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Contribution of PKB/AKT signaling to thyroid cancer

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1. ABSTRACT

The family of serine/threonine kinases B/Akt (hereafter Akt) represents a central node in signalling pathways downstream of growth factors, cytokines, and other cellular stimuli. In mammalian cells the Akt family comprises three highly homologous members -known as Akt1/PKBα, Akt2/PKBβ, and Akt3/PKBγ- that regulate several processes including cell proliferation and survival, growth and response to nutrient availability, migration, tissue invasion and angiogenesis. Aberrant activation of Akt is involved in a variety of human cancers including those arising in the thyroid gland. Here, we review the contribution of Akt-dependent pathway in the proliferation of normal thyrocytes, the different pathogenic mechanisms underlying aberrant Akt signalling in thyroid malignancies as well as the relative roles of Akt substrates that most likely contribute to the onset and/or progression of thyroid cancer. Finally, we discuss the current therapeutic strategies targeting the components of the PI3K/Akt pathway in the context of thyroid malignancy.

2. INTRODUCTION

Thyroid cancer is the most common malignant tumour of the endocrine system and accounts for approximately 1% of all newly diagnosed cancer cases (1, 2). The major histological types of thyroid cancer are derived from the epithelial cells that line follicles and are classified into papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), poorly differentiated thyroid carcinoma (PDC) and anaplastic thyroid carcinoma (ATC) (3, 4). Medullary thyroid cancer derived from para-follicular cells is a relatively rare malignancy that will not be discussed here.

PTC is the most frequent type of thyroid malignancy, and accounts for approximately 80% of all cases (3), FTC accounts for approximately 15% of all thyroid malignancies whereas PDC and ATC are rare (<2% of all thyroid cancer). PDC and ATC can develop directly or from pre-existing well-differentiated PTC or FTC, with

