# Autophagy and thyroid carcinogenesis: genetic and epigenetic links

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## Abstract

Thyroid cancer is the most common cancer of the endocrine system and is responsible for the majority of deaths from endocrine malignancies. Although a large proportion of thyroid cancers belong to well differentiated histologic subtypes, which in general show a good prognosis after surgery and radioiodine ablation, the treatment of radio-resistant papillary-type, of undifferentiated anaplastic, and of medullary-type thyroid cancers remains unsatisfactory. Autophagy is a vesicular process for the lysosomal degradation of protein aggregates and of damaged or redundant organelles. Autophagy plays an important role in cell homeostasis, and there is evidence that this process is dysregulated in cancer cells. Recent *in vitro* preclinical studies have indicated that autophagy is involved in the cytotoxic response to chemotherapeutics in thyroid cancer cells. Indeed, several oncogenes and oncosuppressor genes implicated in thyroid carcinogenesis also play a role in the regulation of autophagy. In addition, some epigenetic modulators involved in thyroid carcinogenesis also influence autophagy. In this review, we highlight the genetic and epigenetic factors that mechanistically link thyroid carcinogenesis and autophagy, thus substantiating the rationale for an autophagy-targeted therapy of aggressive and radio-chemo-resistant thyroid cancers.

**Key Words**
- autophagy
- thyroid cancer
- oncogenes
- epigenetics
- microRNA

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## Introduction

Thyroid cancer accounts for almost 90% of all endocrine-related cancers, and is responsible for the majority of deaths from endocrine malignancies (Siegel *et al*. 2013). Thyroid cancers may arise from either the follicular (thyroid hormone-producing) or the parafollicular (calcitonin-producing) cells (Fig. 1). The large majority of follicular cell-derived thyroid cancers are well differentiated and are classified as papillary (about 80% of all thyroid cancers) or follicular thyroid cancer (PTC and FTC, respectively), and a minor portion show a poorly differentiated or undifferentiated (anaplastic) phenotype (named as poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC), respectively). Thyroid cancers arising from parafollicular cells, named medullary thyroid cancers (MTC), account for 3–5% of all thyroid cancers. PTC is generally associated with favorable outcomes after surgery and radioactive iodine therapy, although 5% of these tumors show radio- and chemo-resistance (Fassnacht *et al*. 2009, Grodski & Delbridge 2009). On the other hand, ATC is extremely aggressive and soon leads the patient to death (Ain 1998, Smallridge 2012, Smallridge *et al*. 2012). MTC also have a generally

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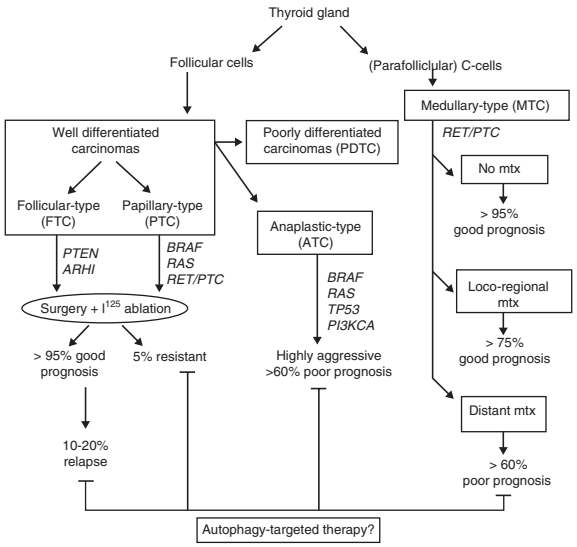


**Figure 1**
Autophagy-targeted opportunities for the therapy of thyroid cancers. Origin and aggressive phenotypes of thyroid cancers and opportunities for autophagy-targeted therapies. The main alterations in oncogenes and oncosuppressor genes in thyroid cancer histotypes are indicated in the boxes. C-cell, calcitonin-producing parafollicular cell; mtx, metastases.

good prognosis, with an overall survival rate at 10 years of ~95% if the tumor is confined to the thyroid gland, which drops to ~40% in the presence of metastases (Roman *et al.* 2006). Patients with an unresectable tumor or with distant metastases can be treated with chemotherapeutics (e.g., doxorubicin, 5-fluorouracil, cisplatin), yet the response rate is very low. Molecular therapy with inhibitors of mitogenic kinases has been disappointing, because it has not substantially improved the survival of patients with aggressive thyroid cancers, while showing an adverse side-effect profile (Gild *et al.* 2011). For instance, the multi-kinase inhibitor vandetanib, which has been approved for the treatment of inoperable or metastatic MTC (Thornton *et al.* 2012), has shown a modest efficacy toward MTC progression, but an extremely toxic profile that includes gastrointestinal, cardiovascular, and neurological disorders (Chau & Haddad 2013). Similarly, the results of a phase II clinical trials on the efficacy of the mammalian target of rapamycin (mTOR) inhibitor everolimus for the treatment of locally advanced or metastatic thyroid cancer have been disappointing (Lim *et al.* 2013). Thus, the lack of efficacious and safe treatment options provokes the search for novel molecular targeted drugs for the cure of such highly malignant thyroid cancers.

Recently, autophagy has emerged as a potential target for the therapy of hematologic and epithelial malignancies (Chen & Karantza 2011, Gundara *et al.* 2012, Wu *et al.* 2012). *In vitro* pre-clinical studies support the possibility of harnessing autophagy for the therapy of thyroid cancers (Lin *et al.* 2009, 2010, 2012*a*,*b*, Lu *et al.* 2012, Jin *et al.* 2013).

Autophagy (literally, 'self-eating') is the process through which damaged or redundant cytoplasmic constituents are degraded within lysosomes. Several oncogenes and oncosuppressor genes regulate the induction of autophagy. Autophagy is also epigenetically regulated through the methylation of autophagy regulatory genes, the activity of histone deacetylases (HDAC), and the expression of microRNAs (miRNAs). Here, we examine the genetic and epigenetic links between autophagy and thyroid carcinogenesis. A better understanding of such mechanistic connections could help to identify new targets for a more accurate diagnostic, prognostic, and therapeutic management of thyroid cancers.

## The autophagy machinery

Macroautophagy is a vesicular-driven process through which protein macroaggregates, large portions of membranes, and entire organelles can be delivered to lysosomes for complete degradation. This process is distinct from chaperon-mediated autophagy and microautophagy in which, respectively, only a single protein at a time or a small amount of cytoplasmic material is internalized in the lysosome. Readers may refer to the many excellent reviews in which the morphological and biochemical features of these processes are described in detail (Orenstein & Cuervo 2010, Yang & Klionsky 2010, Mizushima & Komatsu 2011, Sahu *et al.* 2011). Here, we will focus on macroautophagy (from now on simply named autophagy), as this is the major pathway contributing to the macromolecular turnover and cell homeostasis. In the following paragraphs, we briefly detail the key morphological and regulatory steps of autophagy.

