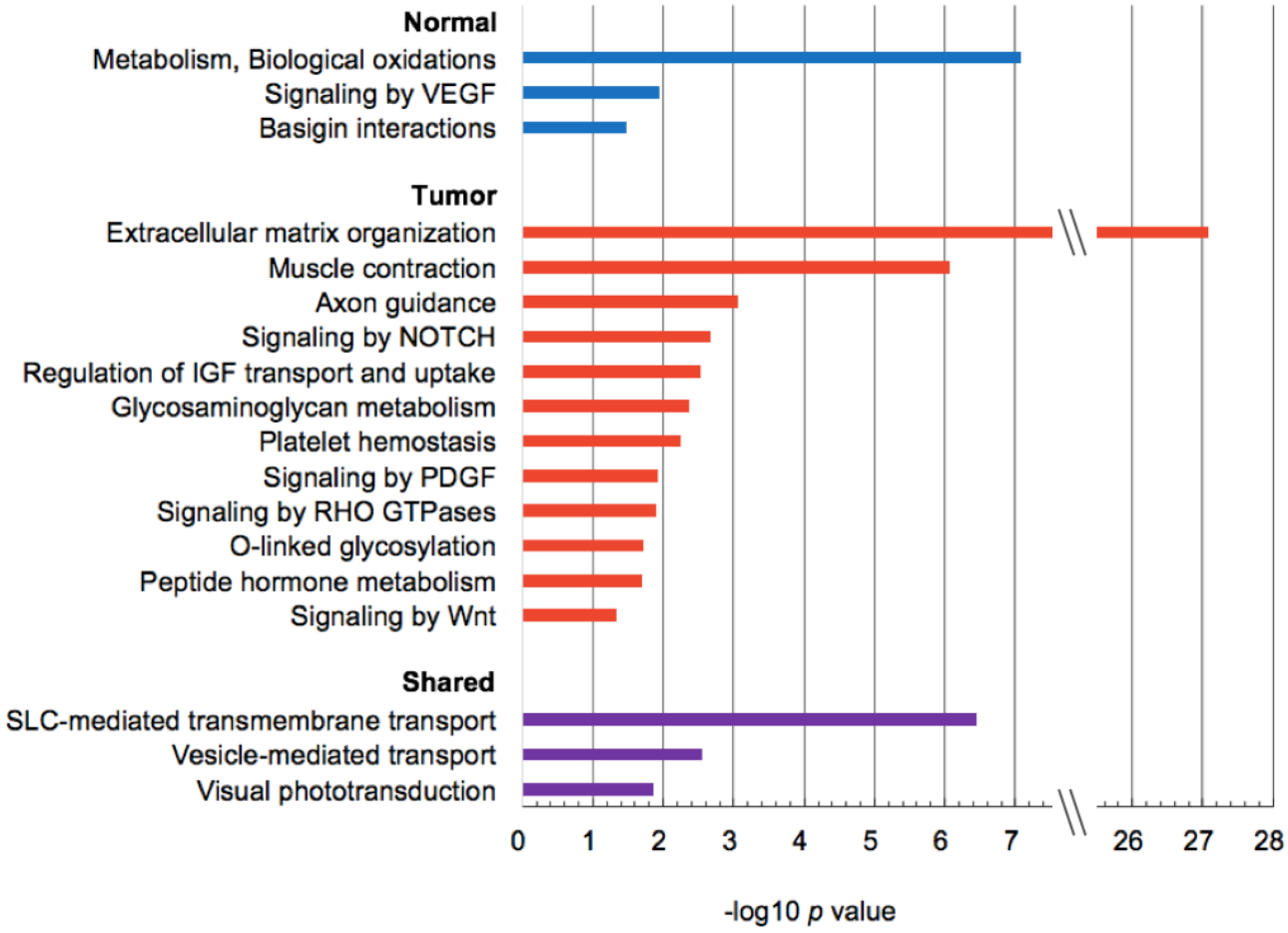
The full list of age-associated genes can be found in Appendix Tables 1a-b.

3.3 Molecular Pathways Affected by Age-associated Gene Expression

To gain insight about cellular functions that may be affected by age-related gene expression, I performed pathway analysis of top genes with the age-associated expression patterns in both datasets, as described in Section 2.4. Among the selected datasets, results from Reactome were found to be most informative. These results are summarized in Figure 8, with full results in Appendix Tables 2a-d.

Figure 8: Age-Associated Pathways

a)



b)

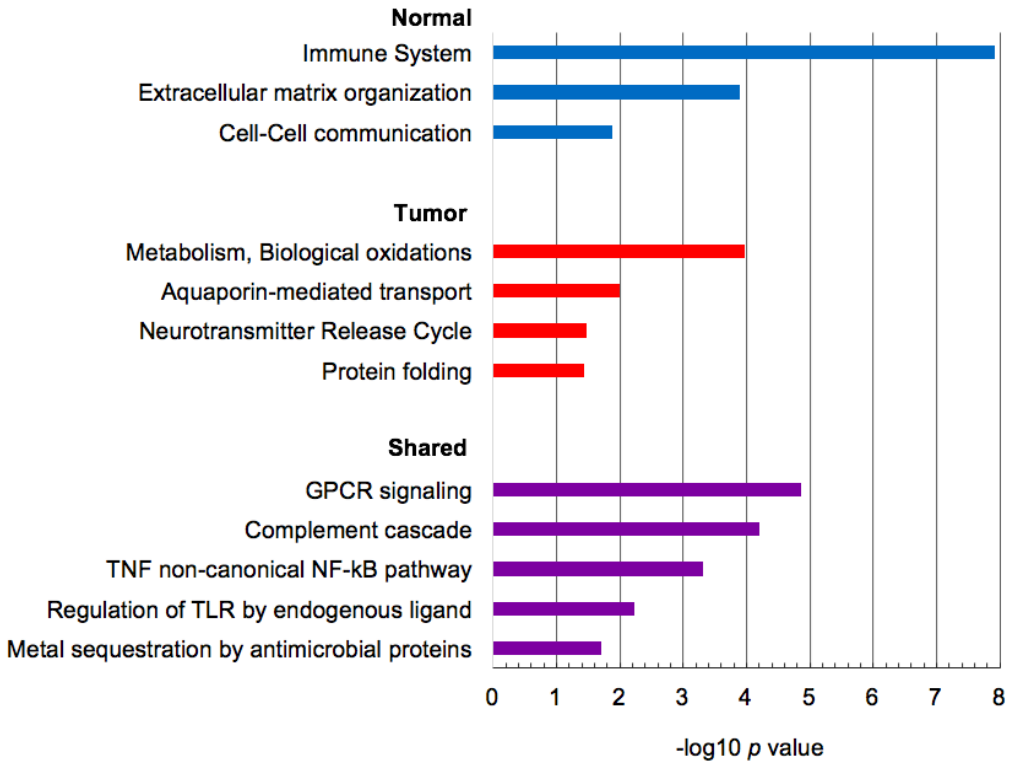


Figure 8: Reactome pathways enriched in genes found downregulated (a) and upregulated (b) with age in both CAGEKID and TCGA datasets. There are notable differences between pathways enriched in normal samples versus tumor samples.

The analysis revealed that pathways that are significantly enriched for age-related gene expression (False discovery rate (FDR) < 0.05) are different between normal and tumor cells. Furthermore, there appeared to be a switch in association with age between normal and tumor cells. For example, whereas ECM and cellular communication pathways were significantly enriched for genes upregulated with age in normal kidney tissue, these pathways showed a significant enrichment for genes which were downregulated with age in tumor samples. Opposite relationships were observed for pathways involved in metabolism and oxidation, which were significantly age-downregulated in normal kidney and age-upregulated in tumor samples.

Among other ccRCC-related pathways, angiogenesis was found to be enriched for genes downregulated with age in tumor cells but not in normal cells. In addition, many immune system functions were found enriched for genes upregulated with age in normal cells, with tumor cells also being enriched for complement cascade, the Tumor Necrosis Factor Receptor 2 (TNFR2) non-canonical NFκB pathway and toll-like receptor regulation.

3.4 Pathway Validation

To further validate these results, I repeated the downstream pathway analysis using a more stringent background (Section 2.4). Extracellular matrix organization pathways remained significantly enriched among genes upregulated with age in normal cells and genes downregulated with age in tumor cells, while metabolism remained significantly enriched among genes downregulated with age in normal cells and genes upregulated with age in tumor cells. Immune system pathways remained significantly enriched among genes upregulated with age in normal cells.

Table 6: Age-Associated Pathways (Stringent Background)

a) Age-Downregulated: Normal Cells

p-value	q-value	Pathway	Source
6.29E-10	3.02E-07	Metabolism	Reactome
3.29E-05	0.007901	Metabolism of amino acids and derivatives	Reactome

b) Age-Downregulated: Tumor Cells

p-value	q-value	Pathway	Source
1.40E-08	5.14E-06	Extracellular matrix organization	Reactome
8.32E-05	0.010208	Protein digestion and absorption	KEGG
8.32E-05	0.010208	Collagen biosynthesis and modifying enzymes	Reactome
0.000145	0.012154	Beta1 integrin cell surface interactions	PID
0.000165	0.012154	Integrins in angiogenesis	PID
0.000251	0.013806	Collagen chain trimerization	Reactome
0.000263	0.013806	Collagen formation	Reactome
0.000746	0.034323	ECM-receptor interaction	KEGG
0.000974	0.039823	Syndecan-1-mediated signaling events	PID
0.001422	0.052348	Axon guidance	Reactome
0.001753	0.058648	Elastic fibre formation	Reactome

c) Age-Upregulated: Normal Cells

p-value	q-value	Pathway	Source
6.39E-10	3.62E-07	Immune system	Reactome
2.20E-06	0.000622	HTLV-I infection	KEGG
8.18E-06	0.001329	Innate immune system	Reactome
9.39E-06	0.001329	Adaptive immune system	Reactome
9.00E-05	0.010187	Chemokine signaling pathway	KEGG
0.000181	0.012776	NOD-like receptor signaling pathway	KEGG
0.000181	0.012776	Th17 cell differentiation	KEGG
0.000181	0.012776	Toxoplasmosis	KEGG
0.000346	0.017643	NF-kappa B signaling pathway	KEGG
0.000346	0.017643	Influenza A	KEGG
0.000374	0.017643	Herpes simplex infection	KEGG
0.000374	0.017643	Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	Reactome
0.000773	0.029183	IL12-mediated signaling events	PID
0.000773	0.029183	Chagas disease (American trypanosomiasis)	KEGG
0.000773	0.029183	Pertussis	KEGG
0.000830	0.029362	Direct p53 effectors	PID
0.001303	0.037643	Rheumatoid arthritis	KEGG
0.001532	0.037643	Staphylococcus aureus infection	KEGG
0.001596	0.037643	Type I diabetes mellitus	KEGG
0.001596	0.037643	Epstein-Barr virus infection	KEGG
0.001596	0.037643	Asthma	KEGG
0.001596	0.037643	Downstream TCR signaling	Reactome
0.001596	0.037643	TCR signaling	Reactome
0.001596	0.037643	TNF signaling pathway	KEGG
0.002197	0.049736	Neutrophil degranulation	Reactome

d) Age-Upregulated: Tumor Cells

p-value	q-value	Pathway	Source
9.24E-08	3.34E-05	Metabolism	Reactome
2.11E-06	0.000382	Metabolism of amino acids and derivatives	Reactome

3.5 Stage 1 Specific Pathway Analysis

Regression analysis was also repeated using only stage 1 patients, to ensure the obtained pathway results were not being influenced by the association between tumor stage and patient age.