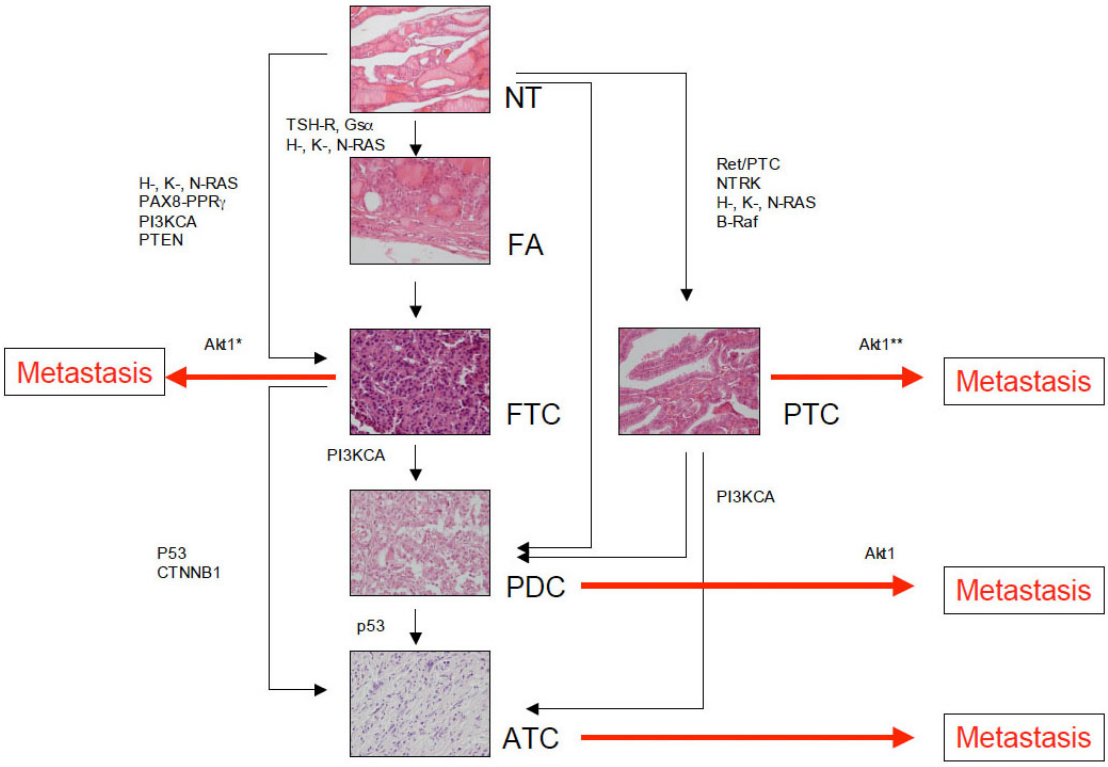

Figure 1. Schematic stepwise model for the origin and the progression of human thyroid cancer. PTC is characterized by genetic alterations that lead to the constitutive activation of the MAPK signalling pathway: these include rearrangement of Ret/PTC, rearrangement of TRKN1, activating gain-of-function mutations of B-Raf or activating gain-of-function mutations in one of the three Ras genes. Ret rearrangements are specific for PTC. Ret/PTC rearrangements are found on average in ~20% of sporadic PTC in adult patients and is more common in patients with a history of radiation exposure (50–80%). The TRK oncogenes occurs in PTC, with a frequency of <10%. Mutations of the B-Raf gene are commonly found in PTC, PDC and ATC (45% of cases) but are rare in FTC. Virtually all mutations involve nucleotide 1799 that results in a valine-to-glutamate substitution at residue 600 (V600E) with consequent activation of B-Raf kinase. Ras mutations are relatively rare in PTC (10-20%) and are most common in FA (20–40%) and conventional FTC (40–50%). Ras mutations have been described also in 18%–27% of PDC and in 50%–60% of ATC. The PAX8/PPARγ rearrangement, typical of FTC, results from t(2;3) (q13,p25) translocation and leads to the fusion of the PAX8 gene, a member of the paired box (PAX) family of transcription factors, with the PPARγ gene that encodes a nuclear receptor protein. PAX8/PPARγ rearrangement occurs in in 2%–10% FA and 35% FTC. Alterations involving the PI3K/Akt signalling pathway, as well as those involving the p53 and CTNNB1 gene, are common in PDC and ATC and thus play a role particularly in later stages of tumour progression. In particular, PIK3CA mutations have been observed in PDC and ATC whereas Akt1 mutations have been observed exclusively in recurrence of PDC, Hürthle cell carcinoma (HCC) and in the follicular variant of PTC (FV-PTC) and/or in the metastatic lesions thereof. Akt1*, mutations in the gene encoding Akt1 in HCC; Akt1**, mutations in the gene encoding Akt1 in FV-PTC. It is of note that the stepwise progression of thyroid cancer presented here is essentially a hypothetical model.

PDC that can further progress into ATC (4) (Figure 1). PTC and FTC are well differentiated, indolent tumours with rather good prognosis that are generally curable with current treatments. In contrast PDC and ATC represent aggressive partially or completely undifferentiated form of thyroid cancer, respectively.

However, aggressive variants of well-differentiated thyroid carcinomas have been identified such as the tall cell variant, columnar cell variant, diffuse sclerosing variant, insular carcinoma, and Hürthle cell (oncocytic, oxyphilic) carcinomas (HCC). HCC are rare entities comprising ~5% of epithelial thyroid tumors that were previously classified as follicular carcinomas by the World Health Organization, though they are now recognized as a distinct clinicopathological entity (5, 6). The Tall Cell Variant of papillary thyroid carcinoma (TCV-PTC), named for its distinct morphology, is characterized by tumour cells being twice as tall as they are wide (5, 6).

Patients with ATC have a mean life expectancy of few months, representing the major therapeutic challenge for thyroid cancer therapy (4, 7, 8). The main cause of thyroid cancer-related mortality is due to the surgical inoperability at diagnosis of many patients and to the frequent insensitivity exhibited by PDC and ATC patients to radioiodine treatment. Therefore, there is a compelling need for ameliorating the comprehension of

Table 1. Cellular models of human thyroid cancer

			MUTATIONS IN ONCOGENES					
HISTOTYPE	**CELL LINE**	**SEX**	**HRAS**	**BRAF**	**PI3K**	**RET**	**TP53**	**CTNBB**
	BCPAP	F		V600E		WT	Asp259Tyr	WT
PTC	KTC-1	M	WT	V600E		WT		
	K1	M	His27His	V600E/WT	Glu549Lys	WT	Arg213Arg	WT
	TPC1	F	His27His	WT		RET/PTC1		WT
	TT2609-CO2	M						
	FT133	M						
FTC	ML1	F						
	WRO82-1	F		V600E/WT			Pro23Leu	
	8505C	F	His27His	V600E		WT	Arg248Gln	WT
	SW1736			V600E		WT		
	Cal-62	F		WT				
	T235	F						
	T238	F						
	Uhth-104	F		V600E				
ATC	ACT-1			WT				
	HTh74	F	His27His	WT		WT		WT
	KAT18			WT		WT		
	TTA1			WT				
	FRO81-2			V600E/WT		WT		
	HTh7			WT		WT		
	C643	M	Gly13Arg	WT		WT	Arg248Gln	WT
	BHT101	F						
	KTC-2	F						

Mutations and rearrangements reported in oncogenes and tumour suppressor genes in human thyroid cancer-derived cell lines (12-14).