### Morphological features and physiological significance

Autophagy comprises the following principal steps: i) the formation of a vacuole, named the autophagosome, that entraps the cargo to be degraded; ii) the fusion of the autophagosome with endosomes and lysosomes that leads to the formation of an autophagolysosome; and iii) the degradation of the autophagy cargo (Fig. 2). The autophagosome is a double-layered vesicle that forms in the proximity of the trans-Golgi network. The progenitor membrane donor of the autophagosome is the smooth



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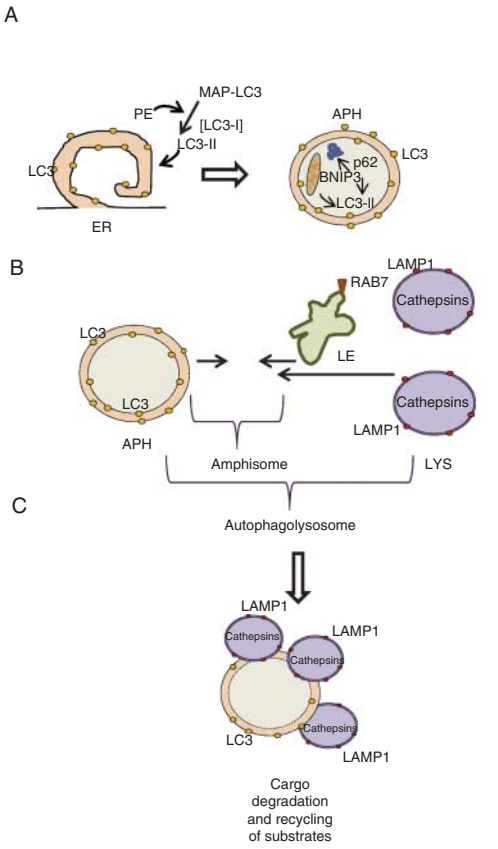


| Key event | Key protein(s) involved |
|---|---|
| Membrane nucleation | ATG2 |
| Membrane expansion | ATG5; ATG9 |
| MAP-LC3 processing | ATG4; ATG5; ATG12; ATG7; ATG3 |
| Cargo sequestration | p62; NBR1 Nix/BNIP3 |
| Vesicle closure | LC3-II; SNARE |
| Vesicle migration | CYTOSKELETON HDAC6 DYNEIN |
| Vesicle docking and fusion | SNARE RAB LAMP1,2 |
| Cargo degradation | Lysosomal hydrolases |
| Substrate export | ATG22 |

**Figure 2**

Morphological features of the (macro)autophagy process. The process can be dissected in three main steps. The key events and the relative protagonists of each step are indicated. (A) Formation of the autophagosomes (APH). Vesicle nucleation and membrane expansion start from the endoplasmic reticulum (ER). The autophagosome is marked by the presence on its internal and external membranes of LC3-PE, arising from MAP-LC3 (MAP-LC3, microtubule associate protein light chain 3; PE, phosphatydylethanolamine). The autophagy cargo includes portions of cytoplasm, protein aggregates, and mitochondrion, which are targeted by specific proteins such as p62, neighbor of BRCA1 (NBR1), and Bcl2/adenovirus E1B 19-kDa interacting protein (BNIP3). (B) Fusion of the autophagosome with late endosomes (LE, identified by RAB7) to form an amphisome, and with lysosomes (LYS, identified by LAMP1) to form an autophagolysosome. This step requires HDAC6-mediated deacetylation of tubulin and the activity of dynein. Tethering of autophagosomes and lysosomes relies on soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor (SNARE) proteins. (C) Degradation of autophagy cargo and recycling of substrates. In the autophagolysosome, the inner membrane of the autophagosome along with its cargo is degraded by lysosomal acid enzymes (essentially the cathepsins). This process is marked by the consumption of LC3 present in the inner membrane of the autophagosome. Substrates are then exported in the cytoplasm and recycled in the biosynthetic pathway. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0271.

endoplasmic reticulum, though membranes from other sources, including the plasma membrane and the outer mitochondrial membrane, are subsequently recruited and contribute to the expansion of this vesicle (Ravikumar *et al*. 2010, Tooze & Yoshimori 2010, Rubinsztein *et al*. 2012, Hamasaki *et al*. 2013). During this process, the lipidated form of light chain 3 (LC3, also known as ATG8) is post-translationally inserted into the expanding autophagosomal membrane through the intervention of several autophagy-related (ATG) proteins (Mizushima *et al*. 2011; box in Fig. 2). The synthesis and membrane translocation of LC3II is considered an hallmark of autophagosome biogenesis (Klionsky *et al*. 2012). Autophagy substrates are specifically sequestered in the lumen of the nascent autophagosome through the intervention of proteins that bridge the substrate to LC3 in the internal membrane (Noda *et al*. 2010; box in Fig. 2). The autophagy process proceeds with the fusion of the autophagosome with several endosomes and lysosomes (at the end an autophagolysosomal vacuole is formed) and the subsequent full



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degradation of the autophagy substrates is conducted by lysosomal acid hydrolases (Eskelinen 2005). Finally, fully degraded substrates are exported to the cytoplasm and reutilized in biosynthetic pathways. It is to be stressed that autophagy has to proceed to completion to exert its prosurvival effects. The production of autophagosomes that do not completely fuse with lysosomes is of no benefit to the cell, because the autophagy substrates are not fully degraded and recycled, and may eventually become toxic. Therefore, to understand the pathophysiological outcome of autophagy, it is important to clearly determine the formation of autophagosome vs the autophagy flux (see section Detection of autophagy in thyroid cancer cells).