Resulting age-downregulated pathways in tumors included ECM organization, collagen metabolism, axon guidance, focal adhesion, integrins and signal transduction pathways including Notch and PI3K-Akt, consistent with previous results. The age-upregulated pathways in stage 1 tumors included metabolism, but were much more enriched with immune-related pathways compared to when all samples were analyzed. Thus, some gene expression patterns were consistent across ccRCC stages (e.g. down-regulation of ECM-related genes in cancer) while others were more unique to early stage disease.

3.6 Sex Specific Pathway Analysis

I also analyzed sex-specific age-related gene expression changes by repeating the regression analysis using only male and female patient data separately. No male specific pathways were found, however immune system pathways (particularly concerning TNF signaling) were found to

be more strongly age-upregulated in females (p=0.01 with FDR=0.069 in males, p=0.002 with FDR=0.024 in females), and Notch pathways were found exclusively age-downregulated in females (p=0.21 with FDR 0.38 in males, p=0.008 with FDR=0.048 in females). This observation suggests that certain molecular features of ccRCC are both age- and sex-dependent.

3.7 Analysis of Association of ECM and Stromal Gene Sets with Patient Age

Given that ECM and immune system pathways were enriched with age-associated gene expression patterns, and in view of the clinical relevance of stroma and immune cell infiltration in RCC,[122,123] I set out to determine to what extent the respective gene signatures were associated with patient age. Yoshihara *et al.*[124] had previously reported on lists of genes whose expression levels represent the stromal and immune compositions of tumor samples. Using these gene sets, they assigned unique stromal and immune scores to each of 329 patient tumors included in the ccRCC TCGA study[88] via Gene Set Expression Analysis (GSEA). My analysis of the relationship between those scores and patient age revealed a negative association between tumor stromal score and patient age (r=-0.186, p=0.00068), while there was no correlation between the immune score and patient age (r=0.001, p=0.98).

To validate these results with an independent method, I used Weighted Gene Co-Expression Network Analysis (WGCNA)[125] to generate stromal and immune scores for gene co-expression profiles in tumor samples of 436 TCGA patients and of 89 CAGEKID patients. WGCNA works by clustering genes with similar expression values (i.e. co-expression) into what are termed modules. The first principle component of each module is termed the eigengene (E), and given a

score based on the strength of co-expression. Downstream pathway analysis was used to determine which of the generated geneset modules most closely corresponded to purely stromal and immune genes, and the results from that module were tested against patient age (Figure 9). This analysis confirmed that tumor stromal E scores are negatively correlated with patients age in both TCGA (r=-0.138; p=0.0038) and CAGEKID (r=-0.26; p=0.013) datasets. Similar analysis using immune E scores corroborated our previous findings on the lack of association between patient age and tumor immune score (r=0.032; p=0.5 for TCGA and r=0.012; p=0.91 for CAGEKID). These results further confirmed that the stromal gene expression signature is indeed negatively associated with patient age.

Figure 9: Patient Age vs. Stromal Eigengene Scores

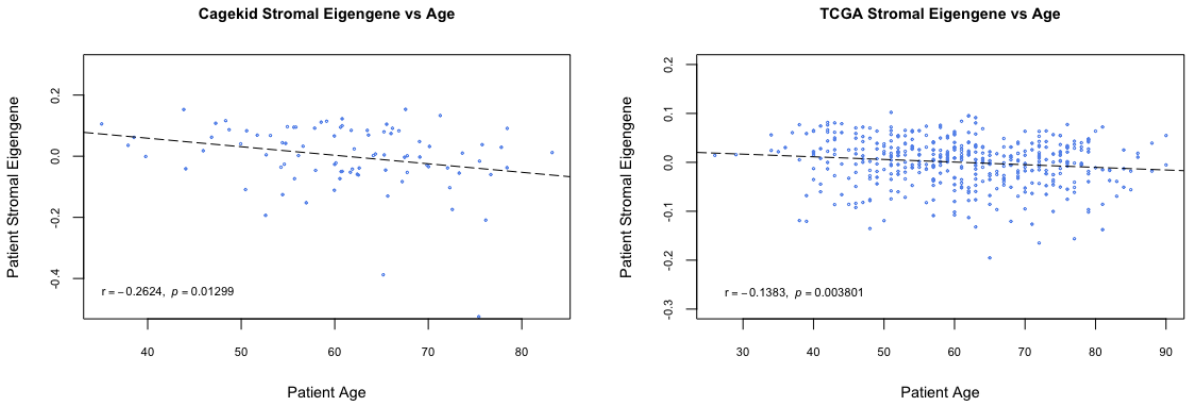
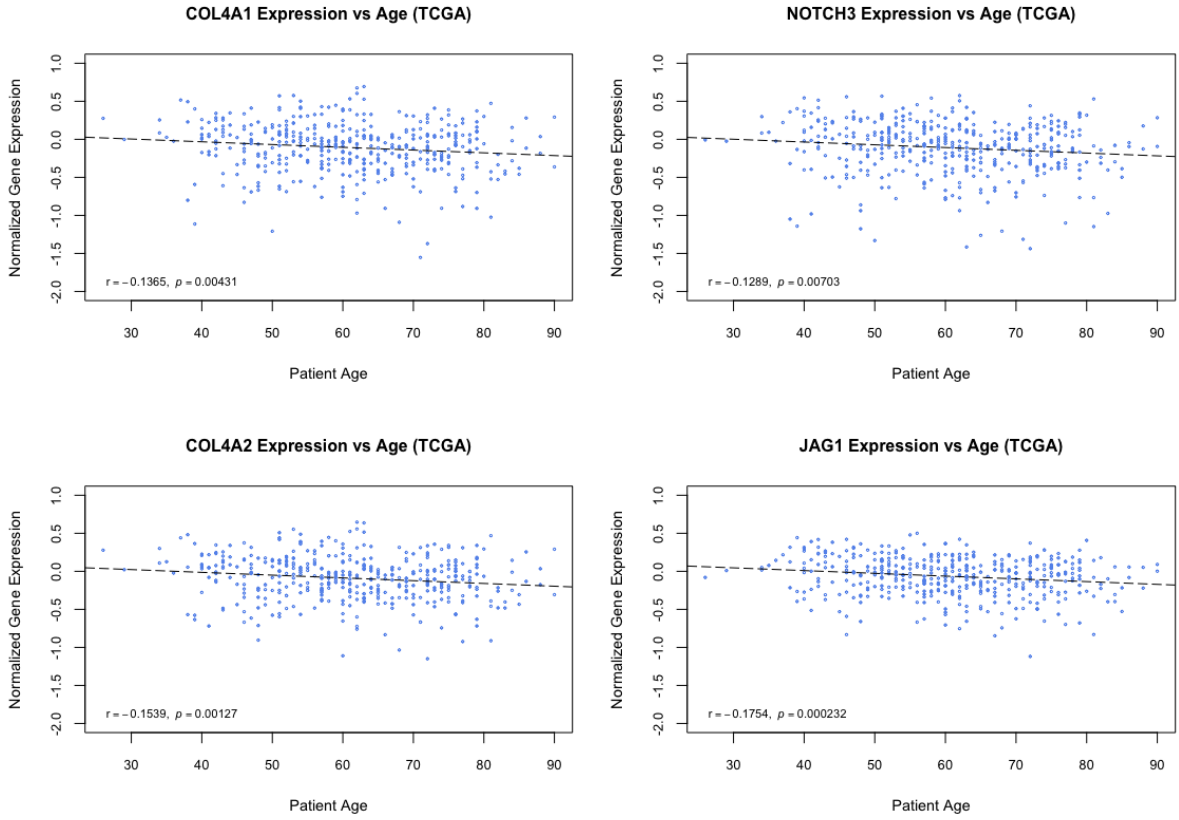


Figure 9: Plots of patient age versus stromal E scores for CAGEKID and TCGA. There is significant correlation between age and stromal score in both datasets, with increased age being associated with lower stromal activity.

This gene expression signature included several genes with previously reported connection to RCC and other cancers. Representative genes in this setting included members of ECM

molecules, especially collagens (COL4A1, COL4A2 and COL18A1), and the members of the Notch/Jagged signaling pathway (NOTCH3, JAG1, DLL1) (Figure 10), many of which are known for their involvement in tumor stromal interactions, angiogenesis and other processes.[5,126,127]

Figure 10: Patient Age vs. ECM/Notch Gene Expression



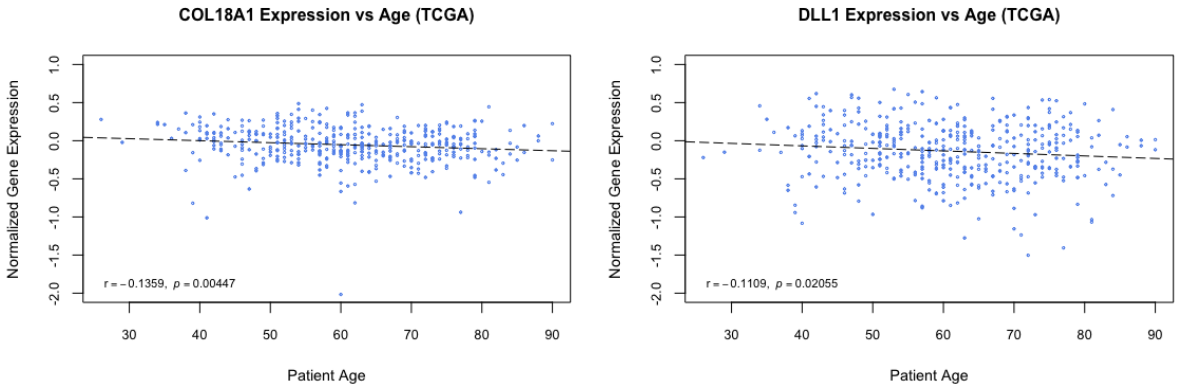


Figure 10: Plots of patient age versus normalized gene expression for COL4A1, COL4A2, COL18A1, NOTCH3, JAG1 and DLL1 (TCGA dataset). Gene expression is significantly downregulated with increased patient age.

3.8 Connectivity Map Analysis of Age-associated Gene Expression

My preceding results demonstrated a reproducible association between age of patients and expression of many genes in ccRCC in two independent data sets. I questioned whether the age-associated gene expression pattern may have a clinical significance by influencing response to drug treatment[7] and by creating new opportunities for drug re-purposing. To address this question, I used the Broad Institute's Connectivity Map (cmap)[128] data sets, which provide information about gene expression patterns in human cell lines as predictors of sensitivity to treatment with different drugs and small molecules. Following the analysis of age-regulated genes in ccRCC against the cmap data set, I identified 32 drugs or small molecules that could be predicted to possess age-dependent anticancer activity (Table 7).