Table 2. Animal models of thyroid cancer

	MOUSE LINE	**STRAIN**	**PATHOLOGY**	**METASTASIS**	**THYROID FUNCTION**
	RET/PTC1	CS57BL/6J	22% with PTC	No	NA
	RET/PTC1	FVB/N	100% with multifocal PTC	No	Normal/hypothyroidism
	RET/PTC3	C3H/He	31% with PTC by 3 months	Lymph Nodes	NA
PTC	*$BRAF^{V600E}$(Tg-BRAF2)*	FVB/N	93% with PTC by 3 months	Lymph Nodes	TSH increased
	$BRAF^{V600E}$(Tg-BRAF3)	FVB/N	25% PTC at 3 months	No	TSH increased
	TRK-T1	B6C3F1	23% with PTC-like cancer	No	NA
	H-Ras^{G12V}	Mixed	3 out of 4 mice developed PTC	Unclear	Normal T4 level
	Prkar1a$^{A2/+}$	Mixed	95% with PTC-like cancer	No	NA
	K-Ras^{G12V}	C57BL/6J,DBA/2	2% with FTC	No	Normal
	Pten $^{-/-}$	FVB/N crossed with 129Sv	FTC in females	No	Normal TSH and T4 levels
	N-$Ras^{Glu61Lys}$	Founders crossed with C57BL/6J	11% adenoma, 29,5% FTC/mixed	Liver, lung, bone	Elevated TSH
FTC	*K-Ras^{G12D} Pten $^{-/-}$*	129SV	100% FTC with local invasion	Lung	Low TSH/High T4
	TRβ$^{PV/PV}$	C57BL/6Jx129SV	100% FTC	Lung	Elevated TSH, T3 and T4
	1b-adrenergic receptor	Founders crossed with C57BL/6J	3 out of 6 lines with goiter and FTC	Lung	Elevated T4
	Rap1b^{G12V}	FVB(Taconic)	FTC after treatment with a goitrogen	No	Normal

Activation of TSHR or PKA is not sufficient to initiate tumourigenesis in mice. Ret/PTC rearrangement and B-Raf V600E mutation are able to initiate thyroid cancer *in vivo*. Ras activation contributes to the development of FTC and PTC. Conditional loss of Pten in the thyroid gland renders the thyrocytes highly susceptible to neoplastic transformation (255). Pten mutant mice develop diffuse goiter characterized by enlarged follicles, in the presence of normal TSH and T4 hormone levels and by 10 months of age, develop follicular adenomas. The concurrent activation of K-Ras (mutation G12D) and PI3K (Pten deletion) in thyroid cells led to aggressive, invasive and metastatic FTC (73).

thyroid tumourigenesis and for improving the treatment of patients with PDC and ATC.

The past decade has witnessed significant expansion in the understanding of the molecular basis of thyroid cancer (9). Cytogenetic and molecular analysis of human thyroid tumours have allowed the identification of several genes that contribute to the development of thyroid carcinomas and have uncovered the association between certain molecular alterations and a specific type of thyroid cancer (9, 10). The use of *in vitro* cellular models of thyroid cancer has provided invaluable tools to study the molecular mechanisms responsible for aberrant activation of intracellular signalling pathways that occurs during the malignant conversion of human thyrocytes (11, 12). More than 30 cell lines derived from human thyroid carcinomas have been established so far (Table 1), though recent studies have reported cross-contaminations and/or misidentifications of some of the most used cell lines (13, 14) that has led to a critical revision of the literature (15). In parallel, multiple genetically modified mice that harbour thyroid-specific activated oncogenes or inactivated tumour suppressor genes have been generated with the aim to model the different types of human thyroid cancer (16). These mouse strains have significantly enhanced our understanding of the mechanisms of thyroid tumour development and dissemination (Table 2). From the use of human and experimental models, it has become apparent that the malignant transformation of the normal thyroid follicular cell involves multiple genetic events that

sequentially activate certain oncogenes (i.e. Ras, Ret/PTC, NTRK1, B-Raf, PIK3CA, Akt1) and inactivate specific tumour suppressors (i.e. p53, PTEN) (9, 10, 12). See Figure 1 for a detailed summary of the known molecular alterations found in thyroid cancer.

