INTRODUCTION

Liver cancer represents 6% and 9% of the global cancer incidence and mortality burden, respectively. It is the second most common cause of cancer related deaths worldwide, the fifth most common cancer in men (554 000 new cases, 8% of the total) and the ninth most common in women (228 000 cases, 3% of the total) ***(Stewart and Wild, 2014).***

In Egypt, liver cancer is the fourth most common cancer and is the second cause of cancer mortality in both sexes ***(Ferlay et al., 2010).***

Most early lesions are recognized in established cirrhosis since mature hepatocellular carcinoma usually follows the development of such late-stage disease ***(Park, 2011)***.

The incidence and mortality rates for HCC are virtually identical, reflecting the poor overall survival rates for patients with this kind of tumor. Most therapies are only effective if HCC is diagnosed at early stages ***(Bruix and Sherman, 2011)***.

Prospective screening, where possible, takes two forms: assessment of serum markers and repeated radiological examination. Radiological examination in the course of late-stage liver disease is informative when possible.In contrast, the most widely used serum marker is α-fetoprotein (AFP) which is ineffective for early lesions ***(El-Serag, 2011)*** because it is criticized as neither sensitive nor specific for HCC, active hepatitis and liver

cirrhosis ***(Wong et al., 2014).*** AFP has a reported sensitivity of 39% to 65% and a specificity of 65% to 94%; approximately one-third of early-stage HCC patients with small tumors (<3 cm) have normal AFP levels ***(Wang et al., 2012a)***. Therefore, it highlights the need for new early detection biomarkers more useful and accurate for HCC ***(Lokman et al., 2011).***

Currently, numerous tissue and circulating markers have been identified; however, few biomarkers are acceptable for clinical utility because of their low predictive accuracy and/or high cost ***(Bai et al., 2009).***

Recently, much attention has been directed to Autophagy related biomarkers as potential diagnostic and prognostic factors for cancer. Autophagy is a survival-promoting pathway that captures, degrades and recycles intracellular proteins and organelles in lysosomes. Autophagy preserves organelle function, prevents the toxic buildup of cellular waste products and provides substrates to sustain metabolism in starvation ***(White, 2015).***

The role of autophagy in cancer biology is unequivocal. While basal (constitutive) autophagy prevents carcinogenesis through the constant elimination of damaged molecules and organelles that may increase the probability of oxidative stress mediated DNA mutation ***(Mathew et al., 2007a),*** induced autophagy can help cancer cells to face adverse situations such as metabolic stress due to hypoxia and hyponutrition or the

damage provoked by anticancer treatment ***(Semenza, 2011; Mathew et al., 2009).***

A group of autophagy-related genes (Atg) demonstrated by gene silencing studies are essential for the process of autophagy. Impaired autophagy causes diverse pathological conditions in human, including liver dysfunction and tumorigenesis ***(Dalby et al., 2010)***.

The actual level of ongoing autophagy in tumour cells is dictated by genetic mutations but also influenced by the epigenetic regulation of gene expression ***(Morani et al., 2013).***

Less than 2% of the mammalian genome can code for protein synthesis and thus called protein coding genes while over 90% of the genome represents noncoding RNA (ncRNA) which are transcribed but do not encode for protein synthesis ***(Li and Wang, 2012).***

In general, ncRNA can be divided into two classes based on their length: short/small ncRNA and long ncRNA. Although comparatively less understood in human cancers, increasing studies indicated that long non coding RNAs(LncRNAs) play active roles in modulating the cancer epigenome and may be important targets for cancer diagnosis and therapy ***(Gupta et al., 2010).***

LncRNAs are arbitrarily defined as transcripts of more than 200 nucleotides in length that lack significant open reading

frames (ORF) and can be localized to both the nucleus and the cytoplasm ***(Van Heesch et al., 2014)***. Often, a significant increase or decrease in LncRNA expression levels is found in tumors compared to normal tissues. Some types of LncRNAs are demonstrated to be present in body fluids, like urine and plasma, which can be easily obtained by the least invasive methods. RNA may be packaged into microparticles, including exosomes, microvesicles, apoptotic bodies and autophagosomes, thus secreted into body fluids. Given this specificity and good accessibility, LncRNAs may be superior biomarkers than many current protein-coding biomarkers.

