Key Molecular Alteration in The PI3K/Akt mTOR Pathway among Egyptian Women With Early Breast Cancer : A Clinico-Molecular Study

Thesis Submitted for Partial Fulfillment of MD Degree in clinical oncology

Presented by

Loay Mohammad Hassan Kassem

M.Sc. Clinical oncology - Cairo University

Under Supervision of

Prof. Dr. Hamdy Abd ELAzim

Professor of Clinical Oncology

Faculty of Medicine-Cairo University

Prof. Dr. Mona Ahmed Abu El Enein

Professor of Clinical Oncology

Faculty of Medicine-Cairo University

Prof. Dr. Thomas Bachelot

Professor of Medical Oncology

Faculty of Medicine-Lyon 1 University

Dr. Shady Elia Anis

Lecturer of Pathology

Faculty of Medicine-Cairo University

Faculty of Medicine

Cairo University

2015

Table of contents

Acknowledgement

First of all, thanks to **ALLAH** the most beneficial and merciful for helping me to complete this work.

I would like to express my deepest gratitude to **my wife and my family** who have helped and inspired me in every step of my work.

I would like to express my sincere appreciation to **Prof. Dr. Hamdy Abd El Azim**, Professor of clinical oncology, Cairo University, for his generous support and guidance to help me to complete this work. It was indeed an honor to work under his supervision.

It is my pleasure to express my unlimited gratitude and deepest thanks to **Prof. Dr.Mona Abuelainin,** Professor of clinical oncology , Cairo University, for her continuous supervision and vast experience she offered me to complete this study. No words of gratitude can equal her help and support.

I am also deeply grateful to **Dr Shady Anis**, lecturer of pathology, Cairo University for his understanding, enthusiasm and assistance to produce this project in the way it is done.

I also wish to thank **Prof. Dr. Thomas Bachelot,** Professor of medical oncology, Centre Léon Bérard for his sincere guidance and his great help that made my work in CLB extremely enjoyable and fruitful.

I also wish to thank **Prof. Dr. Isabelle Treilleux**, Professor of pathology, Centre Léon Bérard, for her valuable advice and personal generosity.

I would also like to acknowledge the great efforts of **Prof. Qing Wang**, Professor of biology, Centre Léon Bérard who helped me develop new ideas within and outside the scope of this work.

I am extremely grateful to all my **friends and colleagues** in the department of clinical oncology, Cairo University for the encouraging and supporting attitude

Also, thanks to my patients who were included in my study for whom this work was carried out.

List of Abbreviations

TOR	Target of rapamycin
mTOR	Mammalian target of rapamycin
PI3K	phosphoinositide 3-kinase
RTKs	receptor tyrosine kinases
GPCRs	G protein coupled receptors
PIP2	phosphatidylinositol diphosphate
PIP3	phosphatidylinositol triphosphate
FOXOs	the forkhead box family of transcription factors
PRAS40	Proline rich AKT substrate 40 kDa
TSC2	tuberous sclerosis 2 protein
PKB	protein kinase B
Raptor	regulatory-associated protein of mTOR
PRAS40	Proline rich AKT substrate 40 kDa
Deptor	DEP-domain-containing mTOR-interacting protein
S6K	S6 kinase
4E-BP1	eIF4Ebinding protein 1
mLST8	mammalian lethal with Sec13 protein 8
Rictor	rapamycin-insensitive companion of mTOR
mSIN1	mammalian stress-activated protein kinase interacting protein
Protor-1	protein observed with Rictor-1
PTEN	phosphatase and tensin homologue
GAP	GTPase-activating protein
Rheb	Ras homolog enriched in brain
eIF4E	the eukaryotic initiation factor 4E
S6K1	p70 ribosomal S6 kinase 1
4E-BP1	the eukaryotic initiation factor 4E (eIF4E)-binding protein 1
PDCD4	programmed cell death 4
eEF2K	eukaryotic elongation factor 2 kinase
PP2A	protein phosphatase 2A
TIF-IA	transcription initiation factor IA
SREBP1	sterol regulatory element binding protein 1 and
PPARg	peroxisome proliferator-activated receptor-g
AMPK	AMP-activated protein kinase