This review -updated to may 2010- is focused on the issues related to Akt signalling that are most relevant to thyroid tumourigenesis: 1) the role of Akt signalling in normal and transformed follicular thyroid cells; 2) the molecular mechanisms that dysregulate Akt signalling in thyroid cancer; 3) the role of Akt substrates in the malignant transformation of thyrocytes; 4) the current therapeutic strategies targeting the components of the PI3K/Akt pathway in the contest of thyroid malignancies.

2. PROTEIN KINASE B/Akt

The PI3K-Akt pathway is a fundamental signalling cascade through which different proliferative, survival and/or differentiative signals are funnelled from tyrosine kinase receptors in multiple cell types, including thyrocytes (17, 18) (Figures 2 and 3). The Akt kinase represents the primary downstream mediator of the effects of the PI3K pathway, and plays a central role in both normal and pathological signalling. In mammalian cells Akt comprises three highly homologous members (>80% protein sequence identity) termed Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, encoded by three different genes located on chromosomes 14q32, 19q13 and 1q43, respectively (Figure 4). Akt kinases have been included into the AGC kinase family, because they show extensive homology within their kinase domains to PKA and PKC (18, 19). Akt kinases share the same structural organization, containing an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory region. The PH domain of Akt can bind specifically to D3-phosphorylated phosphoinositides with high affinity and mediates Akt activation (20, 21). See Figure 4 for a schematic model of Akt kinases.

Despite their sequence similarity however, Akt isoforms are functionally distinct (22), as suggested by the different phenotypes of Akt1−/− (23, 24), Akt2−/− (25, 26), Akt3−/− (27), as well as double mutant Akt1−/−/Akt2−/− (28), Akt1−/−/Akt3−/− (29) and Akt2−/−/Akt3−/− mice (30). *Akt1*-/- mice are smaller and have shorter life span than wild type littermates and exhibit increased spontaneous apoptosis in the testis and thymus (23, 24). In contrast, *akt2-/-* mice show insulin resistance and a diabetes mellitus-like syndrome (24) and *akt3-/-* mice show brain defects (27).

More relevant in the context of the oncogenic properties ascribed to Akt signalling are recent studies showing that Akt1 promotes proliferation and inhibits migration and invasiveness of breast cancer cells in response to oncogenic signals, while Akt2 has apparently the opposite effects (31-34). However, the three Akt isoforms apparently have opposite roles in different cellular contexts, which may have important therapeutic implications. In fact, Akt1 positively stimulates migration in MEF and lung epithelial cells (35, 36). Moreover, nuclear translocation of activated Akt1 is associated with cell invasion and migration in thyroid cancer cells (37).

In this regard, it is of note that the three Akt isoforms differ in their ability to transduce certain oncogenic signals *in vivo* such as those initiated by the Neu and PyMT oncogenes in mammary epithelium (38). Accordingly, ablation of Akt1 inhibits whereas ablation of Akt2 accelerates mammary tumour development initiated by Neu and PyMT oncogenes, while ablation of Akt3 is phenotypically neutral (34). Also the expression of Akt1, Akt2 and Akt3 apparently contribute to the different roles of Akt isoforms. Akt1 and Akt2 are widely expressed while tissue distribution of Akt3 seems to be more restricted, being primarily expressed in brain and testis (39). Accordingly, Akt1 and Akt2 are the principal isoforms expressed in the thyroid gland (40).