Table 7: Connectivity Map Results of Compounds Impacting RCC Age-Related Genes

Compound	p-value	Function
LY-294002	2.2×10^{-16}	PI3K inhibitor
Prestwick-857	0.00004	Antiprotozoal, antifungal
NS-398	0.00026	COX-2 inhibitor
Esculetin	0.00106	Coumarin
Methotrexate	0.0013	Antimetabolite, chemotherapy agent, DMARD
Atractyloside	0.00366	Toxic glycoside
Doxylamine	0.00471	Antihistamine
5707885	0.00843	--
Gemfibrozil	0.00977	Lowers lipid levels
Phenazone	0.01084	NSAID
Griseofulvin	0.01135	Antifungal
Naphazoline	0.0149	Sympathomimetic vasoconstrictor
Monastrol	0.01498	Mitotic kinesin Eg5 inhibitor
STOCK1N-35874	0.01837	--
Etilefrine	0.01892	Antihypertensive
Novobiocin	0.01954	Antibiotic
Cefamandole	0.02005	Second generation cephalosporin antibiotic
Phenacetin	0.02031	NSAID
Quinpirole	0.02109	D2/D3 dopamine receptor agonist
NU-1025	0.02117	Poly ADP ribose polymerase inhibitor
Ionomycin	0.02293	Raises intracellular calcium levels
Perhexiline	0.02435	Carnitine palmitoylCOA transferase inhibitor
Amitriptyline	0.02741	Tricyclic antidepressant
Chlormezanone	0.02759	Muscle relaxant
Alimemazine	0.02926	Antipruritic, prevents itching
Etoposide	0.03489	Chemotherapy agent
Oxymetazoline	0.03853	Decongestant
Pyrantel	0.04344	Antiparasitic
3-nitropropionic acid	0.04424	Toxin, causes brain lesions
Trifluprom azine	0.0445	Antipsychotic
Cromoglicic acid	0.04778	Mast cell stabilizer
Colecalciferol	0.04824	Vitamin D

Among agents whose corresponding gene signature showed a significant overlap with ccRCC age-associated gene expression patterns, the top-ranked compound was LY-294002 ($p<2.2\text{x}10^{-16}$), a known PI3K inhibitor with anti-RCC activity.[129] Although no data on gene expression patterns of a ccRCC cell line was available in cmap dataset, it was observed that treatment of human prostate adenocarcinoma (PC3) cells with LY-294002 resulted in increased expression of

genes downregulated with patient age and decreased expression of genes upregulated with patient age, suggesting that PI3K inhibitors can alter age-related gene expression patterns in cancer.

3.9 Age-related Gene Expression and Cancer Immunotherapy

Recent studies have indicated that treatments with ICIs may prolong survival in some of the patients affected with ccRCC.[130] However, what defines responders to ICI therapy remains poorly understood. Given that outcomes of such treatments depend at least partly on the extent of immune cells infiltration into the tumor mass,[131] which in turn is influenced by ECM organization, I sought to examine if ccRCC age-associated gene expression (including stromal and ECM genes) show different patterns with regards to response to treatment with ICIs.

Although there was no study reporting on gene expression related to responses to such treatment in ccRCC patients, Hugo *et al.*[132] reported on genes differentially expressed between melanoma patients who responded to ICIs, namely anti-PD1 therapy, and those who did not. Their pathway analysis of the 532 genes over expressed in non-responders showed significant enrichment for cell adhesion, ECM organization and angiogenesis GO terms, similar to the pathways which I had found to contain age-downregulated genes in ccRCC. Therefore, using this dataset I performed pathway analysis comparing genes upregulated in ICI non-responders in melanoma and age-downregulated genes in ccRCC. A strong enrichment for ECM genes was observed on both settings (Figure 11). Moreover, I observed that out of the 532 genes associated with ICI resistance, 69 genes are found among the 383 age-downregulated genes in RCC tumors, showing

a significant overlap between these genesets (4.05 fold-enrichment; $p<2.2x10^{-16}$, Fisher's exact test, Figure 12).

Figure 11: Melanoma and RCC Pathways

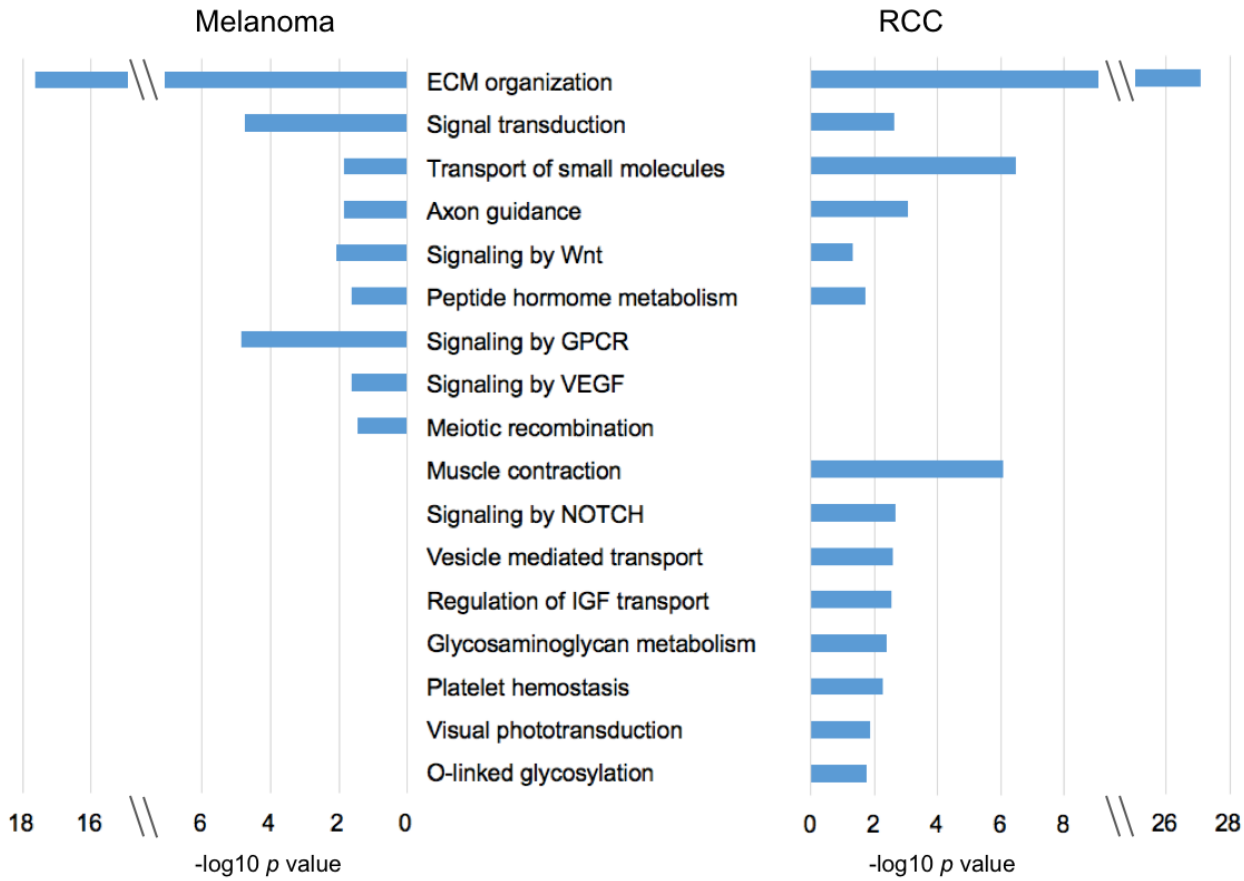
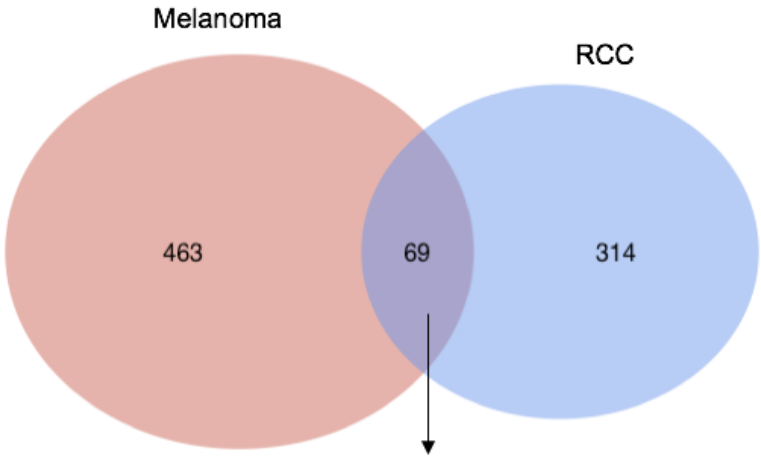


Figure 11: Reactome pathways found significantly enriched in genes upregulated in melanoma nonresponders (Hugo *et al.*) and in genes downregulated with age in RCC. Both sets of genes are particularly enriched for ECM organization.

Figure 12: Overlap of Melanoma and RCC Genes



Enriched Pathways	q-value
ECM organization	5.95×10^{-17}
Axon guidance	6.84×10^{-5}
PI3K-Akt signaling pathway	0.00868
Vesicle-mediated transport	0.0108

Figure 12: Venn diagram of the number of genes significantly upregulated in melanoma nonresponders (Hugo *et al.*) and the number of genes downregulated with age in RCC. 69 genes are found to overlap and are significantly enriched for ECM organization, axon guidance, PI3K-Akt signaling pathway and vesicle-mediated transport.

This result raises the question as to whether older patients might respond better to anti-PD1 therapy than younger patients. When data from the melanoma study was analyzed in this regard, mean and median age was found to be higher in ICI responders, but due to the very small number of patients studied (n=21 for responders and n=17 for non-responders), no statistical analysis to examine the difference was possible. While presently inconclusive, these observations raise the possibility that age of patients may influence the ICI therapy outcome through multiple effects on tumor, stromal and immune cell populations.[133]

3.10 The Impact of Age on the Clinical Course of ccRCC

The aforementioned gene expression studies would be expected to impinge upon age-related clinical characteristics of ccRCC, such as aggressiveness and patient survival. Indeed, my analysis of the data available from the TCGA treatment-naïve ccRCC data set showed a modest, but significant association between patient age and the stage of cancer at the time of presentation (p=0.0051, Mann-Whitney, Figure 13). Thus, patients presenting with stage 3-4 disease where the involvement of lymph nodes and distant organs becomes apparent tended to be older that those with early stage disease. Moreover, in the same cohort, patient age also strongly correlated with poor survival (p=1.88x10^{-5}, logrank test; Figure 14**)**, highlighting the role of age in overall outcomes and in line with previous reports.[134,135]

Figure 13: Patient Age versus RCC Stage (TCGA)

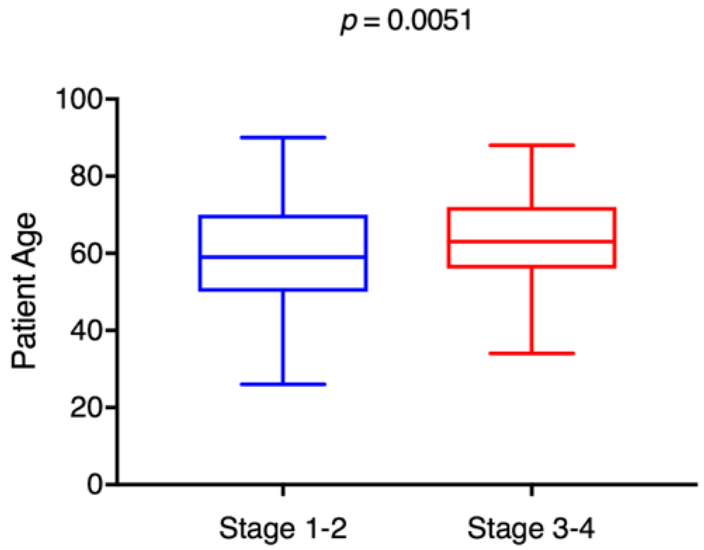


Figure 13: Box plot of patient age versus RCC stage in TCGA dataset. Increased age is significantly associated with higher stage.