ULK1	unc-51-like kinase 1
ATG13	autophagy-related gene 13
FIP200	focal adhesion kinase family-interacting protein of 200 kDa
IKKβ	The inhibitor of κB kinase-β
HIF-1α	Hypoxia inducible factor α
VEGF	Vascular endothelial growth factor
IGF	Insulin like growth factor
IGFR	Insulin like growth factor receptor
RSK1	P90 ribosomal S6 kinase 1
IRS	Insulin receptor substrate
AMPK	Adenosine monophosphate-activated protein kinase
LKB	Liver Kinase B1
REDD1	Regulated in development and DNA damage responses 1
GTPases	Guanosine triphosphatases
GRB10	Growth factor receptor-bound protein 10
MAPK	Mitogen-activated protein kinase
MNKs	Mitogen- interacting kinases
RCC	Renal cell carcinoma
RI-mTORC1	Rapamycin insensitive mTORC1
ERK	Extracellular signal-regulated kinases
TMA	Tissue micro-array
CR	Complete response
SD	Stable disease
PR	Partial response
HR	Hormone receptor
AI	Aromatase inhibitor
PFS	Progression free survival
OS	Overall survival
CBR	Clinical benefit rate

List of Figures

List of Tables

Abstract

Background

The PI3K/AKT/mTOR pathway alterations have significant roles in the development, progression and metastatic potential of breast cancer in addition to enhancing resistance to many of the drugs used to control breast cancer, specially anti hormonal therapies. In this study we aimed to define the correlation between the PI3K mutations and the expression of the phosphorylated forms of different downstream molecules of this pathway in the samples of Egyptian patients with luminal breast cancer.

Methods

Next generation sequencing was used to detect mutations in the PIK3CA hotspots (in exons 10 and 21). Immunohistochemistry (IHC) was performed on TMA blocks prepared from samples of 35 operable luminal (ER positive and HER2 negative) breast cancer patients who presented for postoperative treatment at Cairo University hospitals between 2007 and 2011. The intensity and the percentage of stained tumor cells were taken into consideration in defining high versus low biomarker expression. The cytoplasmic and nuclear stainings were graded separately. Correlation was done between PI3K mutations and the IHC expression of pAKT, LKB1, p4EBP1 and pS6 ribosomal protein (pS6RP) expression with the clinico-pathologic parameters using Pearson's chi-square test. Kaplan-Meier (KM) method was used to estimate disease free survival (DFS) and the difference between the subgroups was evaluated with log-rank test.

Results

Thirty two cases were assessable for LKB1 and pAKT, 33 for p4EBP1 and pS6RP and 24 were assessed for PI3K mutations. Median age at diagnosis was 51.3 years (range: 25 to 82 years). Tumors were larger than 20 mm in 79.2% and 57.9% had axillary lymph node (LN) metastasis. Only 3.5% of the patients had SBR grade I tumors, 71.9% grade II tumors and 24.6% grade III tumors. Estrogen receptors (ER) were found to be negative in 6 patients after central pathology review. Nuclear LKB1, cytoplasmic LKB1, nuclear pAKT, cytoplasmic pAKT, nuclear p4EBP1 and cytoplasmic pS6RP expression was high in 65.6%, 62.5%, 62.5%, 68.8%, 42.4% and 57.6% respectively. PIK3CA mutations were found in 7 patients (29.2%).

PI3K mutations were correlated with decreased cytoplasmic pAKT (p=0.04) and increased nuclear pAKT expression (p=0.10). There was a tendency towards an inverse correlation between PI3K mutations and the expression of pS6RP (p=0.10) and p4EBP1 (p=0.19).
Nuclear LKB1 expression in tumor cells was a marker of good prognosis. It was associated with smaller tumors (p=0.05), more ER positivity (p=0.08) and PR positivity (p=0.002). In the KM model patients with high nuclear LKB1 had longer DFS (HR=0.36; 95%CI: 0.15-1.10; p=0.08). Nuclear pAKT high expression also carried a tendency towards longer DFS (HR=0.51; 95%CI: 0.11-1.16; p=0.13). The expression of p4EBP1, pS6RP and the PI3K mutational status didn't carry any prognostic significance in early breast cancer patients.

Conclusion

Among the studied biomarkers only nuclear expression of LKB1 and pAKT tended to predict better survival in breast cancer patients. PI3K mutation was correlated with the expression of the downstream molecules

Keywords:

(Breast cancer, PI3K/AKT/mTOR, Hormonal treatment)

Review Of Literature

PI3K/AKT/mTOR pathway and breast cancer

Introduction

In 1975, an antibiotic metabolite produced by Streptomyces hygroscopicus bacteria was discovered in the soil of Rapa Nui Island in the South Pacific. It was named "rapamycin" after its place of discovery (Vezina et al., 1975). In addition to its antibacterial and antifungal activity, rapamycin was also proved to have an immunosuppressive effect (Martel et al., 1977). In the 1990s molecular studies have discovered the target of rapamycin (TOR) and in 1994; the mammalian analogue was identified (mTOR) (Brown et al., 1994). Rapamycin was subsequently approved for the prophylaxis of rejection after renal transplantation, as it blocks the signal transduction pathways required for the activation of the helper T cells and that opened the way for researchers in different fields trying to show us the different functions of mTOR in various physiological and pathological conditions. The mTOR pathway integrates signals from nutrients, energy status and extracellular growth factors to regulate many processes, including cell cycle progression, angiogenesis, ribosome biogenesis, and metabolism. (Azim et al., 2010, Laplante and Sabatini, 2009).