Growth factors and cytokines activate Akt through stimulation of PI3K activity (20). Accordingly, PI3K inhibition by chemical inhibitors (wortmannin, LY294002) or PTEN expression results in inhibition of tyrosine kinase receptor-mediated Akt activation (41). Activation of Akt is a multi-step process that involves membrane binding and phosphorylation. Upon activation, PI3K produces increased levels of phosphatidylinositol-3,4,5-trisphosphate (PtdIns-3,4,5-P3) and phosphatidylinositol-3,4-trisphosphate (PtdIns-3,4-P2), which contribute to recruit Akt to the plasma membrane where it binds to the phosphoinositides through its PH domain (41). Once at the cell membrane, Akt activation is obtained through phosphorylation on two critical aminoacids. One such residue lies within the kinase domain activation loop (Thr 308 in Akt1) and is phosphorylated by a PH-domain containing protein, PDK1 (42). This is thought to be the major activating phosphorylation event. Full Akt activity requires the subsequent phosphorylation of a second amino acid in the C-terminus (Ser 473 in Akt1). The identity of the serine 473 kinase is not completely established but this phosphorylation event may results from the rapamycin-insensitive mTOR complex (mTORC2) (43). Upon activation, Akt leaves the plasma membrane and phosphorylates a number of substrates both in the cytoplasm and in the nucleus, which mediates Akt-dependent regulation of cell growth and survival, mitogenesis, migration, glucose metabolism and protein translation (44). Akt targets include: Glycogen Synthase Kinase-3 (GSK-3α and β), tuberous sclerosis complex 2 (TSC-2), the pro-apoptotic Bcl-2 family members Bad and Bim, procaspase-9, IκB Kinases (IκKα and β), the Forkhead family of transcription factors (FOXO), the ubiquitin ligases Mdm2 and Skp2, the CDK inhibitors p21 and p27, the kinases Raf and B-Raf, and others (20, 44). Akt signalling is counteracted by phosphatases PHLPP1 and PP2A (45, 46).

3. AKT ACTIVATION: UPSTREAM PATHWAYS

The central core of the Akt pathway is composed of upstream regulators of Akt function that include PI3K and the Phosphatase Tensin Homolog Deleted on