### Biochemical regulation of autophagy at a glance

The induction and progression of autophagy are controlled by a complex network of signaling pathways that involve a number of protein- and lipid-kinases, protein- and lipid-phosphatases, and monomeric and trimeric GTPases (Mehrpour *et al*. 2010, Chen & Klionsky 2011). The pathways illustrated in Fig. 3 represent an obvious, though efficacious, oversimplification. The master signal that triggers autophagy comes from the Unc51-like kinase 1 (ULK1, homolog of yeast Atg1) complex (Wong *et al*. 2013). Two upstream kinases, namely the mTOR-raptor complex 1 (mTORC1) and AMPk, control the activation of ULK1, the former acting as a repressor (Ganley *et al*. 2009, Jung *et al*. 2009) and the latter acting as an activator (Egan *et al*. 2011). mTORC1 integrates the signals from: i) the phosphatydilinositol-3-kinase (PI3k) class I/AKT pathway, which senses the presence of growth factors; ii) the AMPk pathway, which senses the lack of energy; and iii) the Rag A/B (a Ras-related GTPase) complex, which senses the availability of amino acids. In the presence of growth factors, the PI3k/AKT pathway negatively regulates autophagy through a tonic activation of mTORC1 via the tuberous sclerosis complex (TSC) and Rheb (Ras homolog enriched in the brain) (Petiot *et al*. 2000, Arico *et al*. 2001, Inoki *et al*. 2002). The lipid phosphatase activity of phosphatase and tensin homolog (PTEN) shuts down this pathway, thus abolishing the mTOR-mediated repression of ULK1. When amino acids are abundant, the RagA/B complex activates mTORC1 (Sancak *et al*. 2010), while the lack of amino acids is sensed by the RAS–BRAF–ERK1/2 pathway that triggers autophagy through the stimulation of heterotrimeric GTP proteins (Ogier-Denis *et al*. 1995, 2000). Glucose depletion and other metabolic stresses that reduce the production of ATP or provoke the production of reactive oxygen species (ROS) activates the AMPk pathway, that in turn represses mTORC1 and activates ULK1 (Alers *et al*. 2012), thus initiating autophagy (Alexander *et al*. 2010, Castino *et al*. 2011, Janda *et al*. 2012, Wong *et al*. 2013). Downstream to ULK1, class III PI3k (also known as Vps34) produces phosphatydil-3-phosphate (PI3P), the starting platform for the biogenesis of the autophagosome (Noda *et al*. 2010). Vps34 is activated through its interaction with Beclin1 (also known as ATG6 or Vps30) and p150 (homolog of Vps15), besides other regulating proteins (He & Levine 2010). This pathway is impaired when Beclin1 is sequestered through the binding with BCL2 (Pattingre *et al*. 2005).

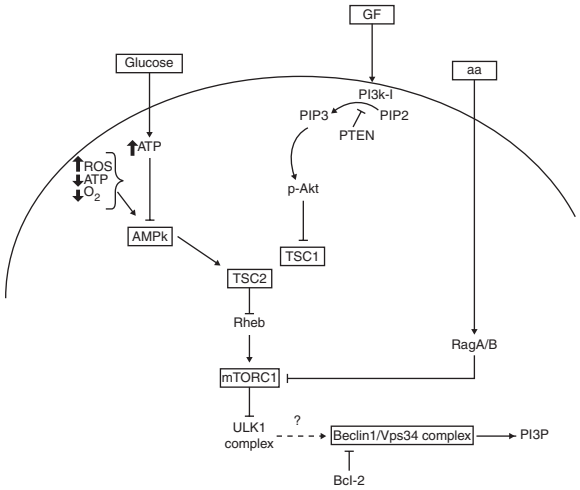


**Figure 3**
Biochemical regulation of the autophagy process. The scheme illustrates the main signaling pathways that control the induction of autophagy. Growth factors (GF) stimulate the activity of class I PI3k, which produces PIP3 and leads to activation of the AKT–mTOR axis. PTEN reduces the availability of PIP3, and therefore prevents the activation of the AKT–mTOR axis. Amino acids signal through the RagA/B pathway to keep mTOR active. The activation of mTORC1 negatively impinges on the ULK complex, thus inhibiting the induction of autophagy. In the absence of growth factors and of amino acids (starvation) this pathway is switched off, and mTOR is inactive, thus allowing the rise in the level of basal autophagy. The lack of glucose and of oxygen, as well as the presence of pro-oxidant species (e.g., ROS), activate the AMPk pathway, which results in the inhibition of mTOR and direct activation of the ULK complex and ultimately in the rise of autophagy. Largely unknown is the link between the ULK complex and the BECLIN1–Vps34 complex. The latter is inhibited when BCL2 binds to BECLIN1. Once activated, Vps34 produces PI3P.

## Role of autophagy in cancer development and progression

In quiescent and appropriately fed cells, autophagy runs at a constant basal level that ensures the homeostatic macromolecular turnover (Ravikumar *et al*. 2010), and it is



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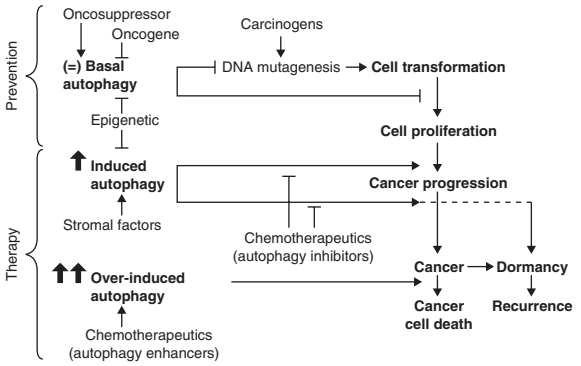


**Figure 4**
Double-face role of autophagy in the carcinogenic process and role of genetics and epigenetics in its regulation. Basal autophagy exerts a protective action in cells by eliminating molecules that could lead to DNA mutagenesis and cell transformation. Primitive mutations in certain oncogenes or oncosuppressors, as well as epigenetic regulation of certain autophagy-related genes, could limit the benefit of autophagy at this preventive step. Once cancer is established, autophagy may confer the advantage to cancer cells to face metabolic stresses (such as nutrient depletion, hypoxia, and chemotherapy-induced damages) and possibly to survive in a dormant state. At this stage, a chemotherapeutic regimen including autophagy-inhibiting drugs could elicit cancer cell death. On the other hand, chemotherapeutics that increases autophagy to a level beyond the point-of-no-return could also elicit cell death.

upregulated above the baseline when the lack of nutrients or of energy imposes the degradation of redundant self-constituents to recover substrates and energy necessary for cell survival, or when it is necessary to eliminate a cell component that has been damaged by an extracellular insult (a toxic drug, radiation, or oxidative stress) (Kroemer *et al.* 2010, Ravikumar *et al.* 2010). Given these housekeeping functions, it is logical to suspect that autophagy is deregulated in cancer or, conversely, that deregulation of autophagy can promote carcinogenesis. However, the role of autophagy in cancer is not unequivocal, because, paradoxically, it may either prevent cell transformation or favor the survival of cancer cells, depending on how autophagy is regulated in the various steps of the carcinogenic process (White & Di Paola 2009; Fig. 4). On one hand, autophagy opposes cell transformation by helping to get rid of mutagenic pro-oxidant molecules and by cooperating with the DNA repair system (Robert *et al.* 2011, Rodriguez-Rocha *et al.* 2011). Yet, this same process can turn to the advantage of cancer cells subjected to the genotoxic stress imposed by radiotherapy and chemotherapy. In addition, in growing tumors with defective vascularization there are areas in which the supply of nutrients and of oxygen is insufficient, and here autophagy is upregulated allowing cancer cells to survive despite the prohibitive metabolic conditions (Degenhardt *et al.* 2006), possibly in a dormant state (Lu *et al.* 2008). These cells resist to chemo- and radio-therapeutic treatments, and eventually give rise to cancer relapse. Further, a transient upregulation of autophagy is observed during the epithelial–mesenchymal transition (EMT; Akalay *et al.* 2013), and this function prevents the cell death by anoikis of cancer cells which have detached from the basement membrane to invade the extracellular matrix (Fung *et al.* 2008). Thus, autophagy is differently regulated during the carcinogenic process and its actual level in the cell could vary in subclones, depending on the acquisition of new oncogenic assets. The situation is far more complicated considering that autophagy is influenced by epigenetic factors (see below) and by extracellular factors such as oxygen, glucose, nutrients, growth factors, hormones, and cytokines.