Growth and proliferation of normal and malignant cells is mediated through the binding of certain growth factors (as insulin-like growth factor, epidermal growth factor and vascular endothelial growth factor) to their trans-membrane receptors and activating specific enzymes called tyrosine kinases. Those tyrosine kinases, through series of phosphorylation cascades including phosphoinositide 3-kinase (PI3K)/AKT induce signal transduction to the nucleus. Activated AKT can promote proliferation and growth via several mechanisms, one of the most important is the mTOR activation. MTOR can regulate (through its downstream effectors) cellular nutrient metabolism, angiogenesis, ribosome biogenesis, cell growth, survival and proliferation. (Foster and Fingar, 2010, Azim et al., 2010)

Several studies have shown a connection between mTOR deregulation and different types of malignancies and in different stages of

carcinogenesis (Zoncu et al., 2011, Fruman and Rommel, 2014). As a result of such discoveries, the PI3K/AKT/mTOR axis was recognized as an important target for anti-cancer therapies and many new drugs inhibiting this pathway were introduced to cancer care in the last decades. At the moment, mTOR inhibitors are approved for treatment of advanced RCC, advanced HR-positive breast cancer (combined with everolimus) and advanced neuroendocrine tumors (Kunz et al., 2013, Ciruelos et al., 2013, Escudier et al., 2012). Ongoing trials are testing different inhibitors of PI3K/mTOR in almost all cancer types. However, the therapeutic benefit of mTOR inhibitors in the clinical setting is still limited by the absence of predictive markers for response in addition to the added toxicity. In the next chapters we will discuss the PI3K/AKT/mTOR signaling, mechanisms of mTOR activation, the structure of the mTOR complex and its physiological functions, the role of the PI3K/AKT/mTOR pathway in breast cancer and various clinical trials testing mTOR inhibitors in advanced breast cancer in addition to the efforts done to determine molecular biomarkers predictive of response.

Chapter 1

The PI3K/AKT/MTOR upstream activators

The main mechanism by which mTOR is activated is through the PI3K activation. PI3Ks family has 3 classes, the most important of which is the class I enzymes which are activated by the growth factors receptors (Katso et al., 2001). Class I PI3Ks are further classified to class IA enzymes, which are activated by receptor tyrosine kinases (RTKs), G protein coupled receptors (GPCRs) and RAS, and class IB enzymes, which are regulated only by GPCRs.

Class IA PI3Ks consist of a p110 catalytic subunit and a p85 regulatory subunit (FIG. 2a). The regulatory subunit (p85) mediates receptor binding, activation, and localization of the enzyme to the cell membrane in response to growth factor binding. The activated p110 catalytic subunit phosphorylates phosphatidylinositol diphosphate (PIP2) to phosphatidylinositol triphosphate (PIP3) which is responsible for AKT activation.

AKT (also known as protein kinase B (PKB)) is a serine-threonine protein kinase that has three isoforms: AKT1, AKT2 and AKT3. AKT activation is mediated by PIP3 that recruits AKT by translocating it to the plasma membrane. The resulting conformational change in AKT exposes two crucial amino‐acid residues for phosphorylation (Stephens et al., 1998, Liu et al., 2009a). Activated AKT is responsible for phosphorylation of many proteins such as mTORC1, glycogen synthase kinase 3 and FOXOs (the forkhead box family of transcription factors) (Manning and Cantley, 2007).

AKT can then activate mTOR by phosphorylating both Proline rich AKT substrate 40 kDa (PRAS40) and tuberous sclerosis 2 protein (TSC2) to attenuate their inhibitory effects on mTOR (Inoki et al., 2002).

MTOR structure and organization

MTORs are a family of large (290 kDa, 2549 amino acids) serine-threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)

related kinase super-family. The mTOR signaling receives inputs from five major intracellular and extracellular stimuli which are: growth factors, stress, energy status, oxygen, and amino acids. Different interactions with these stimuli regulate many major cellular processes, including protein and lipid synthesis, proliferation and autophagy. MTOR has 2 multi-protein complexes: mTORC1 and mTORC2. Figure 1 shows the signals affecting mTORC1 &2 and their functions.