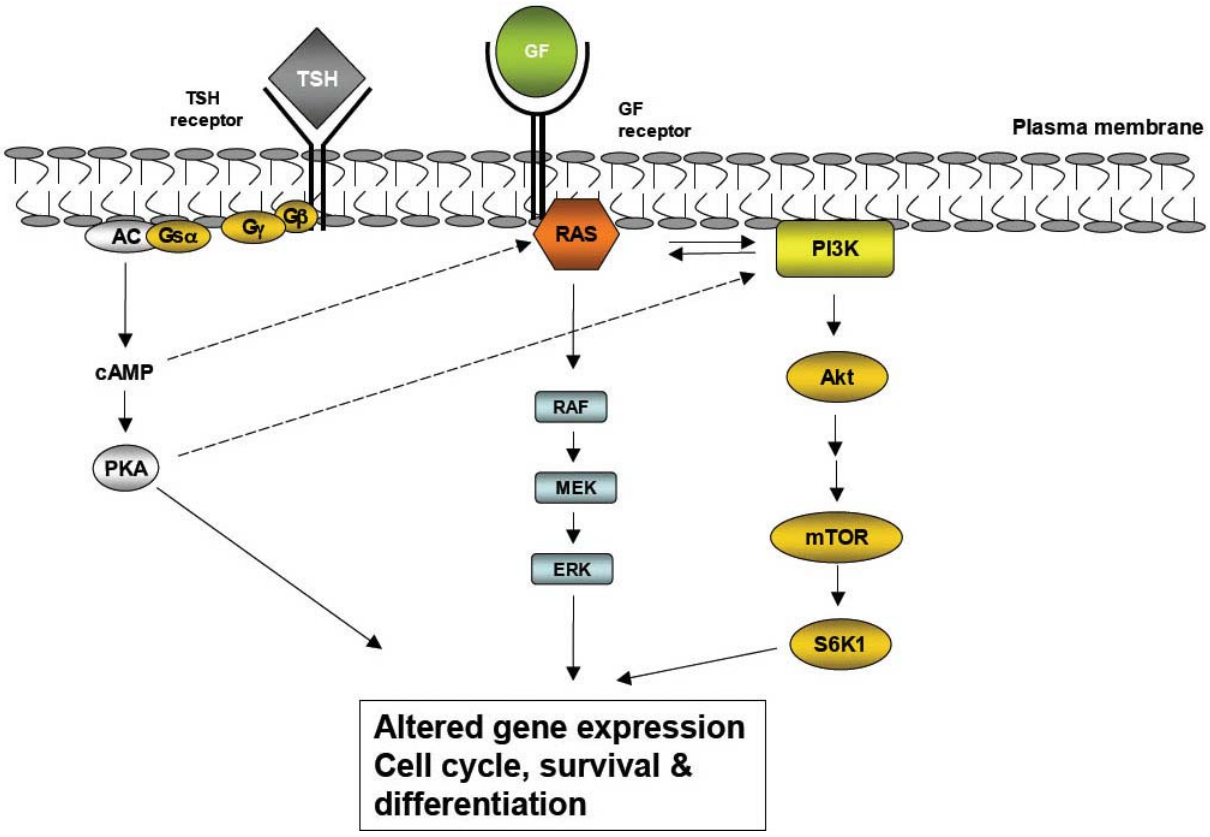

Figure 2. Proliferative pathways in thyroid cells. The cellular *in vitro* models that have been used to investigate the mechanisms involved in the proliferation of thyroid cells include rat thyroid cells lines (FRTL-5, WRT, and PC Cl3 cells) and short-term primary cultures of dog and human thyrocytes. DNA synthesis in canine thyrocytes requires the simultaneous presence of TSH and insulin/IGF1. Insulin or IGF1 alone have minimal effects on DNA replication, but they support DNA synthesis and cell cycle progression induced by TSH, EGF, bFGF, or phorbol. HGF is the only growth factor that acts as a full mitogen in dog thyrocytes, stimulating proliferation also in the absence of insulin/IGF-I. In rat thyroid cells, TSH and insulin/IGF-I cooperates to synergistically promote proliferation; insulin/IGF1 represent powerful mitogens whereas TSH is not able to induce DNA synthesis in the absence of insulin. The role of TSH in the G1 progression is to make cells competent to respond to insulin/IGF-I, which leads to the activations of MAPK and PI3K. In human thyroid cells TSH is able to induce DNA synthesis in serum-free primary cultures of adult. The mitogenic effect of TSH is increased by the presence of IGF-I or insulin, which alone weakly stimulate DNA synthesis (11). TSH is by far the most important physiological regulator of growth and function of thyrocytes. By binding to its cognate heterotrimeric G protein-coupled receptor, the TSH receptor (TSH-R), TSH causes dissociation of the G protein into α and βγ subunits and activates the adenylyl cyclase/cAMP cascade. cAMP activates protein kinase A (PKA), which in turn, is required for thyroid cell differentiation and proliferation. In dog and human thyroid cells TSH does not activate Ras, PI3K, Akt or MAPK, and thus requires insulin- and/or IGF1-dependent activation of PI3K and its effectors, Akt and p70S6 kinase (p70S6K). In rat cells, at difference with dog and human thyrocytes, TSH-induced proliferation apparently requires Ras, Akt and PI3K. Ras activity appears to be necessary for TSH to induce the transition from quiescence to G1. Growth factors (i.e. EGF, HGF) regulate cell cycle progression of dog thyrocytes with different mechanisms that differ from those elicited by TSH. EGF and HGF activate Ras, the MAPK cascade but differ in their ability to activate the parallel PI3K/Akt pathway. EGF is not a full mitogen for dog thyrocytes because it potently activates the MAPK pathway but only weakly Akt; HGF is unique in triggering proliferation of thyrocytes in the absence of insulin because it is the only growth factor that can strongly activate both Ras-MAPK and PI3K-Akt. Akt apparently mediates the effects exerted by insulin/IGF1 as well as those exerted by serum on cell cycle progression of thyrocytes.

Chromosome Ten (PTEN) as well as downstream effectors including kinases [i.e. the mammalian Target of Rapamycin (mTOR), GSK3α and β], transcription factors (i.e. FOXO, NF-κB), and CDK inhibitors (p21 and p27) (20, 44). See Figure 3 for a detailed description. In this section we will review those molecules that may play a role in the activation of Akt in thyroid cancer.

3.1. The phosphatidylinositol 3-kinase

PI3Ks are a family of intracellular lipid kinases that generate the lipid second messenger PtdIns-3,4,5-P3 and PtdIns-3,4-P2. To date, several members of the PI3K family have been isolated in mammalian cells and they are grouped into three classes according to structure and substrate specificity (47). Class I PI3Ks phosphorylate