Figure 14: Kaplan-Meier Curve of Younger vs Older Patients (TCGA)

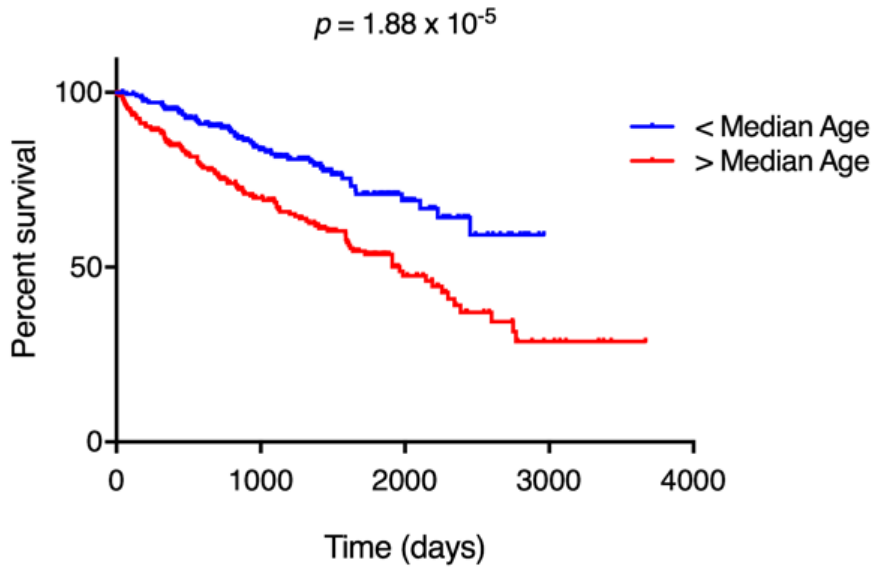


Figure 14: Kaplan-Meier survival curve of younger patients (< median age) versus older patients (> median age) in TCGA dataset. Older age is significantly associated with lower overall survival.

CHAPTER 4: DISCUSSION

This study explored the role of organismal aging in shaping the transcriptome of ccRCC, the most prevalent renal cancer in the human population. In this regard, I made several novel observations. First, using two different large datasets (TCGA and CAGEKID), I uncovered consistent and significant dysregulation of gene expression patterns as a function of patient age. Second, I described age-related dysregulation patterns of genes assigned to (and likely involved in) several major regulatory pathways including regulation of the ECM, stroma, metabolism, oxidation and other networks. Of note, ECM and stromal genes were downregulated in older ccRCC patients while metabolic genes were upregulated, a reversal of the pattern found in normal cells. Third, I established that at least some of these differences were maintained upon adjustment for tumor stage (especially for stage 1). Fourth, I identified age-related differences in genes that are specific to female patients (TNF, Notch pathways). Fifth, I identified *in silico* that a PI3K inhibitor can serve as a putative age-specific agent to target ccRCC. Sixth, using gene co-expression algorithms I obtained preliminary suggestion that age may affect genes involved in cancer immunotherapy, an emerging modality in the treatment of metastatic ccRCC. Seventh, these molecular observations were paralleled by age-related changes in clinical characteristics, such as stage and survival, of ccRCC cases included in TCGA dataset. To the best of my knowledge such a comprehensive analysis of the interrelationship between human life cycle and renal cancer has not been described to date.

My analysis has several implications. Incidence of many human cancers is strongly age-dependent,[3] with specific disease types and molecular subtypes often virtually confined to

pediatric, adult or elderly patient populations.[136-138] The contribution of genetic predisposition, environmental factors, mutational burden, cell senescence, susceptible stem cell pools, chronic inflammatory processes and failing homeostatic mechanisms, such as growth control and immunity, have all been implicated in this diversity.[139] In this regard ccRCC represents an interesting case where histologically and clinically similar disease may occur in patients as young as 20, or as old as 80 years of age (25 to 90 years in the cohort analyzed), which places the time of ccRCC onset over nearly six decades of life. Moreover, the commonality of VHL mutations and the resulting prominent role of hypoxia and angiogenesis pathways in ccRCC strongly link the pathogenesis of this disease with vascular and stromal host tissue responses. Cellular populations contributing to these responses undergo profound changes during the life time of an individual, as exemplified by age-related decline in the proficiency of angiogenesis and immunity.[4,133] As these populations are of great interest as targets of current therapies in ccRCC[140,141] their biological changes due to aging are of considerable practical interest.

My study reflects the impact of these processes on the cancer cell transcriptome. The mechanisms by which aging hosts may influence the transcriptome of the cancer cell population are presently unknown, and while selective analysis of gene expression profiles of tumor-associated stromal cells could be informative, such analyses are presently lacking. However, some inferences could be made from the comparisons between corresponding normal and neoplastic kidney at different ages in ccRCC patients. In my own analysis of normal kidney tissue, functional annotation of genes up-regulated with age revealed enrichment in immune-response pathways, whereas genes down-regulated with age were enriched in oxidative

phosphorylation. These results corroborate previously published data,[142] and reflect on age-associated activity of immune and metabolic pathways in individuals.

In tumor samples, I observed a decrease in stromal gene expression signature and genes involved in ECM organization. Of the genes functionally related to ECM, a large proportion encoded collagen family of proteins (COL). Some of these downregulated genes were also associated with angiogenesis, as might be expected given the role of collagen fragments as angiogenesis inhibitors.[126] Dysregulation of collagens has been found to be actively involved in tumor progression[143] and may control ECM stiffness, mechanosignaling and metastasis.[144] Further exploration of the role of collagens in modulating invasion and metastasis as a function of age is warranted. Among genes related to ECM organization, those mapped to the JAG/Notch pathway were also notably affected. DLL1 expression was previously found to be significantly higher in tumors of younger RCC patients,[5] albeit mainly in precapillary endothelial cells. Future analyses involving single cell sequencing approaches may enable separation of signals from the cancer and stromal cell compartments, including the vasculature.

I observed that genes associated with angiogenesis are downregulated with age in RCC. This is in line with previous reports on the effects of aging on vascular integrity and function,[5] and would suggest that targeted therapies blocking angiogenic pathways may differ in their effects in younger and older patients. Preclinical data suggests that indeed, the effects of sunitinib are greater in old-atherosclerotic mice than in younger animals which can mount a robust mobilization of bone marrow cells associated with resistance to VEGF inhibitors.[7] While clinical efficacy of these agents is thought to be maintained across the age spectrum of ccRCC

patients,[145] clinical trials tend to include relatively few elderly patients making it difficult to evaluate efficacy given limited data. In one attempt, Van den Brom *et al.* found no significant difference in efficacy of anti-angiogenic drugs such as sunitinib and sorafenib among elderly patients, however side effects were generally more prevalent.[146] Since my study documents age-related changes in targets and modulators of therapeutic antiangiogenesis it is possible that, while active, these agents may exert mechanistically different effects and patterns of resistance in patients of different age. Likewise, alternatives to VEGF inhibition, such as elements of the Notch pathway,[127] may also be different in this setting. Further data are needed to address these questions.

Interestingly, my sex-specific analysis revealed exclusive age-downregulation of the Notch pathway in females. As this signal may reflect vascular or stem cell contributions, it is possible that younger females with ccRCC may exhibit different corresponding phenotypes[147] than older patients, with opportunities to develop age/sex-matched therapies. Similarly intriguing is the exclusive age-upregulation of immune system pathways in female tumors. In this light, it is of great interest to assess the sex-related responses to ICIs in female patients of different age, as PD-1/PD-L1 inhibitors firmly enter the therapeutic armamentarium in metastatic ccRCC.[148]

When focusing on stage 1 tumors only, I found increased enrichment of immune-related pathways among age-upregulated genes, similar to my results from analyzing normal kidney tissue samples. This is of great interest as an indication of early involvement of immune response in development of ccRCC, which may be affected by patient age, and later suppressed by immune evasion mechanisms. Indeed, immune-related genes were no longer prominent when

tumors of all stages were included in the analysis, suggesting that a therapeutic 'rescue' of this potential should be considered, and indeed, may be the basis of efficacy in the contexts of ICI treatment in metastatic ccRCC.

With regards to anti-PD1 therapy, the results from Hugo *et al.*'s data indicating greater effectiveness in older patients would need to be validated by a significantly larger study. Given that ccRCC age-dependent alteration in ECM expression might well affect drug efficacy, this is worthy of further investigation. It is also interesting that an agent pertaining to the PI3K pathway was the top hit according to the cmap analysis, since The Cancer Genome Atlas had previously suggested this pathway to be a clinical target.[88] It now appears that PI3K targeting alters age related gene expression patterns in RCC tumors, which may be a key to future effectiveness against RCC.

In section 1.8, I had previously discussed the role of the p53/Rb pathways and INK4a/ARF locus in inducing senescence, a process associated with age. It was therefore of interest to see whether any genes known to be involved in regulating senescence were also age-associated. Among the genes found to be age-downregulated in normal cells was PRODH, a gene directly implicated in senescence as a downstream effector of p53 by Nagano et al[149]. The only other gene found by this study to be directly affected by p53, DAO, was age-upregulated in tumors along with TP53TG1 (TP53 target 1), a long noncoding RNA critical for proper DNA damage response.[150] Given these findings, it would be of interest to further investigate and understand the impact of RCC on age-related patterns of the p53 pathway.

It was not surprising that older age was found to be associated with decreased overall survival. As the TCGA data did not list cause of death, it was unfortunately not possible to measure disease-specific survival. Nor was there information provided on age-related co-morbidities such as cardiovascular disease in either dataset, with the exception of hypertension status (present/not present) in CAGEKID. As noted in section 1.3.8, inflammation can directly affect development of RCC. Atherosclerosis is itself an inflammatory condition in which angiogenesis is promoted.[151,152] Such pre-existing disease could therefore be likely to impact on RCC development and prognosis and potentially alter therapeutic outcomes and should therefore be evaluated in the future.

CHAPTER 5: CONCLUDING REMARKS AND PERSPECTIVES

Overall, my study reveals the impact of age on the molecular repertoire of ccRCC, including alterations in gene expression in normal and cancer tissues. Knowing the impact that ECM organization has on cancer progression, the influence of age particularly found over ECM-related pathways can rightly be considered of potential clinical relevance. Age-related alterations were additionally found in pathways relevant to the hallmarks of cancer previously discussed, including immune system/inflammation, angiogenesis and metabolism, lending further validity to the concept of patient age affecting the development of RCC.