As for HCC, some LncRNAs may be useful as novel potential biomarkers for diagnosis, prognosis and prediction of response to therapy ***(Huang et al., 2013).***

AIM OF THE WORK

- To retrieve a LncRNA and a selected target autophagy related gene involved in HCC development from public microarray databases.
- To evaluate their usefulness as molecular biomarkers for early HCC detection.
- To explore their relation to different clinico-pathological factors of HCC.

Chapter One

AUTOPHAGY

Autophagy is the major intracellular degradation system by which cytoplasmic materials are delivered to and degraded in the lysosome. However, the purpose of autophagy is not the simple elimination of materials, but instead autophagy serves as a dynamic recycling system that produces new building blocks and energy for cellular renovation. Thus it's considered the cellular strategy for maintaining proteostasis and responding to nutrient stress ***(Mizushima and Komatsu, 2011; Mizushima et al., 2011; Bankaitis, 2015).***

Mechanism of Autophagy:

The autophagic pathway begins with the sequestration of organelles and portions of the cytoplasm via a double-membrane termed the isolation membrane (or phagophore) which can be derived from several cellular compartments including the endoplasmic reticulum [ER], Golgi complex, ER-Golgi intermediate compartment [ERGIC], mitochondria, or ER-mitochondria associated membranes [MAMs] as well as the plasma membrane ***(Tooze and Yoshimori, 2010)***. The isolation membrane expands to completely envelop the isolated contents in a double-membrane vesicle called the autophagosome which then undergoes maturation through fusion with lysosomes to form autolysosomes where

lysosomal enzymes degrade the contents within the autolysosome ***(Eskelinen, 2005; Lennemann and Coyne, 2015).***

Types of autophagy:

There are roughly three classes of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy in mammalian cells ***(Glick et al., 2010)***.

- ***Macroautophagy:***

Macroautophagy is the main autophagic pathway that uses the intermediate organelle "autophagosome" where an isolation membrane (also termed phagophore) sequesters a small portion of the cytoplasm including soluble materials and organelles to form the autophagosome. The autophagosome then fuses with the lysosome to become an autolysosome ***(Mizushima and Komatsu, 2011).*** A hallmark of macroautophagy is the autophagic flux in which lysosomal enzymes degrade the contents within the autolysosome. Alternatively, early/late endosomes can fuse with autophagosomes forming amphisomes that can then mature to autolysosomes in which both endosomal and autophagosomal contents are degraded ***(Lennemann and Coyne, 2015).*** Degradation by autophagy can be either nonselective for bulk intracellular components (canonical) or selective for cargo such as damaged organelles (mitophagy), invasive pathogens (xenophagy) or protein aggregates (aggrephagy) ***(Stolz et al., 2014).***

- ***Microautophagy:***

In microautophagy, the lysosome itself engulfs small components of the cytoplasm by inward invagination of the lysosomal membrane. Membrane dynamics during microautophagy may be quite similar or identical to that of endosomal sorting complex required for transport (ESCRT)-dependent multivesicular body formation which occurs in the late endosome ***(Sahu et al., 2011)***. Microautophagy is important in the maintenance of organellar size, membrane homeostats and cell survival under nitrogen restricted conditions ***(Li et al., 2012a).***

- ***Chaperone-mediated autophagy (CMA):***

This class does not involve membrane reorganization;but instead substrate proteins directly translocate across the lysosomal membrane. The chaperone protein Hsc70 (Heat shock cognate 70) and cochaperones specifically recognize cytosolic proteins that contain a KFERQ-like pentapeptide ***(Orenstein and Cuervo, 2010)***. The transmembrane protein Lamp-2A (lysosomal-associated membrane protein 2A); which is an isoform of Lamp-2, acts as a receptor on the lysosome and unfolded proteins are delivered into the lysosomal lumen through a multimeric translocation complex ***(Mizushima and Komatsu, 2011).***