From the above considerations it appears clear that autophagy has a great impact on the progression of tumors and on the response to therapeutic treatments, and therefore influences the prognosis. Consistently, certain autophagy-related proteins have been shown to be of prognostic value. For instance, the hyperexpression of BECLIN1 and of LC3 in general associates with a better prognosis in patients with glioblastoma (Pirtoli *et al.* 2009), colorectal cancer (Li *et al.* 2009, Koukourakis *et al.* 2010), lymphomas (Nicotra *et al.* 2010, Huang *et al.* 2011), or duodenal adenocarcinoma (Wu *et al.* 2013). Conversely, low expression of BECLIN1 or of LC3 associates with poor prognosis in patients with hepatocarcinoma (Ding *et al.* 2008), glioblastoma (Huang *et al.* 2010), colorectal cancer (Koukourakis *et al.* 2010), lymphoma (Nicotra *et al.* 2010), or lung carcinoma (Won *et al.* 2012).

## Autophagy and thyroid cancer

A systematic study addressing the prognostic value of autophagy in thyroid cancers has not yet been performed. Still, some *in vitro* studies have proven the involvement of autophagy in the cytotoxic response of thyroid cancer cells to anti-tumor drugs (Table 1). In this section, we provide some technical tips for assessing the presence of autophagy in thyroid cancer biopsies and discuss the mechanistic links between autophagy and thyroid carcinogenesis.

### Detection of autophagy in thyroid cancer cells

The expression of autophagy protein markers can be demonstrated in *ex-vivo* thyroid tumor tissue and in



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**Table 1** Studies involving autophagy in thyroid cancer cells

| Thyroid cancer histotype | Anti-tumor treatment | Autophagy manipulation | Autophagy involvement | Conclusions | Reference |
|---|---|---|---|---|---|
| Papillary (TPC1 and 8505-C cells) | Doxorubucin (anthracycline antibiotic); radiation | 3-MA inhibition of PI3K | Inhibition of autophagy promotes resistance to doxorubicin and radiation | Autophagy may be useful for the treatment of refractory papillary thyroid cancer | Lin *et al.* (2009) |
| Papillary (TPC1 and 8505-C cells) | RAD001 (rapamycin analogue, also known as everolimus or afinitor)<br><br>Other treatments: doxorubicin, desatinib, PHA665752 | RNAi knockdown of ATG5; transfection with EGFP-LC3 plasmid; 3-MA inhibition of PI3K | ATG5 RNAi knockdown abrogates the effects of RAD001. Autophagic activation resulted in Src phosphorylation and Met dephosphorylation<br>Src inhibition did not reverse the effects of RAD001, whereas MET inhibition reversed the effects of autophagy blockade on chemosensitivity | RAD001 induces autophagy that enhances the therapeutic response to doxorubicin and external beam radiation | Lin *et al.* (2010) |
| Follicular (WRO cells) | Reversine (2,6-disubstituted purine ATP-analog) | Transfection with pEGFPC1-LC3; 3-MA inhibition of PI3K | Autophagy promotes anti-cancer activity through cell-cycle arrest and apoptosis | Reversine is effective to induce autophagy (autophagosome formation) and reduce the activation of Akt/mTOR pathway | Lu *et al.* (2012) |
| Medullary (MTC1.1 and TT cells) | Sunitinib, sorafenib (RET inhibitors)<br><br>Everolimus, trehalose (autophagy activators) | Transfection with ATG5 siRNA | Silencing of ATG5 diminishes the antiproliferative effects of sunitinib and sorafenib, and abrogates both everolimus and trehalose-induced increases in tyrosine kinase inhibitor efficacy | Activation of autophagy potentiates the anti-cancer effect of sunitinib and sorafenib<br><br>Autophagy activators should be combined with targeted RET protooncogene therapy in MTC | Lin *et al.* (2012*a*) |
| Papillary (TPC1 cells) and anaplastic (FRO cells) | Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) | Transfection with ATG7 siRNA | Inhibition of autophagy renders TPC1 cells resistant to the effect of TRAIL, while it sensitizes FRO cells to TRAIL-induced apoptosis | Modulation of autophagy could be combined with TRAIL for the treatment of thyroid cancer | Jin *et al.* (2013) |

3-MA, 3-methyladenine.