Further exploration is needed to elucidate the relationship between age and ccRCC progression in view of host and tumor cell subsets populating these complex and heterogeneous lesions.[1] While currently existing sequencing data has been of great use, it would be best if single-cell sequencing data could be obtained in order to truly ascertain that the genomic signals are from tumor cells and not surrounding stroma. It is also of interest to separate aging as such from age-related diseases, especially those affecting therapeutic targets in ccRCC such as the vasculature and the immune system. This study did not have this capacity. Thus, further efforts are needed to improve our understanding of ccRCC biology and devise a better, more personalized and more age-appropriate care for this daunting disease. Given that the majority of RCC therapeutic targets are primarily involved in tumor microenvironment (i.e. angiogenesis and immune cells), our finding that stroma, as a major component of tumor microenvironment, is affected by aging raise the prospect that patient's age may be a key determinant of response to current treatments for RCC.

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APPENDIX

Appendix Tables

Table 1a: Genes age-downregulated in both CAGEKID and TCGA regression analyses

Normal Cells	Tumor Cells
A1CF	ABI3BP
AASS	ACAN
ABAT	ACTA2
ABLIM3	ADAMTS2
ACMSD	ADAMTSL2
ACOT7	ADH1B
ACSF2	AEBP1
ACSM2A	AFAP1L2
ACSM2B	AGRN
ACSM5	AJAP1
ACY1	AKAP12
ACY3	ALDH8A1
ADH6	ANGPTL3
ADM2	ANO1
AFM	ANTXR1
AFP	AOC3
AGMAT	APCS
AGXT	APLNR
AGXT2	ARHGAP23
AGXT2L1	ARID5B
ALB	ARL10
ALCAM	ASPN
ALDH1L1	ATP1B2
ALDH2	AUTS2
ALDH4A1	BEX1
ALDOB	BEX4
ALS2CL	BGN
AMN	BMP4
AMOT	BSPRY
ANPEP	C11orf95
AOX1	C1orf172
APLN	C1QTNF3
APLNR	C7
APLP1	C9orf150
APOE	C9orf71
APOH	CACNA1H
APOM	CACNB2
ARHGAP23	CAMK1G
ARHGAP28	CCDC3
ARSF	CCDC80
ASB15	CCL18
ASL	CCL21
ASPDH	CD93
ASPG	CDHR2

ASS1	CECR1
ATP10A	CENPV
ATP1B2	CGN
AUTS2	CHST1
AZGP1	CLEC18A
BAIAP2L2	CLEC18B
BHMT	CLEC18C
BMP7	CLIC6
C10orf116	CMTM8
C10orf125	CMYA5
C14orf37	CNN1
C17orf61	COL12A1
C19orf69	COL14A1
C1QL1	COL15A1
C2orf54	COL16A1
C5orf27	COL18A1
C6orf115	COL1A1
C8orf80	COL1A2
CACHD1	COL21A1
CACNA2D1	COL3A1
CACNB2	COL4A1
CALB1	COL4A2
CAPN12	COL4A3
CCBL1	COL4A4
CCDC68	COL5A1
CCL15	COL5A2
CD248	COL6A1
CDC14A	COL6A2
CDH6	COL6A3
CDHR5	COLEC12
CES3	CPXM1
CETP	CRISPLD2
CGREF1	CRMP1
CHI3L1	CSDC2
CHRDL1	CTGF
CISH	CTSK
CLDN2	CYBRD1
CLEC18A	CYP39A1
CLEC18B	DAAM2
CLEC18C	DACT1
CLIC5	DACT3
CMYA5	DCDC2
COCH	DCHS1
COL4A3	DCN
COL6A1	DES
COLEC11	DLL1
COTL1	DLX5
CPN2	DMKN
CPNE6	DOCK9
CPXM1	DPEP1
CRABP1	DSP
CRB2	DTX3
CRHBP	EBF1
CRIPAK	ECM1
CRYAA	ECM2

CRYBB3	EDIL3
CTSL2	EDNRA
CUBN	EFNB2
CYP17A1	EHD3
CYP2B6	ELFN1
CYP2C8	ELN
CYP3A7	EMILIN1
CYP4A22	EPAS1
CYP4F2	EPHA4
CYP4F3	F2RL3
DAB2	F3
DACH1	F5
DAO	FAM178A
DCXR	FAM83D
DDC	FBLIM1
DDN	FBLN2
DEPDC1B	FBLN5
DEPDC7	FBLN7
DHDH	FBN1
DHRS3	FBXO32
DHRS4L2	FGF1
DIO1	FGF13
DNAJC12	FHL3
DNMT3L	FHL5
DOK6	FILIP1
DPEP1	FMO3
DPYS	FMOD
DTX4	FN1
DUSP2	FOXC1
EHD3	FRAS1
EMCN	FRMD4A
ENO3	FRY
ENOSF1	FST
ENPEP	FXYD1
ENPP6	FZD7
ERRFI1	G6PC
ESPN	GEM
ETNK2	GGT5
EYA2	GJC1
F2RL3	GLT8D2
F3	GOLGA8A
FABP1	GPC3
FAM132A	GPR124
FAM151A	GPRASP1
FAM24B	HAVCR2
FAM40B	HEG1
FAM83D	HEYL
FAM86B1	HMCN1
FCGR3B	HPGD
FCN3	HRC
FERMT1	HSPB6
FGF1	HSPB7
FGFR4	HSPG2
FLT4	IGF2
FN3K	IGFBP5

FUT6	INHA
FYN	INHBA
GAS2	INPP4B
GATSL3	IRF6
GCAT	ISLR
GDA	ITGA11
GGTLC2	ITGA8
GIMAP8	ITGB1BP3
GJB2	ITGBL1
GLIS1	ITPR3
GLYAT	JAG1
GLYATL1	KAL1
GOLIM4	KANK2
GPD1	KCNE4
GPT	KIAA0240
GPX3	KIRREL
GRB10	KLF12
GSTA1	KLF5
GSTA2	KLF7
GSTT2	KRT19
HAAO	LAD1
HAO2	LATS1
HAVCR2	LCN12
HBA1	LEFTY1
HBA2	LEPREL2
HBB	LHFP
HECW1	LILRB5
HIST1H2BG	LINGO1
HMOX1	LMOD1
HNF1A	LOXL2
HNF4A	LRRC10B
HPD	LRRC17
HSD17B14	LTBP1
HTRA1	LTBP2
IGF2BP2	LTBP4
IL13RA2	LTF
IL1RL1	LUM
IL22RA1	LYVE1
IP6K3	MACF1
IQSEC2	MAP1B
IRS2	MAP9
ISM1	MARVELD3
ITGA8	MCF2L
IYD	MDK
KCNAB2	MEST
KCNH6	MFAP2
KDR	MFAP4
KHK	MFGE8
KL	MIB1
KLF15	MICAL2
KLHL3	MLANA
KLK1	MLL3
KLK6	MMP2
KLK7	MOXD1
KMO	MRC1

LBP	MRVI1
LGALS2	MST1P9
LMX1B	MTSS1L
LOX	MXRA5
LRP2	MYH10
MAF	MYH11
MAPT	MYH8
MEG3	MYL9
MIOX	MYO1D
MLXIPL	MYO7B
MME	MYSM1
MPST	NAV1
MT1F	NEIL1
MT1G	NES
MT1H	NEURL1B
MTSS1L	NEXN
MYH8	NFASC
MYLK4	NID2
NAT8	NOG
NCCRP1	NOTCH2
NECAB2	NOTCH3
NEK6	NPR2
NES	NR2F1
NFASC	NR2F2
NPFF	NRIP2
NPHS1	NTRK2
NPL	NYNRIN
NPY6R	OBSL1
NR1H3	OLFML1
NTNG1	OLFML2A
NXNL2	OLFML2B
NXPH2	OR2A7
OCEL1	OR2T10
OLFML2A	PALLD
OLR1	PARM1
OPN1SW	PAWR
OXER1	PBX1
PAH	PCOLCE
PARD6G	PDE3A
PC	PDE5A
PCOLCE2	PDGFRB
PCTP	PDLIM4
PDE10A	PDZRN3
PDE7A	PELI2
PDLIM2	PHGDH
PDPN	PIGR
PDZD3	PKHD1
PDZK1IP1	PLAGL1
PHYHIP	PLAT
PIPOX	PLCL1
PKLR	PLEKHH1
PLA2G12B	PLEKHH2
PLA2R1	PLG
PLCE1	PLN
PLCH2	PODN

PLG	PODXL
PM20D1	POSTN
PNPLA1	PPAPDC3
PODXL	PPP1R12B
POSTN	PRELP
POU5F1	PRKAR1B
PPP1R14D	PRKG1
PRAP1	PRKY
PRLR	PRND
PRODH2	PROM1
PRR5	PRR15L
PRX	PRRX1
PSORS1C3	PTGIR
PTGDS	PTK7
PTH1R	PTN
PTPRB	PTPRB
PTPRD	PTPRG
PTPRO	PTPRN2
RAB11FIP3	PVRL1
RAB3IL1	PYGM
RASSF4	QPCT
RBP4	RAMP1
RBP5	RAPH1
RDH5	RASAL2
REEP6	RBP4
REN	REM1
RGS7	RGS5
RIN3	RNF144A
RNF186	RNF180
SARDH	RNF183
SCRN2	RNF212
SEMA5A	RNF38
SERPINA4	ROBO1
SERPINC1	RPS2P32
SERPINF2	SATB1
SH3BP2	SCARF2
SLC12A3	SCD5
SLC13A2	SCG5
SLC13A3	SCGB2A1
SLC16A9	SDR42E1
SLC17A4	SEMA3G
SLC22A13	SEMA5A
SLC22A18AS	SFRP1
SLC22A3	SFRP2
SLC22A6	SGCA
SLC22A7	SGIP1
SLC22A8	SH3PXD2A
SLC23A1	SLAIN1
SLC25A45	SLC14A1
SLC26A4	SLC22A2
SLC26A6	SLC29A2
SLC26A9	SLC30A2
SLC28A1	SLC34A1
SLC2A2	SLC35E2
SLC30A2	SLC41A3

SLC30A8	SLC44A4
SLC34A1	SLC6A1
SLC34A3	SLC9A3
SLC36A2	SLIT3
SLC5A10	SMOC2
SLC5A11	SNED1
SLC5A12	SORBS3
SLC5A2	SOX7
SLC5A9	SRPX
SLC6A18	SSC5D
SLC6A19	SST
SLC7A13	STAC2
SLC7A7	SULF1
SLC7A8	SUSD1
SLC7A9	SVIL
SLC8A1	SYNE2
SLC9A3R1	SYNPO2
SLIT2	SYT9
SMTN	TAGLN
SNHG9	TBX3
SOBP	TCEAL7
SOST	TCF21
SPARC	TEK
SPOCK1	TEKT2
SPOCK2	TF
SREBF1	TGFB2
ST3GAL1	THBS2
ST3GAL6	TIMP2
ST6GALNAC3	TM4SF4
STRA6	TMC4
SULF1	TMEM119
SUSD2	TMEM178
SUSD3	TMEM25
SYNPO	TMEM47
TBXA2R	TMEM98
TCEAL2	TMOD2
TCL6	TMPRSS3
TFEC	TNC
TGFBR3	TRIM63
THPO	TRNP1
TINAG	TRO
TM6SF2	TSPAN7
TMEM130	TSPYL5
TMEM150A	TUB
TMEM174	UGT3A1
TMEM52	UMOD
TMEM82	VIL1
TMEM86B	VIP
TMSB15A	WASF1
TNFRSF25	WFDC1
TNNI1	ZFP28
TNNT2	ZIK1
TPCN1	ZMIZ1
TRIM14	ZNF192
TRIM50	ZNF385B