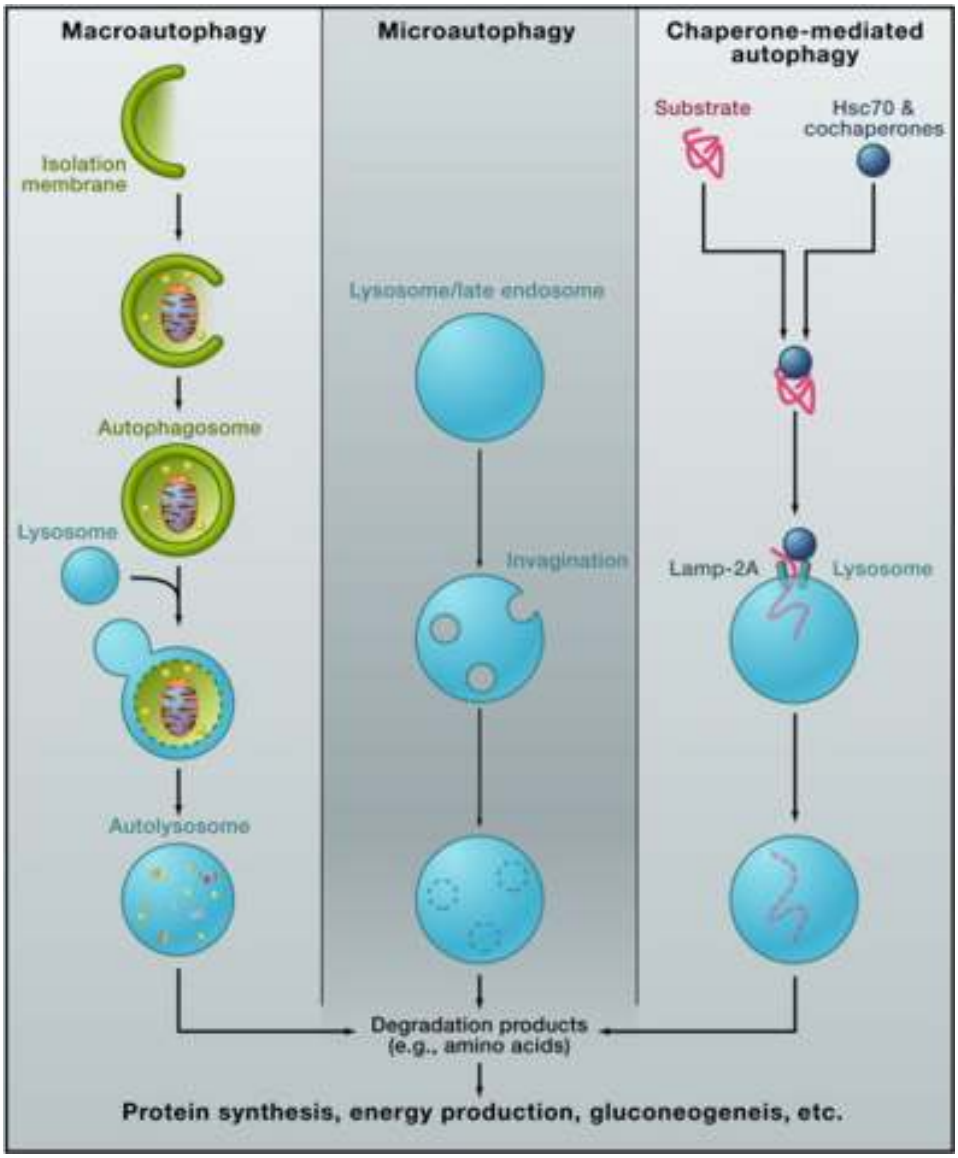

Figure (1): Types of Autophagy in mammalian cells
(Mizushima and Komatsu, 2011).

Role of autophagy in protein degradation:

In eukaryotic cells, proteins are degraded through two major proteolysis systems: the proteosome degradation system and autophagy. The ubiquitin-proteosome system is the major catabolic pathway for short-lived proteins, while autophagy is a process through which long-lived proteins, damaged organelles and other waste intracellular materials are delivered to lysosomes for degradation. Approximately 1%–1.5% of cellular proteins are catabolized per hour by autophagy even under nutrient-rich conditions in the liver.However,the rate by which basal autophagy contributes to macromolecule synthesis and energy production in the steady state by supplying amino acids, glucose and free fatty acids is unclear. Basal autophagy acts as the quality-control machinery for cytoplasmic components and it is crucial for homeostasis of various post mitotic cells such as neurons and hepatocytes ***(Mizushima and Komatsu, 2011).***

Autophagy is constitutively active at low levels in order to preserve cellular homeostasis but strongly induced by stressful conditions such as nutrient deprivation, growth factor depletion, oxidative stress, hypoxia, irradiation and anticancer drug medications. Under these stressful conditions, autophagy is believed to act primarily as a first protective response. Nevertheless, autophagy may also participate in cell death constituting an alternative caspase-independent cell death mechanism called type II or macroautophagy-related

programmed cell death ***(Meijer and Codogno, 2004; Mariño et al., 2014; Naponelli at al., 2015)***.