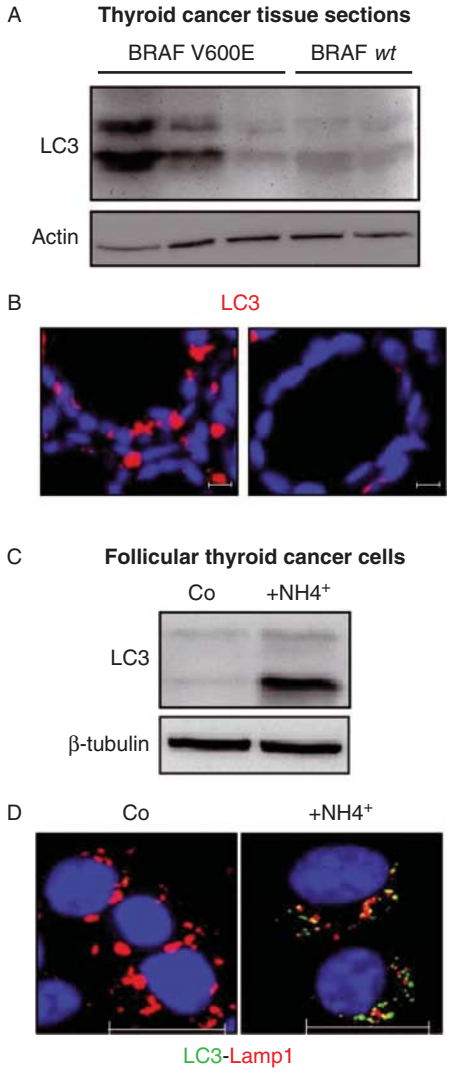


**Figure 5**
Detection of autophagy in thyroid cancer. Representative images showing the detection of markers of autophagy in thyroid cancer tissues and cells. (A and B) Analysis of the expression and accumulation of LC3 in thyroid cancer tissues. (A) Western blotting of LC3 in homogenates of frozen biopsies from BRAF-mutated and BRAF wt thyroid cancers. The presence of the LC3II isoform (lower band) is indicative of autophagy. In a preliminary study conducted on 19 cases (12 of which with mutated BRAF), we have not found a statistically significant correlation between the mutation of BRAF and the level of LC3II in thyroid cancer. (B) Immunofluorescence of LC3. The puncta in the cytoplasm of cancer cells are indicative of the presence of autophagosomes. (C and D) Expression of LC3 in cultured thyroid cancer cells (FTC133 cell line) incubated or not for 24 h with ammonium chloride ($NH_4^+$). (C) Western blotting; (D) immunofluorescence co-staining of LC3 and LAMP1. It is evident the accumulation of LC3 in the cells incubated with ammonium chloride which in fact prevented the fusion of autophagosomes with lysosomes (as can be appreciated in panel D) and the degradation of LC3II (as can be appreciated in panel (C). Microscope magnification: (B) 40×; (D) 63×. Scale bars: (B) 10 μm; (D) 20 μm. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0271.

cultured thyroid cancer cells. Paraffin-embedded and cryostatic tissue sections can be processed for immunohistochemistry and immunofluorescent detection of autophagy markers, while western blotting analysis is better performed in freshly isolated or frozen biopsy tissues. Both these techniques are complementary and should be employed.

As an example, we report our preliminary observations on the detection of autophagy markers in tissue biopsies and in cultured cells of thyroid cancer origin. Western blotting of LC3 reveals the presence of the autophagosome-bound LC3II isoform, which arises from LC3I (Fig. 5A and C). One important caveat is that infiltrating fibroblasts, macrophages, and mastocytes might also express autophagy markers. This should be taken into consideration when performing a western blot with the whole homogenate of a biopsy, unless cancer cells are isolated by laser microdissection. In immunofluorescence, nontumor cells can be discriminated by using appropriate markers for stromal cells. A faintly detectable diffuse cytoplasmic signal of LC3 is considered as negative background, while a pattern of many LC3-positive puncta (in general, more than 10 per cell) is indicative of ongoing autophagy (Fig. 5B and D). Immunocostaining with multiple markers (e.g., LC3, p62, BECLIN1, lysosome-associated membrane protein (LAMP1)) is recommended for a better assessment of the autophagy flux and of the signaling molecules involved. The true level of ongoing autophagy is much easier assayed in cultured cells through the pharmacologic or genetic manipulation of the autophagy flux. For instance, in the presence of drugs that alkalinize the lysosome pH (e.g. ammonium chloride) all the autophagosomes produced during the incubation time accumulate in the cell as they do not fuse with lysosomes (Kawai *et al*. 2007, Klionsky *et al*. 2012), and this reflects in the accumulation of LC3II protein (Fig. 5C and D).

## The genetic connection: oncogenes and oncosuppressor genes

Numerous oncogenes and tumor suppressor genes regulate autophagy (Maiuri *et al*. 2009). In general, oncogenic proteins exert a negative activity and oncosuppressor proteins exert a positive activity on autophagy induction and progression. Several oncogenes and oncosuppressors implicated in thyroid carcinogenesis also play a role in the regulation of autophagy.

The main signaling pathways that link thyroid carcinogenesis with autophagy deregulation are the



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RAS–RAF–ERK and the class I PI3k–AKT–mTOR pathways. The RAS/RAF/MEK/ERK pathway controls the mTOR-dependent pathway by sensing the absence of amino acids (Ogier-Denis *et al*. 2000). Aberrant signaling through the RAS/RAF/MEK/ERK cascade has been implicated in thyroid tumor initiation and development. For instance, the *RET/PTC* rearrangement, which leads to the RET/PTC fusion oncoprotein, activates the RAS–RAF–MAPK cascade (Knauf *et al*. 2003, Santoro *et al*. 2004). However, the most frequent aberration of this signaling pathway in thyroid cancer is associated with the oncogenic activation of BRAF. The BRAF V600E mutation, which leads to constitutive activation of BRAF kinase, is frequently found in PTC (Xing 2007) and, though less frequently, also in ATC (Nikiforova *et al*. 2003, Takano *et al*. 2007) and PDTC (Begum *et al*. 2004). In melanomas, oncogenic BRAF has been associated with inhibition of mTOR and upregulation of basal autophagy (Maddodi *et al*. 2010). However, another study showed that in metastatic melanomas oncogenic BRAF opposed the induction of autophagy by chemotherapeutics or rapamycin (Armstrong *et al*. 2011). Another oncogene of this same pathway which is mutated in a large percentage of thyroid cancers is the *RAS* oncogene (Motoi *et al*. 2000, Nikiforova & Nikiforov 2009). Activating mutations of RAS are associated with aggressive phenotypes of thyroid cancer and poor prognosis (Garcia-Rostan *et al*. 2003). Remarkably, the oncogenic mutants Ha-RAS and K-RAS have been shown to confer a metabolic advantage to cancer cells through the upregulation of basal autophagy (Guo *et al*. 2011, Kim *et al*. 2011). The above findings on the effects of active BRAF and Ha-RAS on autophagy seem to contradict the general rule that oncogenes signal to downregulate autophagy. Besides the fact that basal and stress-induced autophagy should be distinguished, it is likely that the true effect of oncogenic BRAF and RAS on autophagy regulation is cell context dependent and also reliant on the extracellular trigger. In addition, it should be considered that oncogenic RAS can signal through either the RAF–MEK–ERK1/2 pathway or the PI3K/AKT pathway, with a different impact on the regulation of autophagy (see below). Therefore, the final outcome on autophagy regulation by oncogenic RAS will depend on which downstream pathway will predominate.

The class I PI3k-(PTEN)–AKT–mTOR pathway is the other oncogenic pathway aberrantly hyperactive in thyroid cancer cells and is also known to regulate autophagy (see Fig. 3). Growth factors activate class I PI3k, which then phosphorylates phosphatidylinositol-3,4-diphosphate into phosphatidylinositol-3,4,5-triphosphate (PIP3), the phosphate donor needed for the phosphorylation of AKT. Active AKT then phosphorylates a number of downstream targets that ultimately regulate various cellular functions, including cell survival, proliferation, autophagy, protein synthesis, angiogenesis, and migration. Genetic alterations in the PI3k/AKT signaling pathway have been linked to thyroid cancers (Garcia-Rostan *et al*. 2005, Shinohara *et al*. 2007, Wang *et al*. 2007). The PIK3CA gene (encoding the catalytic subunit of p110α of class I PI3k) has been found amplified or mutated in thyroid carcinomas (Wu *et al*. 2005, Wang *et al*. 2007). Increased AKT activity has been associated with the aggressive behavior of FTCs and PTCs (Ringel *et al*. 2001, Shinohara *et al*. 2007). AKT negatively regulates autophagy through the mTOR pathway (Arico *et al*. 2001, Castino *et al*. 2008) and, directly, through phosphorylation of BELCIN1 (Wang *et al*. 2012). The class I PI3k/AKT pathway may be abnormally upregulated as a consequence of PTEN loss-of-function. The lipid phosphatase activity of PTEN removes the phosphate in position 3 from PIP3, thus limiting the availability of PIP3 needed for the activation of AKT. By shutting down the activation of AKT, PTEN relieves the AKT–mTOR block on autophagy (Arico *et al*. 2001). It is worth noting that mutations or deletions of the tumor suppressor gene *PTEN* have been recognized as an important step in the development of thyroid gland carcinomas (Dahia *et al*. 1997, Eng 2002).