TRIM6	ZNF404
TRIM63	ZNF407
TSKU	ZNF418
TSPAN2	ZNF462
TST	ZNF471
TTC36	ZNF577
TTTY14	ZNF703
TUBA4B	ZNF711
TUBAL3	ZNF780B
TYRO3	
UGT1A9	
UGT2A1	
UGT2B7	
ULK4	
UPB1	
UPP2	
USH1C	
USP9Y	
VDR	
VEGFA	
VSIG2	
VSIG8	
WFDC1	
WNK1	
WT1	
XPNPEP2	
XYLB	
ZNF814	

Table 1b: Genes age-upregulated in both CAGEKID and TCGA regression analyses

Normal Cells	Tumor Cells
ABCA3	ABHD6
ACTG2	ACSM2A
ADAMTS1	ACSM2B
ADAMTS15	ACSM5
ADH1B	ACY1
ADH1C	ADI1
AEBP1	ADRB2
AEN	ADSSL1
AGFG2	AEN
AGR2	AGMAT
AJAP1	AGXT2
AKR1B1	AIFM1
AKR1C1	AIMP2
AKR1C2	AKR1B10
ALOX5	ANGPTL4
ALOX5AP	AP1M2
AMBP	APOBEC3H
AMICA1	AQP7
ANGPTL2	AQP9
ANGPTL4	ARG2
ANXA1	ARHGEF37
ANXA3	ASL
AOAH	ATP8B3
APCDD1	AZGP1
AQP2	B3GNT4
AQP6	BAIAP3
ARL4C	BCL7B
ASPHD2	BEX5
ATHL1	BIRC3
B4GALNT2	BNIP1
B4GALT5	C10orf128
BAK1	C11orf75
BCL2A1	C11orf86
BCL6	C12orf44
BIN2	C12orf62
BIRC3	C17orf107
C10orf47	C17orf89
C12orf34	C19orf24
C1orf114	C19orf70
C1orf162	C1orf170
C1orf216	C1orf53
C1orf38	C1QL1
C1QA	C5orf46
C1QB	C7orf30
C1QC	C8orf22
C1QTNF3	C9orf66
C1R	CABP1
C1S	CCDC150
C3AR1	CCDC24
C6orf105	CCDC64B
C7	CCDC91

C7orf29	CCL17
C9orf167	CCL2
CALCA	CCL28
CAPN6	CCL3
CARD6	CD70
CASP1	CDK2AP2
CASP4	CFB
CCDC109B	CFD
CCDC3	CISD3
CCL11	CITED4
CCL19	CKMT2
CCL2	CLEC2B
CCL21	CNFN
CCL23	COL9A2
CCL3	CPN2
CCL4	CREB3L3
CCL4L2	CRYAA
CCL5	CRYM
CCND2	CTH
CD14	CTXN3
CD163	CX3CR1
CD1C	CXCL1
CD2	CXCL10
CD27	CXCL11
CD300A	CXCL5
CD36	CYP24A1
CD37	DAO
CD3D	DCLK1
CD3E	DCXR
CD48	DDC
CD52	DECR2
CD53	DGCR6
CD69	DNAJB1
CD79A	DNAJC12
CD79B	DPYS
CD82	EFCAB4A
CD86	EFNA5
CDKN1A	EIF4EBP1
CDR2L	ELF3
CFHR1	ENPEP
CH25H	ETHE1
CHST9	ETV7
CLDN14	FABP6
CLDN3	FABP7
CLDN4	FAM131C
CLDN7	FAM151A
CLEC10A	FAM158A
CLIP2	FAM173A
CLU	FAM195A
CNN1	FAM20A
COL14A1	FAM26F
COL16A1	FBXL21
CORO1A	FCGR1B
CP	FCGR3A
CPA3	FCGR3B

CPE	FCRL6
CPVL	FGFBP2
CSF1R	FGG
CST7	FICD
CTHRC1	FSTL3
CTSK	GC
CTSS	GCHFR
CXCL16	GCNT3
CXCL2	GCSH
CXCL9	GDA
CYBB	GDPD1
DARC	GLRX
DDB2	GLYAT
DDIT4L	GOLT1A
DEGS2	GPD1
DNASE1	GPT
DOCK11	GPX4
DOK2	GSTA1
DTX1	GSTA2
EHF	GZMH
ELF3	HAGH
ELF5	HAGHL
EMP1	HAPLN1
ENO2	HLA-DRB6
ERAP2	HMBS
EVI2A	HOXD9
EVI2B	HP
F10	HPD
F2RL1	HPR
FAM109B	HRCT1
FAM26F	HSD3B7
FAM57A	HSPB1
FAM83F	HSPB8
FAS	IDO1
FBLIM1	IFI27L2
FBLN2	IGJ
FBXO2	IL1B
FCER1A	IL1R2
FCER1G	IL1RL1
FCGBP	IL20RB
FES	IL27RA
FGL2	IL32
FHL2	IL4I1
FIBIN	IL6
FMO2	IRF1
FMOD	ISOC2
FNDC4	KCNK1
FOLR2	KCNN1
FOXQ1	KIAA1324L
FPR3	KISS1R
FXYD3	KRT80
GABRP	LAGE3
GALNT3	LBP
GBP2	LENG9
GCNT3	LGALS4

GGT6	LIN7B
GLIPR2	LIX1
GNAZ	LOX
GNE	LRG1
GPIHBP1	LYG1
GPM6B	LYRM1
GPR110	MANEAL
GPR143	MAOA
GZMA	MAPK12
GZMH	MARCO
GZMK	MEGF6
HABP2	MEOX2
HCK	METRN
HCLS1	MGST1
HCST	MLYCD
HGF	MMRN1
HIF1A	MRPL41
HIGD1B	NCAM1
HLA-DMB	NDUFA4L2
HLA-DPA1	NDUFS6
HLA-DPB1	NECAB2
HLA-DQA1	NECAB3
HLA-DQB1	NEGR1
HLA-DRA	NFE2L3
HLA-DRB6	NGEF
HN1	NME4
HOPX	NOVA1
HS6ST1	NPTX2
HSD11B1	NR1H4
HSPA7	NRN1
HSPB6	NUDT8
HUNK	NUPR1
ID1	P2RX4
IFI27L2	PAH
IFITM1	PARD6A
IGJ	PFKFB4
IGSF6	PGF
IKBKE	PI3
IL10RA	PLA2G12B
IL1R2	PLEKHF1
IL27RA	PLIN2
IL2RG	PNCK
IL34	POF1B
INPP5D	PPAP2C
IQCA1	PPFIA3
IRAK2	PPP1R14D
IRF5	PPP1R1A
IRF8	PPP1R3C
ITGB4	PRAME
ITGB6	PRDX4
KCNJ5	PRELID1
KCNJ8	PSMG3
KCNK13	PSORS1C1
KDELR3	PTHLH
KLF5	PXMP2

KLHL13	PYCRL
KLRB1	RAB7L1
KRT18	RAC3
LAIR1	RARRES2
LAMB3	RBP5
LAMC2	RCAN1
LAPTM5	REPS2
LAT2	RGS14
LCK	RGS7
LCN2	RHBDL1
LCP1	RHOD
LGALS9	RILP
LGI2	RNASET2
LIPG	RPL13P5
LIPH	RTN2
LIX1	RTN4RL1
LMOD1	RUNDC3A
LOXL4	S100A8
LRG1	S100A9
LSP1	SAA1
LST1	SCNN1B
LTB	SEC11C
LTBP1	SERPINF2
LTF	SERTAD1
LUM	SFN
LY86	SLC17A3
LY96	SLC22A18AS
LYPD6B	SLC25A20
LYZ	SLC2A2
MACC1	SLC2A5
MANEAL	SLC5A1
MFI2	SLITRK4
MMP7	SNAP25
MNS1	SNCG
MOXD1	SNORD17
MPEG1	SNTA1
MRAS	SNX10
MRPS6	SOD2
MS4A4A	SPAG1
MS4A6A	SPAG4
MSR1	SPINK5
MTHFD1L	SPOCK1
MYOF	SPR
NCF2	SRA1
NCKAP1L	SSSCA1
NFATC4	ST20
NIPAL1	TCEB2
NNMT	TEX11
NOV	TFPI2
NUPR1	THAP7
ORAI2	THRSP
PAQR8	TLCD1
PDE6G	TMEM160
PFKFB3	TMEM19
PGM5	TMEM27

PITPNM1	TMEM54
PLAU	TNFRSF12A
PLCB1	TNFSF14
PLEK	TNNT1
PLEKHB1	TP53TG1
PLK3	TRAF4
PPAP2C	TRIB3
PPL	TRIM54
PRELP	TRPC2
PROM1	TRPV4
PRSS22	TTLL6
PTAFR	TUBA1C
PTGS1	TUBA3D
PYCARD	TUBA3E
QPCT	UBL4A
RAC2	UGT1A6
RAMP1	UGT1A9
RANBP3L	UGT2B7
RAP2B	UPB1
RARRES1	VEPH1
RASAL1	VKORC1
RASD1	ZNF323
RCAN3	ZNF688
REG1A	
RELB	
RGS1	
RGS16	
RGS19	
RGS2	
RHOD	
RHOV	
RNASE6	
RNF144B	
RNF24	
RRAD	
RTP4	
S100A1	
S100A13	
S100A14	
S100A3	
S100A4	
S100A6	
SAMSN1	
SASH3	
SCARA3	
SCCPDH	
SCGB1D2	
SCGB2A1	
SDCBP2	
SECTM1	
SELE	
SEMA4A	
SERP2	
SERPINA3	
SFN	

SFRP1 SFRP2 SFTA2 SGPP2 SLC14A1 SLC14A2 SLC16A5 SLC38A1 SLC38A4 SLC43A3 SLC4A7 SLC5A8 SLC6A12 SLC9A3 SLCO4A1 SLPI SMAGP SOCS3 SOX9 SP140L SPARCL1 SPI1 SRPX2 STK17A SULT2B1 SYT11 SYTL2 SYTL4 TAC1 TACSTD2 TBC1D16 TES TESC TFF3 THNSL2 TM4SF1 TMC6 TMEM125 TMEM54 TMPRSS4 TNC TNFAIP8 TNFAIP8L2 TNFRSF11B TNFRSF12A TNFRSF1B TPSAB1 TPSB2 TREM2 TRIM22 TRIM55 TTC39A TTC9 TUBA1A TYROBP	