Molecular mechanisms of autophagy:

Although autophagy has been extensively studied at the cellular level for more than four decades, its molecular mechanisms have just started to be elucidated in the past few years. Three major steps consisting of initiation, nucleation and elongation in autophagosome formation were described ***(Pyo et al., 2012).*** To date, 32 genes involved in autophagy have been identified in mammals and these have been termed as autophagy-related genes (Atg) ***(He and Klionsky, 2009; Pyo et al., 2012)***. Among these, 16 genes (Atg 1-10, 12-14, 16 and 18) are required for all types of autophagy ***(Xie and Klionsky, 2007; Longatti and Tooze, 2009; Pyo et al., 2012)***. These Atg proteins function at several physiologically continuous steps in autophagy mainly induction, nucleation and expansion of autophagosome formation ***(Mizushima, 2010; Pyo et al., 2012)***.

- ***Molecular Regulation of Induction of autophagy:***

Several extracellular (e.g. nutrient status, hormonal and therapeutic treatment) and intracellular (e.g. metabolic stressors and accumulation of misfolded proteins) stimuli are able to activate autophagy and many signaling pathways are involved in the regulation of the autophagic process. The Mammalian Target of Rapamycin (mTOR) pathway is the most studied pathway

regulating autophagy. The mTOR pathway involves two functional complexes: the mTOR complex 1 (mTORC1) that is an important controller of cell growth and proliferation and plays a major role in controlling autophagy and the mTOR complex 2 (mTORC2) that is not directly implicated in autophagy modulation. The mTORC1 pathway is a key sensor of nutrient and energy status and is regulated by signals such as growth factors, amino acids and stressors. Mainly under nutrient-rich conditions, mTORC1 directly interacts with the ULK1 kinase complex (ULK1-mAtg13-FIP200-Atg101) and phosphorylates ULK1 and mAtg13, and thus inhibits the membrane targeting of the ULK1 kinase complex. During starvation conditions, on the other hand, the inactivated mTORC1 dissociates from the ULK1 kinase complex and results in the ULK1 kinase complex free to phosphorylate components such as mAtg13 and FIP200 in the ULK1 kinase complex leading to autophagy induction ***(Mizushima, 2010; Russell et al., 2014; Naponelli et al., 2015).***

In addition to mTORC1, AMP-activated protein kinase (AMPK), another cell key energy sensor can play a major role in transmitting autophagic signaling. AMPK is activated by the increase in cellular AMP/ATP ratio occurring during nutrient deprivation or hypoxia, and positively regulates the ULK complex by both direct phosphorylation of ULK1 and inhibition of mTORC1 via a pathway involving tuberous sclerosis complex 1 and 2 (TSC1/2) ***(Russell et al., 2014; Naponelli et al., 2015).***

- ***Molecular Regulation of autophagosome nucleation:***

In mammals, the class III PI3K complex plays an essential role in isolation membrane nucleation during autophagy ***(Mariño and López-Otín, 2004; Pyo et al., 2012)*** while the class I PI3K pathway is involved in autophagy regulation through insulin signaling cascade to activate mTOR and PKB ***(Yang and Klionsky, 2009; Pyo et al., 2012).*** The class III PI3K (Vps34) is associated with Beclin1 (Atg6) and p150 to form the class III PI3K core complex. As the first step of autophagosome formation, autophagosome nucleation requires Beclin1. Mammalian Beclin1, which was identified as an interaction partner of Bcl-2 ***(Liang et al., 1998; Pyo et al., 2012)*** associates with the class III PI3K core complex to generate PI(3)P ***(Funderburk et al., 2010; Pyo et al., 2012)***. The interaction of Beclin1 with Vps34 is known to promote the catalytic activity of VPS34 and increase levels of PI(3)P, but it is dispensable for the normal function of Vps34 in protein trafficking or recruitment of endocytic events ***(Wurmser et al., 1999; Zeng et al., 2006; Pyo et al., 2012).***