Another oncogene that might link autophagy deregulation with thyroid carcinogenesis is c-MET. This proto-oncogene encodes a membrane tyrosine kinase receptor for the hepatocyte growth factor (HGF), which is a potent mitogen for epithelial cells and promotes cell motility and invasion in carcinoma cells (Stella *et al*. 2010). A large cohort study revealed that about 50% of PTCs are characterized by MET overexpression (Di Renzo *et al*. 1992), and this represents a sign of more aggressive disease (Ramirez *et al*. 2000, Mineo *et al*. 2004). Very recently, it has been shown that c-MET overexpression or its activation by HGF negatively regulates autophagy in A549 carcinoma cells (Liu *et al*. 2012). In the context of thyroid cancer, it has been reported that induction of autophagy by RAD001-mediated inhibition of mTOR sensitized PTC cells to chemo- and radio-therapy through the inhibition of c-MET (Lin *et al*. 2010).

What about other tumor suppressors, besides PTEN, that are deleted or mutated in thyroid cancers and might play a role in the regulation of autophagy?

The haplo-insufficient tumor suppressor gene *BECLIN1* was the first oncosuppressor gene that proved the link between autophagy and cancer susceptibility



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(Liang *et al*. 1999, Qu *et al*. 2003, Yue *et al*. 2003). *BELCIN1* has been found mutated or monoallelically deleted in a large proportion of a variety of epithelial cancers, including breast and ovary carcinomas (Qu *et al*. 2003, Yue *et al*. 2003). Predictably, similar gene alterations could also be found in thyroid cancers. However, as yet no studies have addressed this issue.

The other tumor suppressor that may link autophagy and thyroid cancer progression is p53. Contrary to what is seen in many other cancers, TP53 loss-of-function mutations occur late in thyroid tumorigenesis. TP53 mutations are practically absent in differentiated thyroid cancers, while their prevalence is high (17–38%) in PDTC and is even higher (55–88%) in ATC (Fagin & Mitsiades 2008, Smallridge *et al*. 2009). The regulatory activity of p53 in the autophagy pathway is quite ambiguous (Maiuri *et al*. 2010). As a transcription factor, wild-type p53 promotes autophagy by directing the transcription of certain autophagy genes, including damage-regulated autophagy modulator (DRAM) and ULK1/2 (Crighton *et al*. 2006, Gao *et al*. 2011). However, in many different cell types the lack of p53 has been shown to stimulate the autophagic flux, suggesting an inhibitory function of this tumor suppressor. In line with this finding, certain DNA-binding deficient p53 mutants that fail to relocate to the nucleus, and instead reside in the cytoplasm, have been shown to repress the induction of autophagy by metabolic stresses.

The ras-homolog GTPase aplasia Ras homolog member I (*ARHI*) (or *DIRAS3*) is another oncosuppressor that potentially links autophagy to thyroid carcinogenesis. ARHI positively regulates the autophagy-mediated dormancy of tumor cells (Lu *et al*. 2008). Of interest, ARHI is found underexpressed in FTCs (Weber *et al*. 2005).

## The epigenetic liaisons: the role of DNA methylation, histone deacetylates, and miRNAs

Epigenetics refers to heritable (i.e., transmitted via meiosis or mitosis) changes in the expression of a gene or a set of genes not dependent on variations in the primary DNA sequence. Given that all cells in the same organism bear the same genome, epigenetics explains the different cellular phenotypes in the body as a result of the silencing of different subsets of genes. It is now clear that epigenetics plays a role in cancer, as cancer cells have their own epigenome. During cell proliferation, the epigenome is transmitted to daughter cells, although novel epigenetic signatures may emerge in the progeny as a consequence of (micro)environmental interference, thus explaining the appearance of clones with different behavior in the context of the tumor (Timp & Feinberg 2013). There are four epigenetic mechanisms known to regulate gene expression: DNA methylation in correspondence to the promoter region, histone conformation changes (essentially dictated by acetylation and deacetylation of certain lysine residues), chromatin remodeling (both histone conformation changes and chromatin remodelling affect DNA accessibility), and miRNAs (short, noncoding mRNAs that impair RNA translation by hybridizing to specific domains of the UTR of target mRNAs).

In recent years, a large body of evidence has accumulated showing the pivotal role of epigenetic changes in thyroid tumorigenesis driven by DNA methyl transferases (DNMTs), HDAC, and miRNAs (reviewed in Pallante *et al*. (2010), Braun & Hüttelmaier (2011), Russo *et al*. (2011) and Catalano *et al*. (2012*a*)). In this section, we provide a brief overview of the proteins and of the mechanisms involved in the epigenetic regulation in thyroid cancer that also have an impact on the regulation of autophagy.

For instance, the hypermethylation of the *PTEN* promoter region sporadic thyroid cancers has been reported (Alvarez-Nuñez *et al*. 2006). The lack of PTEN expression maintains active AKT in thyroid cancer cells, and consequently autophagy in these cells will be repressed.

The promoter of death-associated protein kinase (DAPk) has also been found to be hypermethylated in a large proportion of thyroid cancers (Hoque *et al*. 2005). This kinase induces autophagy by disrupting the BECLIN1–BCL2 complex (Zalckvar *et al*. 2009), and its epigenetic silencing in thyroid cancer cells might impair the induction of autophagy under stress conditions.

An intriguing epigenetic liaison between autophagy and thyroid carcinogenesis could involve the tumor suppressor ARHI, a Ras homolog that was recently shown to induce autophagy and autophagy-mediated dormancy in ovarian cancer cells (Lu *et al*. 2008). *ARHI* is generally monoallelically expressed (from the paternal allele), as the maternal allele is inherited in the hypermethylated (and therefore silenced) state. *ARHI* maps to 1p31, a region that is frequently deleted in thyroid cancers, particularly in FTCs (Weber *et al*. 2005). It has been hypothesized that silencing of ARHI expression, as a result of combined hypermethylation of the maternal allele and deletion of the paternal allele, is pivotal to thyroid carcinogenesis (Weber *et al*. 2005).