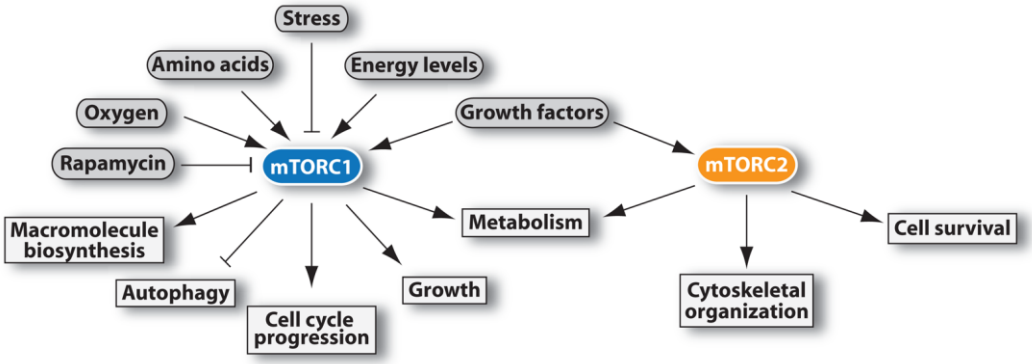

Figure 1: Different signals leading to mTOR activation and its results.(Laplante and Sabatini, 2012)

MTORC1 has five components: mTOR, which is the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8, also known as GbL); prolinerich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) (Peterson et al., 2009). RAPTOR and PRAS40 are specific to mTORC1, while the rest are shared with mTORC2 (Zoncu et al., 2011).

Raptor, a 150 KDa protein is responsible for recruiting the downstream substrates of mTOR: p70 S6 kinase (S6K) and the translational repressor eIF4Ebinding protein 1 (4E-BP1) leading to stimulation of cell growth and proliferation (Beauchamp and Platanias, 2013). PRAS40 and Deptor are negative regulators of mTORC1 (Peterson et al., 2009). The role of mLST8 in the mTOR signaling is still unknown.

MTORC2 consists of 6 different components: mTOR; rapamycin-insensitive companion of mTOR (Rictor); mammalian stress-activated protein kinase interacting protein (mSIN1); protein observed with Rictor-1 (Protor-1); mLST8; and Deptor. MTORC2 was originally thought to be insensitive to rapamycin (Sarbassov et al., 2004, Jacinto et al., 2004).

However, some cell types showed inhibition of mTORC2 signaling with long term treatment with rapamycin. There is evidence that mTORC2 plays a role in stabilizing the cytoskeletal structure of the cell which determines the cell shape and knockdown of rictor resulted in the loss of actin polymerization and cell spreading (Sarbassov et al., 2004, Jacinto et al., 2004).

MTORC2 signaling is insensitive to nutrients, but responds to growth factors as insulin via a PI3K dependent mechanism that is not clearlu understood (Laplante and Sabatini, 2013). One potential mechanism involves a novel role for ribosomes, as ribosomes are needed for mTORC2 activation and mTORC2 binds them in a PI3K-dependent fashion (Laplante and Sabatini, 2013, Zinzalla et al., 2011).

MTORC2 can also regulate cell metabolism, survival, apoptosis, growth and proliferation by direct phosphorylation of AKT on Ser473 (Sarbassov et al., 2005). Similar to its role in mTORC1, Deptor negatively regulates mTORC2 activity (Peterson et al., 2009).

Down regulators of the mTOR

The tumor suppressor PTEN (phosphatase and tensin homologue) is the most important negative regulator of the PI3K signaling pathway. PTEN opposes the effect of PI3K by dephosphorylating PIP3 back into PIP2 and hence, reducing the intracellular levels of PIP3, decreasing AKT activation and balancing the cellular processes initiated by the PI3K signaling as proliferation and angiogenesis. Loss of PTEN results in unrestrained signaling by the PI3K pathway, leading to cancer (Cully et al., 2006).

One of the most important upstream regulators of this pathway comes through tuberous sclerosis 1 (TSC1) and TSC2 which acts as a GTPase-activating protein (GAP) for the Ras homolog enriched in brain (Rheb) GTPase. The GTP-bound form of Rheb directly interacts with mTORC1 and strongly stimulates its kinase activity. As a Rheb GAP, TSC1/2 negatively regulates mTORC1 by converting Rheb into its inactive GDP-bound state (Inoki et al., 2003, Tee et al., 2003).