UBD UCP2 UPP1 VAV1 VCAM1 VCAN VGLL1 VMO1 VSIG4 VTCN1 VWF ZNF486 ZNF853 ZNF883	

Table 2a: Age-Downregulated Pathways - Normal Cells

q-value	Pathway	Source
8.45E-08	Metabolism	Reactome
3.48E-07	SLC-mediated transmembrane transport	Reactome
3.83E-07	Biological oxidations	Reactome
8.46E-07	Retinol metabolism - Homo sapiens	KEGG
7.15E-06	Conjugation of carboxylic acids	Reactome
7.15E-06	Amino Acid conjugation	Reactome
7.15E-06	Conjugation of salicylate with glycine	Reactome
4.12E-05	Transport of inorganic cations/anions and amino acids/oligopeptides	Reactome
4.38E-05	Transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds	Reactome
8.28E-05	Drug metabolism - cytochrome P450 - Homo sapiens	KEGG
0.000126	Mineral absorption - Homo sapiens	KEGG
0.000142	Pentose and glucuronate interconversions - Homo sapiens	KEGG
0.000375	Transmembrane transport of small molecules	Reactome
0.000453	Histidine, lysine, phenylalanine, tyrosine, proline and tryptophan catabolism	Reactome
0.000472	Metabolism of xenobiotics by cytochrome P450 - Homo sapiens	KEGG
0.000500	Protein digestion and absorption - Homo sapiens	KEGG
0.000650	Chemical carcinogenesis - Homo sapiens	KEGG
0.000937	Phase II conjugation	Reactome
0.001568	Conjugation of benzoate with glycine	Reactome
0.001568	Amine-derived hormones	Reactome
0.001655	FOXA2 and FOXA3 transcription factor networks	PID
0.001733	Maturity onset diabetes of the young - Homo sapiens	KEGG
0.001733	Fatty acids	Reactome
0.002052	Ascorbate and aldarate metabolism - Homo sapiens	KEGG
0.002280	Alanine, aspartate and glutamate metabolism - Homo sapiens	KEGG
0.002756	Binding and Uptake of Ligands by Scavenger Receptors	Reactome
0.002756	Scavenging of heme from plasma	Reactome
0.002756	Multifunctional anion exchangers	Reactome
0.002953	Amino acid transport across the plasma membrane	Reactome
0.003582	Synthesis of Leukotrienes (LT) and Eoxins (EX)	Reactome
0.004379	Arachidonic acid metabolism - Homo sapiens	KEGG
0.004856	Cytochrome P450 - arranged by substrate type	Reactome
0.004856	Endocrine and other factor-regulated calcium reabsorption - Homo sapiens	KEGG
0.004856	Glycine, serine and threonine metabolism - Homo sapiens	KEGG
0.005753	Renin-angiotensin system - Homo sapiens	KEGG
0.005753	HDL-mediated lipid transport	Reactome
0.005866	Pyrimidine catabolism	Reactome
0.007538	Tryptophan metabolism - Homo sapiens	KEGG
0.007565	Organic anion transport	Reactome
0.008257	Phenylalanine metabolism - Homo sapiens	KEGG
0.009682	Arachidonic acid metabolism	Reactome
0.010224	Drug metabolism - other enzymes - Homo sapiens	KEGG
0.010661	Phase 1 - Functionalization of compounds	Reactome
0.010661	Metabolism of Angiotensinogen to Angiotensins	Reactome
0.010661	Organic cation/anion/zwitterion transport	Reactome
0.011262	Metabolism of amino acids and derivatives	Reactome
0.011449	VEGF binds to VEGFR leading to receptor dimerization	Reactome
0.011449	VEGF ligand-receptor interactions	Reactome
0.011449	Inositol transporters	Reactome
0.011449	Miscellaneous substrates	Reactome

0.011691	Peptide hormone metabolism	Reactome
0.011958	Amino acid and oligopeptide SLC transporters	Reactome
0.013613	Visual phototransduction	Reactome
0.013948	The retinoid cycle in cones (daylight vision)	Reactome
0.013948	CYP2E1 reactions	Reactome
0.013948	Thyroxine biosynthesis	Reactome
0.015963	Arginine biosynthesis - Homo sapiens	KEGG
0.016199	VEGF and VEGFR signaling network	PID
0.016199	Erythrocytes take up oxygen and release carbon dioxide	Reactome
0.016199	Eicosanoids	Reactome
0.022353	Steroid hormone biosynthesis - Homo sapiens	KEGG
0.022469	Lipoprotein metabolism	Reactome
0.023949	Tryptophan catabolism	Reactome
0.029947	Lipid digestion, mobilization, and transport	Reactome
0.030241	Aflatoxin activation and detoxification	Reactome
0.031370	Xenobiotics	Reactome
0.031370	Erythrocytes take up carbon dioxide and release oxygen	Reactome
0.031370	O2/CO2 exchange in erythrocytes	Reactome
0.031370	Metallothioneins bind metals	Reactome
0.031370	Response to metal ions	Reactome
0.031674	Sodium-coupled sulphate, di- and tri-carboxylate transporters	Reactome
0.031674	Type II Na+/Pi cotransporters	Reactome
0.033535	Basigin interactions	Reactome
0.041135	Hemoglobins chaperone	BioCarta
0.047237	Tyrosine metabolism - Homo sapiens	KEGG
0.047237	Steroid hormones	Reactome
0.047237	Sialic acid metabolism	Reactome

Table 2b: Age-Downregulated Pathways - Tumor Cells

q-value	Pathway	Source
8.24E-28	Extracellular matrix organization	Reactome
4.21E-21	Collagen chain trimerization	Reactome
1.73E-18	Collagen biosynthesis and modifying enzymes	Reactome
4.85E-16	Protein digestion and absorption - Homo sapiens	KEGG
7.61E-16	Collagen formation	Reactome
3.08E-13	Beta1 integrin cell surface interactions	PID
4.36E-10	ECM-receptor interaction - Homo sapiens	KEGG
1.64E-09	NCAM1 interactions	Reactome
3.24E-09	Integrins in angiogenesis	PID
1.05E-08	Syndecan-1-mediated signaling events	PID
1.40E-08	Elastic fibre formation	Reactome
2.91E-08	Integrin cell surface interactions	Reactome
5.91E-08	Molecules associated with elastic fibres	Reactome
5.91E-08	Assembly of collagen fibrils and other multimeric structures	Reactome
8.67E-07	Muscle contraction	Reactome
2.85E-06	Beta3 integrin cell surface interactions	PID
4.72E-06	ECM proteoglycans	Reactome
8.52E-05	Scavenging by Class A Receptors	Reactome
8.55E-05	Degradation of the extracellular matrix	Reactome
0.000223	Focal adhesion - Homo sapiens	KEGG
0.000339	NOTCH2 Activation and Transmission of Signal to the Nucleus	Reactome
0.000339	Collagen degradation	Reactome
0.000549	Regulators of bone mineralization	BioCarta
0.000883	Axon guidance	Reactome
0.000963	Vascular smooth muscle contraction - Homo sapiens	KEGG
0.001221	NCAM signaling for neurite out-growth	Reactome
0.001381	Glycosaminoglycan metabolism	Reactome
0.001391	intrinsic prothrombin activation pathway	BioCarta
0.001391	Activation of Matrix Metalloproteinases	Reactome
0.001531	AGE-RAGE signaling pathway in diabetic complications - Homo sapiens	KEGG
0.002111	Signaling by NOTCH2	Reactome
0.002218	Developmental Biology	Reactome
0.002274	A tetrasaccharide linker sequence is required for GAG synthesis	Reactome
0.002452	Signal Transduction	Reactome
0.002559	Amoebiasis - Homo sapiens	KEGG
0.002985	Regulation of Insulin-like Growth Factor (IGF) transport and uptake by Insulin-like Growth Factor Binding Proteins (IGFBPs)	Reactome
0.003393	PI3K-Akt signaling pathway - Homo sapiens	KEGG
0.004333	Keratan sulfate biosynthesis	Reactome
0.004699	Cardiac conduction	Reactome
0.005531	cGMP effects	Reactome
0.006000	Binding and Uptake of Ligands by Scavenger Receptors	Reactome
0.006862	Receptor-ligand binding initiates the second proteolytic cleavage of Notch receptor	Reactome
0.006862	Constitutive Signaling by NOTCH1 HD Domain Mutants	Reactome
0.006862	Signaling by NOTCH1 HD Domain Mutants in Cancer	Reactome
0.008919	Amine compound SLC transporters	Reactome
0.009215	Dilated cardiomyopathy - Homo sapiens	KEGG
0.009359	TGF-beta signaling pathway - Homo sapiens	KEGG
0.009718	Keratan sulfate/keratin metabolism	Reactome
0.010647	Nitric oxide stimulates guanylate cyclase	Reactome

0.011152	Syndecan-4-mediated signaling events	PID
0.011949	Signaling by PDGF	Reactome
0.012790	RHO GTPases Activate ROCKs	Reactome
0.014595	Notch signaling pathway	PID
0.016846	Smooth Muscle Contraction	Reactome
0.018718	O-glycosylation of TSR domain-containing proteins	Reactome
0.019658	Glycoprotein hormones	Reactome
0.019658	Peptide hormone biosynthesis	Reactome
0.023872	Arrhythmogenic right ventricular cardiomyopathy (ARVC) - Homo sapiens	KEGG
0.025215	Proteoglycans in cancer - Homo sapiens	KEGG
0.027716	Signaling by NOTCH3	Reactome
0.028020	Hypertrophic cardiomyopathy (HCM) - Homo sapiens	KEGG
0.029046	RHO GTPases activate PAKs	Reactome
0.032647	Heparan sulfate/heparin (HS-GAG) metabolism	Reactome
0.032736	Striated Muscle Contraction	Reactome
0.032736	cGMP-PKG signaling pathway - Homo sapiens	KEGG
0.032736	Signaling by NOTCH	Reactome
0.032736	Antagonism of Activin by Follistatin	Reactome
0.033774	Complement and coagulation cascades - Homo sapiens	KEGG
0.035316	Activated NOTCH1 Transmits Signal to the Nucleus	Reactome
0.035316	Retinoid metabolism and transport	Reactome
0.035316	Breast cancer - Homo sapiens	KEGG
0.040343	Chondroitin sulfate/dermatan sulfate metabolism	Reactome
0.045581	amb2 Integrin signaling	PID
0.047078	Pre-NOTCH Processing in the Endoplasmic Reticulum	Reactome
0.047078	Platelet amyloid precursor protein pathway	BioCarta
0.047078	Negative regulation of TCF-dependent signaling by WNT ligand antagonists	Reactome
0.049469	Actions of nitric oxide in the heart	BioCarta