Histone deacetylation promoted by HDACs negatively affects gene transcription. There is evidence that certain HDACs (namely SIRT1, HDAC1, HDAC2, HDAC6) can



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regulate the autophagy process at the level of gene transcription and of protein function (Moresi *et al*. 2012, True & Matthias 2012, Füllgrabe *et al*. 2013). As histone deacetylation decreases DNA accessibility, the inhibitors of HDACs promote gene transcription. Indeed, the treatment with the HDAC inhibitor suberoylanide hydroxamic acid was shown to induce autophagy in several cell types (Shao *et al*. 2004, Gammoh *et al*. 2012). There is promising evidence that HDAC inhibitors can be used in combination with other chemotherapeutics to improve their effectiveness in the treatment of thyroid cancers. HDAC inhibition was shown to increase the expression of E-cadherin, thus promoting cell–cell adhesion and reducing the migration of *in vitro* cultured thyroid cancer cells (Catalano *et al*. 2012*b*). E-cadherin is downregulated during the EMT process that precedes the metastasization of cancer cells, and, it is worth noting, the actual level of autophagy in cancer cells increases at this step in concomitance with E-cadherin silencing (Akalay *et al*. 2013). Whether the reexpression of E-cadherin following HDAC inhibition also associates with repression of autophagy in thyroid cancer cells has yet to be proven. Besides histones, some HDACs also deacetylate target cytoplasmic proteins, among which are also some proteins involved in the initiation and progression of autophagy (Lee *et al*. 2008, 2010, Lin *et al*. 2012*a*,*b*, Yi *et al*. 2012). For instance, SIRT1 deacetylates ATG5, ATG7, and ATG8 (Lee *et al*. 2008), which are essential for the formation of autophagosomes, while HDAC6 deacetylates the cytoskeletal protein tubulin, thus favoring the fusion of autophagosome with lysosomes (Lee *et al*. 2010).

The third epigenetic mechanism that potentially links autophagy and thyroid cancer are the miRNAs, a class of noncoding RNAs of 20–24 nucleotides that control gene expression at the post-transcriptional level (Ghildiyal & Zamore 2009). The link between miRNAs and epigenetics is bidirectional, as on one hand the expression of miRNAs is dictated by the methylation and acetylation status of DNA, and on the other hand miRNAs themselves regulate the expression of DNMTs and HDACs (Iorio & Croce 2009). For simplicity, we will only refer to those miRNAs that interfere with the expression of proteins involved in the regulation of autophagy and that are abnormally expressed in thyroid cancers.

Several miRNA profiling studies have identified changes in miRNA patterns occurring during thyroid cancer development and progression, opening a new field for the understanding of this disease and providing improved diagnostic, prognostic, and therapeutic approaches (reviewed in Pallante *et al*. (2010), Braun & Hüttelmaier (2011), De la Chapelle & Jazdzewski (2011) and Gundara *et al*. (2012)). Autophagy in itself, as a metabolic process regulated by an intricate network of proteins, is regulated by miRNAs at various levels (Gundara *et al*. 2011, Frankel & Lund 2012, Zhai *et al*. 2013). In principle, any miRNA targeting a signaling pathway involved in the control of autophagy would exert its regulatory activity on this pathway. For instance, miR221/222 and miR21, which target PTEN and consequently sustain the hyperactivation of AKT (Garofalo *et al*. 2009, Chun-Zhi *et al*. 2010), indirectly have a negative impact on induction of mTOR-dependent autophagy. With relevance to the present context, miR21 was found upregulated in PTCs and ATCs (Braun *et al*. 2010, Frezzetti *et al*. 2011), and miR221/222 cluster was found to be upregulated in PTCs (He *et al*. 2005, Pallante *et al*. 2006, Visone *et al*. 2007, Sheu *et al*. 2010). The loss of miR200 expression marks the progression of thyroid carcinomas culminating in tumor growth factor beta (TGFβ) dependent EMT and elevated invasiveness (Braun *et al*. 2010). Members of the miR200 family (comprising miR141, 200a, 200b, 200c, and 429) are potent suppressors of EMT through the downregulation of the expression of EMT-promoting factors like ZEB1, ZEB2, SNAI2, SMAD2, TGFβR1, and TGFβ2, and by antagonizing the transcriptional repression of E-cadherin (Gregory *et al*. 2008, Park *et al*. 2008, Braun *et al*. 2010). Of note, the actual level of autophagy in cancer cells was found to increase during EMT in concomitance with E-cadherin downregulation (Akalay *et al*. 2013). Thus, one can hypothesize that in thyroid cancer cells with low-levels of miR200, autophagy is upregulated to oppose anoikis in invading cells.

More recently, miRNAs specifically targeting the mRNA of autophagy proteins are being identified. Here, we will mention only those miRNAs involved in both the regulation of autophagy and the progression of thyroid cancers. The first miRNA described in this field is miR30a, which downregulates the expression of Beclin1 (Zhu *et al*. 2009). In the same family, miR30d was shown to target the mRNA of various autophagy proteins including BECLIN1, Bcl2/adenovirus E1B 19-kDa interacting protein (BNIP3L, which plays a role in mitophagy), ATG2, ATG5, and ATG12 (Yang *et al*. 2013). miR30a and miR30d were found to be expressed at a low-level in PTCs (Tetzlaff *et al*. 2007) and at a very low-level in ATCs (Schwertheim *et al*. 2009), respectively. It is intriguing to note that the miR30 family also antagonizes TGFβ-induced EMT and *in vitro* invasiveness of ATC-derived cells (Braun *et al*. 2010). miR130a, one of the most significantly downregulated miRNAs in thyroid cancer cells with BRAF mutation (Cahill *et al*. 2007),



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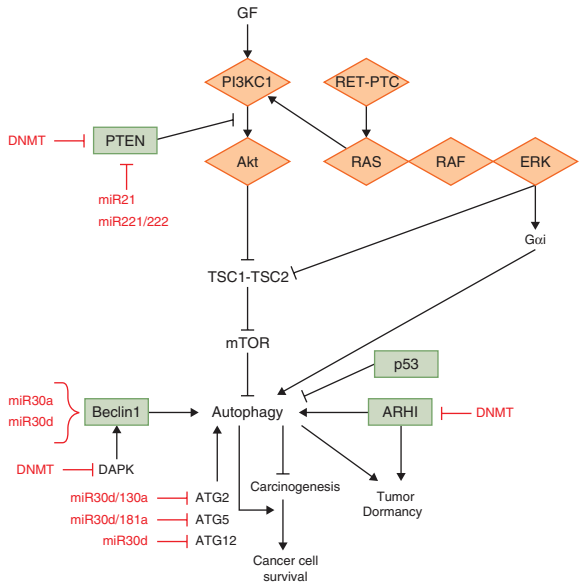


**Figure 6**
Genetic and epigenetic links between thyroid carcinogenesis and autophagy regulators. The scheme summarizes the potential links between autophagy and thyroid cancer progression at both genetic and epigenetic levels. Oncogenes are represented by rhombus and oncosuppressor genes by rectangles. Epigenetic regulations by DNMTs and miRNAs are indicated. GF, growth factor. Full colour version of this figure available via http://dx.doi.org/10.1530/ERC-13-0271

is known to downregulate the expression of ATG2B (Kovaleva *et al.* 2012), while miR181a, which is expressed at a very low level in the presence of *RET/PTC1* rearrangement (Cahill *et al.* 2006), is known to downregulate ATG5 (Huang *et al.* 2012, Tekirdag *et al.* 2013). Finally, miR183 was recently shown to negatively regulate the expression of LC3 in MTC cells (Abraham *et al.* 2011).