- ***Molecular Regulation of autophagosome expansion and elongation:***

The expansion of the isolation membrane is basically the simultaneous elongation and nucleation of little cistern. It is not known yet how the Atg12-Atg5 complex recruits additional membranes, but two ubiquitin-like protein conjugation systems are involved in the expansion of autophagosome membranes

and are essential for the formation of pre-autophagosomes. Once autophagosome formation is completed, Atg proteins are released back to the cytoplasm by a yet uncharacterized mechanism ***(Kuma et al., 2002; Pyo et al., 2012).***

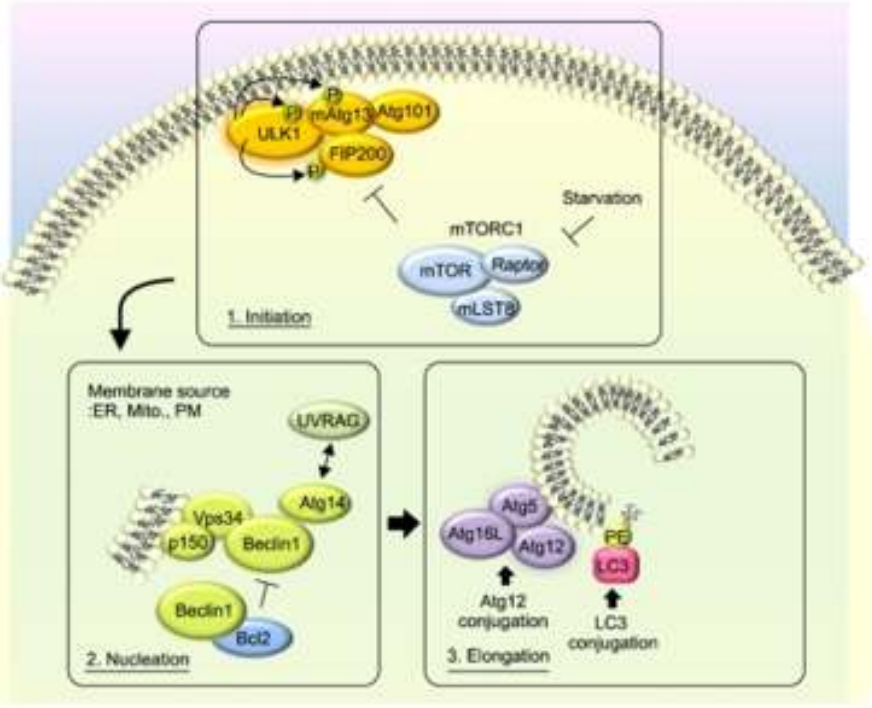

Figure (2): Molecular regulation of autophagosome formation in mammalian macroautophagy. Three major steps consisting of initiation, nucleation and elongation in autophagosome formation are described ***(Pyo et al., 2012).***

Crosstalk between Autophagy and Apoptosis:

When autophagy is excessively induced, it can result in autophagic cell death, so-called type II programmed cell death (PCD), which is distinct from type I PCD (apoptosis) and from

necrosis ***(Chen et al., 2010; Platini et al., 2010; Chen and Klionsky, 2011)***.

As both autophagy and apoptosis are important for the development and prevention of human diseases, the crosstalk between these two pathways has received increased attention and several observations help to summarize this interplay in mammalian cells. Under certain conditions, autophagy and apoptosis are two independent processes ***(Eisenberg-Lerner et al., 2009; Chen and Klionsky, 2011)*** whereas in other situations, the activation of autophagy inhibits apoptosis ***(Platini et al., 2010)*** or autophagy occurs upstream of apoptosis ***(Eisenberg-Lerner et al., 2009;Chen and Klionsky, 2011)***. Furthermore, regulators of apoptosis, such as Bcl-2 family members (Bcl-2 and Bcl-x_L) ***(Levine et al., 2008; Chen and Klionsky, 2011)*** and CASP8 and FADD-like apoptosis regulator ***(Djavaheri-Mergny et al., 2010)*** can regulate autophagy and proteins involved in autophagy, such as Atg5, beclin 1 and Atg4D can also have a role in apoptosis ***(Fimia and Piacentini, 2010).*** However, apoptosis-inducing factors might also have a role in autophagy, as for example, caspases are involved in autophagy in mammalian cells; the expression of caspase 9 is mediated by the expression of beclin 1, but the role of caspase 9 in autophagy has not yet been investigated ***(Wang et al., 2007; Chen and Klionsky, 2011)***.