Table 2c: Age-Upregulated Pathways - Normal Cells

q-value	Pathway	Source
1.21E-08	Cytokine-cytokine receptor interaction - Homo sapiens	KEGG
1.21E-08	Staphylococcus aureus infection - Homo sapiens	KEGG
1.21E-08	Immune System	Reactome
5.10E-08	Hematopoietic cell lineage - Homo sapiens	KEGG
8.90E-08	Asthma - Homo sapiens	KEGG
5.18E-07	Translocation of ZAP-70 to Immunological synapse	Reactome
3.50E-06	PD-1 signaling	Reactome
3.50E-06	Phosphorylation of CD3 and TCR zeta chains	Reactome
4.92E-06	Systemic lupus erythematosus - Homo sapiens	KEGG
4.92E-06	Classical antibody-mediated complement activation	Reactome
4.92E-06	Immunoregulatory interactions between a Lymphoid and a non-Lymphoid cell	Reactome
7.08E-06	Type I diabetes mellitus - Homo sapiens	KEGG
7.23E-06	Cell adhesion molecules (CAMs) - Homo sapiens	KEGG
1.35E-05	Peptide ligand-binding receptors	Reactome
1.41E-05	Autoimmune thyroid disease - Homo sapiens	KEGG
1.41E-05	Graft-versus-host disease - Homo sapiens	KEGG
1.41E-05	Allograft rejection - Homo sapiens	KEGG
1.92E-05	Classical complement pathway	BioCarta
1.92E-05	Complement and coagulation cascades - Homo sapiens	KEGG
1.92E-05	NF-kappa B signaling pathway - Homo sapiens	KEGG
2.70E-05	Generation of second messenger molecules	Reactome
3.44E-05	Class A/1 (Rhodopsin-like receptors)	Reactome
3.87E-05	G alpha (i) signalling events	Reactome
3.98E-05	IL12-mediated signaling events	PID
3.98E-05	Creation of C4 and C2 activators	Reactome
5.40E-05	GPCR downstream signaling	Reactome
5.68E-05	Rheumatoid arthritis - Homo sapiens	KEGG
6.18E-05	Complement cascade	Reactome
0.000102	Innate Immune System	Reactome
0.000105	Downstream TCR signaling	Reactome
0.000131	GPCR ligand binding	Reactome
0.000131	Extracellular matrix organization	Reactome
0.000177	Chemokine signaling pathway - Homo sapiens	KEGG
0.000314	Intestinal immune network for IgA production - Homo sapiens	KEGG
0.000320	Chemokine receptors bind chemokines	Reactome
0.000470	Th17 cell differentiation - Homo sapiens	KEGG
0.000489	The co-stimulatory signal during t-cell activation	BioCarta
0.000489	Direct p53 effectors	PID
0.000489	Phagosome - Homo sapiens	KEGG
0.000489	TNFR2 non-canonical NF-kB pathway	Reactome
0.000523	Costimulation by the CD28 family	Reactome
0.000549	IL4-mediated signaling events	PID
0.000692	Pertussis - Homo sapiens	KEGG
0.000750	Adaptive Immune System	Reactome
0.000751	Activation of csk by camp-dependent protein kinase inhibits signaling through the t cell receptor	BioCarta
0.000866	TCR signaling	Reactome
0.000866	Degradation of the extracellular matrix	Reactome
0.001046	Initial triggering of complement	Reactome
0.001640	lck and fyn tyrosine kinases in initiation of tcr activation	BioCarta

0.001640	Toll-like receptor signaling pathway - Homo sapiens	KEGG
0.001644	Th1 and Th2 cell differentiation - Homo sapiens	KEGG
0.002029	Viral myocarditis - Homo sapiens	KEGG
0.002444	Osteoclast differentiation - Homo sapiens	KEGG
0.002741	Collagen formation	Reactome
0.002886	Toxoplasmosis - Homo sapiens	KEGG
0.002886	Inflammatory bowel disease (IBD) - Homo sapiens	KEGG
0.002984	HTLV-I infection - Homo sapiens	KEGG
0.003657	Antimicrobial peptides	Reactome
0.003848	Activation of pkc through g-protein coupled receptors	BioCarta
0.003848	Assembly of collagen fibrils and other multimeric structures	Reactome
0.003923	Tuberculosis - Homo sapiens	KEGG
0.004030	Signaling by GPCR	Reactome
0.004370	Influenza A - Homo sapiens	KEGG
0.004370	Primary immunodeficiency - Homo sapiens	KEGG
0.004370	African trypanosomiasis - Homo sapiens	KEGG
0.004370	CXCR4-mediated signaling events	PID
0.004476	Transcriptional misregulation in cancer - Homo sapiens	KEGG
0.005017	Cytokine Signaling in Immune system	Reactome
0.006075	MHC class II antigen presentation	Reactome
0.006164	Malaria - Homo sapiens	KEGG
0.006980	Neutrophil degranulation	Reactome
0.007007	Regulation of TLR by endogenous ligand	Reactome
0.007447	Chagas disease (American trypanosomiasis) - Homo sapiens	KEGG
0.011710	The AIM2 inflammasome	Reactome
0.012783	Eicosanoid metabolism	BioCarta
0.013456	Cross-presentation of particulate exogenous antigens (phagosomes)	Reactome
0.013456	Type I hemidesmosome assembly	Reactome
0.013456	Formyl peptide receptors bind formyl peptides and many other ligands	Reactome
0.014092	Cell junction organization	Reactome
0.017296	Antigen processing and presentation - Homo sapiens	KEGG
0.017296	Prion diseases - Homo sapiens	KEGG
0.017296	NOD-like receptor signaling pathway - Homo sapiens	KEGG
0.019503	TNFs bind their physiological receptors	Reactome
0.019503	Collagen degradation	Reactome
0.020662	TCR signaling in naive CD4+ T cells	PID
0.022632	Validated targets of C-MYC transcriptional repression	PID
0.022632	ECM-receptor interaction - Homo sapiens	KEGG
0.023604	IL12 signaling mediated by STAT4	PID
0.025545	CD28 dependent Vav1 pathway	Reactome
0.025545	RHO GTPases Activate NADPH Oxidases	Reactome
0.025545	Alpha6 beta4 integrin-ligand interactions	PID
0.025545	Pertussis toxin-insensitive ccr5 signaling in macrophage	BioCarta
0.025545	G-protein signaling through tubby proteins	BioCarta
0.025550	Antigen processing-Cross presentation	Reactome
0.025550	Fc epsilon RI signaling pathway - Homo sapiens	KEGG
0.025550	Leishmaniasis - Homo sapiens	KEGG
0.026104	Anchoring fibril formation	Reactome
0.027954	Natural killer cell mediated cytotoxicity - Homo sapiens	KEGG
0.027966	ECM proteoglycans	Reactome
0.030459	Leukocyte transendothelial migration - Homo sapiens	KEGG
0.030578	Cytosolic DNA-sensing pathway - Homo sapiens	KEGG
0.030578	Keratan sulfate biosynthesis	Reactome
0.030578	IL23-mediated signaling events	PID
0.030578	Corticosteroids and cardioprotection	BioCarta

0.031392	TNF receptor superfamily (TNFSF) members mediating non-canonical NF-kB pathway	Reactome
0.036985	T cell receptor signaling pathway	BioCarta
0.037874	p73 transcription factor network	PID
0.038991	G alpha (q) signalling events	Reactome
0.042360	Role of mef2d in t-cell apoptosis	BioCarta
0.043656	Synthesis of Lipoxins (LX)	Reactome
0.043656	Terminal pathway of complement	Reactome
0.043656	CD22 mediated BCR regulation	Reactome
0.043656	Metal sequestration by antimicrobial proteins	Reactome
0.044865	Cell-Cell communication	Reactome
0.047246	Tight junction interactions	Reactome
0.047246	TNF signaling pathway - Homo sapiens	KEGG
0.048729	Activation of IRF3/IRF7 mediated by TBK1/IKK epsilon	Reactome

Table 2d: Age-Upregulated Pathways - Tumor Cells

q-value	Pathway	Source
0.000106	Phenylalanine metabolism - Homo sapiens	KEGG
0.000106	Cytokine-cytokine receptor interaction - Homo sapiens	KEGG
0.000106	Metabolism	Reactome
0.000106	Conjugation of carboxylic acids	Reactome
0.000106	Amino Acid conjugation	Reactome
0.000106	Conjugation of salicylate with glycine	Reactome
0.000214	Biological oxidations	Reactome
0.000223	Chemokine receptors bind chemokines	Reactome
0.000256	Phase II conjugation	Reactome
0.000870	Peptide ligand-binding receptors	Reactome
0.000905	Drug metabolism - cytochrome P450 - Homo sapiens	KEGG
0.000968	Metabolism of amino acids and derivatives	Reactome
0.001597	Pentose and glucuronate interconversions - Homo sapiens	KEGG
0.005680	Metabolism of xenobiotics by cytochrome P450 - Homo sapiens	KEGG
0.005811	Arginine and proline metabolism - Homo sapiens	KEGG
0.005811	Chemical carcinogenesis - Homo sapiens	KEGG
0.005964	Arginine biosynthesis - Homo sapiens	KEGG
0.005964	Regulation of TLR by endogenous ligand	Reactome
0.006470	Class A/1 (Rhodopsin-like receptors)	Reactome
0.008164	Drug metabolism - other enzymes - Homo sapiens	KEGG
0.010033	Transport of glycerol from adipocytes to the liver by Aquaporins	Reactome
0.010033	G alpha (i) signalling events	Reactome
0.010033	Alanine, aspartate and glutamate metabolism - Homo sapiens	KEGG
0.011661	TNF signaling pathway - Homo sapiens	KEGG
0.012298	Histidine, lysine, phenylalanine, tyrosine, proline and tryptophan catabolism	Reactome
0.012298	GPCR ligand binding	Reactome
0.014405	Phenylalanine and tyrosine catabolism	Reactome
0.014405	Pyrimidine catabolism	Reactome
0.015952	Tyrosine metabolism - Homo sapiens	KEGG
0.016310	Glycine, serine and threonine metabolism - Homo sapiens	KEGG
0.018251	Glucuronidation	Reactome
0.019621	Metal sequestration by antimicrobial proteins	Reactome
0.019621	Conjugation of benzoate with glycine	Reactome
0.019765	African trypanosomiasis - Homo sapiens	KEGG
0.021218	Salmonella infection - Homo sapiens	KEGG
0.021218	Norepinephrine Neurotransmitter Release Cycle	Reactome
0.021567	IL23-mediated signaling events	PID
0.026318	TNF receptor superfamily (TNFSF) members mediating non-canonical NF-kB pathway	Reactome
0.029015	Metabolism of nucleotides	Reactome
0.029015	Passive transport by Aquaporins	Reactome
0.029015	Alternative complement activation	Reactome
0.029015	Phenylalanine, tyrosine and tryptophan biosynthesis - Homo sapiens	KEGG
0.029015	Dopamine Neurotransmitter Release Cycle	Reactome
0.029015	Ascorbate and aldarate metabolism - Homo sapiens	KEGG
0.029803	GPCR downstream signaling	Reactome
0.031158	Toll-like receptor signaling pathway - Homo sapiens	KEGG
0.031158	Porphyrin and chlorophyll metabolism - Homo sapiens	KEGG
0.033910	Metabolism of polyamines	Reactome
0.033910	Neurotransmitter Release Cycle	Reactome
0.033910	Staphylococcus aureus infection - Homo sapiens	KEGG

0.033910	Rheumatoid arthritis - Homo sapiens	KEGG
0.036270	Tryptophan metabolism - Homo sapiens	KEGG
0.036270	downregulated of mta-3 in er-negative breast tumors	BioCarta
0.036270	Post-chaperonin tubulin folding pathway	Reactome
0.036593	Amoebiasis - Homo sapiens	KEGG
0.037300	Vitamin D (calciferol) metabolism	Reactome
0.042663	Metabolism of fat-soluble vitamins	Reactome
0.047846	IL27-mediated signaling events	PID
0.047846	Metabolism of porphyrins	Reactome
0.049718	Chemokine signaling pathway - Homo sapiens	KEGG
0.049718	TNFR2 non-canonical NF-kB pathway	Reactome
0.049718	Urea cycle	Reactome

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