The scheme in Fig. 6 summarizes the potential links between autophagy and thyroid cancer progression at both genetic and epigenetic levels.

## Can we exploit autophagy for the therapy of thyroid cancers?

Autophagy-targeted therapy is nowadays considered a valuable strategy to combat radio- and chemo-resistant cancers (Chen & Karantza 2011, Yang *et al.* 2011, Gundara *et al.* 2012). Accumulating experimental data suggest that the efficacy of such therapies is strictly dependent on the actual level of ongoing autophagy in tumor cells, which is dictated by both genetic mutations and epigenetic phenomena, besides the dynamic influence of the tumor microenvironment.

The first report suggesting the involvement of autophagy in the response to therapeutic treatments of thyroid cancer dates back only a few years. Lin *et al.* (2009) found that both doxorubicin and radiation induced autophagy and cell death in cultured PTC cells. As the inhibition of Vps34-mediated autophagy by 3-methyl adenine increased the resistance toward these treatments, these authors concluded that autophagy was instrumental to cell toxicity in both treatments.

With regard to the therapy of thyroid cancer, besides radiation, new molecular therapies are emerging which employ kinase inhibitors, proteasome inhibitors, and epigenetic modulators (Catalano *et al.* 2010), and some of these drugs have been shown to also affect the autophagy process. For instance, sorafenib and sunitinib, two small molecules that inhibit the activity of RET kinase and that are commonly used for the treatment of MTCs, were shown to induce autophagosome accumulation in MTC cultured cells (Lin *et al.* 2012*a*,*b*). Apoptosis of MTC cells induced by these two drugs was abrogated when the essential autophagy protein ATG5 was silenced, and conversely it was enhanced when autophagy was concomitantly upregulated by co-treating with the mTOR inhibitor, everolimus (Lin *et al.* 2012*a*,*b*). Everolimus and other mTOR inhibitors are being tested for their potential efficacy against thyroid cancers (Gild *et al.* 2013, Lim *et al.* 2013). Everolimus is a rapamycin analog and it was introduced to the clinical management of epithelial cancers because of its ability to halt the biosynthetic and proliferative pathways downstream of the AKT–mTOR axis, but it is now recognized that rapamycin analogs may also elicit their beneficial effects via induction of autophagy. Consistently, sensitization of PTC cells to doxorubicin and radiotherapy by everolimus (known also as RAD001) was shown to strictly depend on autophagy (Lin *et al.* 2010).

Statins, when used for the treatment of thyroid cancers, may also act through the induction of autophagy. In a study, a xenograft of ATC in mice was successfully treated by combining paclitaxel, a cytoskeleton disrupting agent, with combretastatin A4 phosphate, a vascular-inhibiting drug, and it was found that the latter drug induced autophagy in cancer cells, besides inducing apoptosis of endothelial cells (Yeung *et al.* 2007). In addition, rosuvastatin, a statin with antiproliferative activity, was shown to induce autophagy and to promote the switch from pro-survival to pro-death autophagy in PTC cells (Zeybek *et al.* 2011).

Further, reversine, a synthetic purine analog with promising therapeutic potential, has recently been shown to induce growth arrest and apoptosis in FTC cells through the induction of autophagy (Lu *et al.* 2012).



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More recently, autophagy was shown to mediate the resistance to apoptosis induced by tumor necrosis factor-related apoptosis inducing ligand (TRAIL) in PTC and ATC cells (Jin *et al*. 2013).

As for epigenetic modulators, the use of HDAC inhibitors for autophagy therapy in cancer has been proposed (Yang *et al*. 2011). The HDAC inhibitor valproic acid (VPA) has been successfully employed in a combination therapy in ATC (Noguchi *et al*. 2009). VPA sensitized ATC cells to paclitaxel (Catalano *et al*. 2007) and to doxorubicin (Catalano *et al*. 2006). The small molecule lithium chloride was shown to synergize with VPA to inhibit the growth of *in vitro* cultured MTC cells (Adler *et al*. 2010). It is interesting to note, in this context, that lithium chloride is an inducer of mTOR-independent autophagy (Fornai *et al*. 2008), and that VPA, by inhibiting HDAC6, negatively interferes with the formation of the autophagolysosome (Lee *et al*. 2010).

Demethylating agents could also work in this manner. For instance, decitabine (2′-deoxy-5-azacytidine) has been shown to favor apoptosis by conventional chemotherapeutics in leukemic cells through the hyperinduction of autophagy (Schnekenburger *et al*. 2011).

Finally, in the near future we might be able to exploit miRNA-targeting autophagy for the cure of thyroid cancers (Gundara *et al*. 2012).

## Concluding remarks

The paradoxical role of autophagy in cancer development and progression has important clinical implications in therapy, because it suggests that autophagy-inducer drugs may have benefits in preventing the development and growth of cancer cells while autophagy-inhibitor drugs should improve the efficacy of anti-cancer therapies in developed and metastatic cancers. Indeed, both pro- and anti-autophagy therapeutic drugs have shown their efficacy in *in vitro* and *in vivo* models of cancer, as well as in clinical trials (Chen & Karantza 2011, Gundara *et al*. 2012, Wu *et al*. 2012). These contradictory results could be explained by considering that the actual level of autophagy in cancer cells was likely to be different in the different experimental models because of differences in the genetic and epigenetic background, and also because of the influence of stromal factors (e.g., vascularization, presence of cytokines, interactions with fibroblasts and macrophages).

Whether autophagy can represent a valuable target for the therapy of thyroid cancers remains speculative. A deeper knowledge of the mechanistic links between autophagy and thyroid carcinogenesis is expected to clarify the diagnostic and prognostic potential of autophagic-related biomarkers and could greatly contribute to a more rational use of novel therapeutic approaches based on the modulation of autophagy to cure thyroid cancers.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review.

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