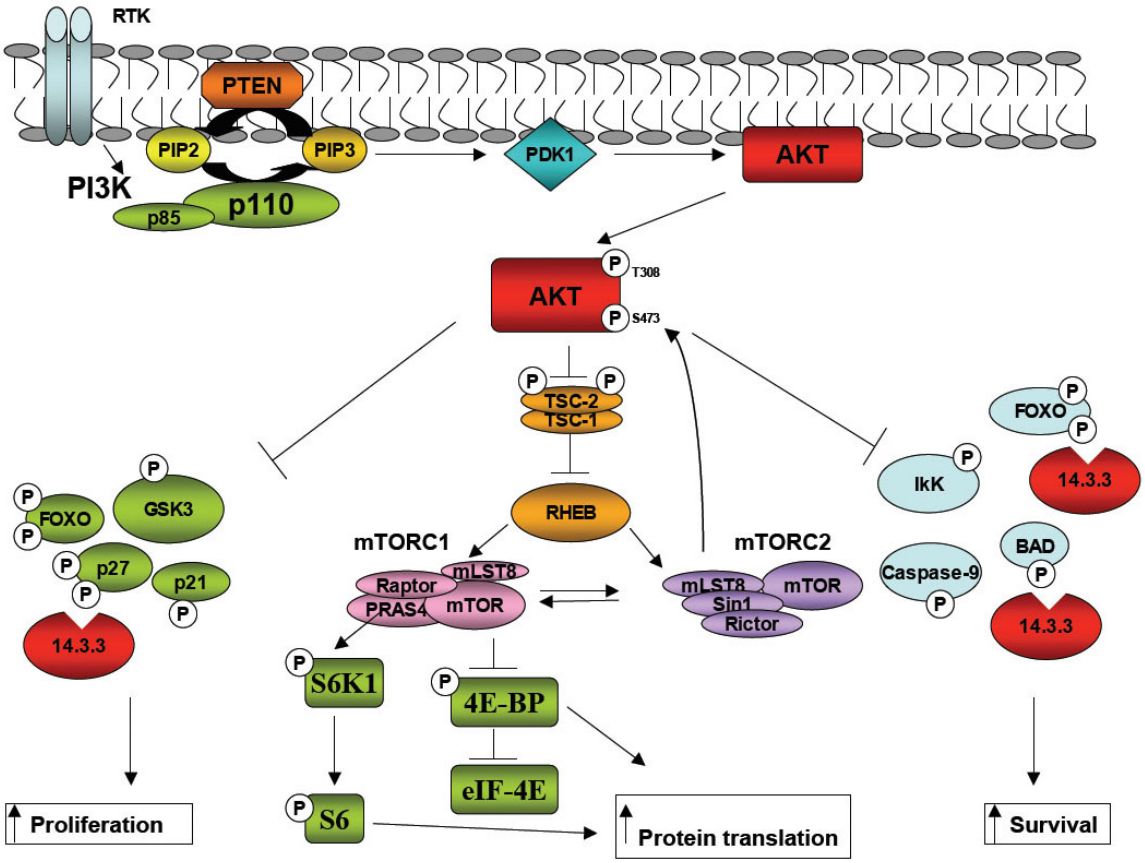

Figure 3. The PI3K-Akt pathway. Upstream activation of Akt by the growth factor receptor (RTK) pathway and cellular functions of some Akt substrates. Once activated, Akt phosphorylates a number of substrates in the cytoplasm and in the nucleus. Akt targets include: glycogen systhesis kinase-3 (GSK-3α and β), tuberous sclerosis complex 2 (TSC-2), the pro-apoptotic Bcl-2 family members Bad and Bim, procaspase-9, IκB Kinases (IκKα and β), the forkhead family of transcription factors (FOXO), the ubiquitin ligases Mdm2 and Skp2, the CDK inhibitors p21 and p27, the kinases Raf and B-Raf, and others. Nuclear proteins: FOXO, p27, p21; cytoplasmic proteins: GSK3, IκK, TSC2, caspase-9, S6K, 4E-BP; mitochondrial proteins: BAD.

PtdIns-3,4-P3 whereas class II and III PI3Ks use phosphatidylinositol as a substrate. Class I PI3Ks are further subdivided according to the signalling receptors that activate them: usually class IA PI3Ks are activated by growth factor receptor tyrosine kinases (RTKs); class IB PI3Ks are activated by G protein–coupled receptors (GPCRs) (48). Class I PI3Ks are heterodimeric molecules composed of a catalytic subunit known as p110 and a regulatory subunit denoted p85, which contains two SH2 (Src homology) domains that allow interaction with phosphotyrosines on activated tyrosine kinase receptors. This results in recruitment of the protein to the plasma membrane and activation of the enzymatic activity. There are three variants of the p110 catalytic subunit designated p110α, β, or δ, expressed by separate genes (PIK3CA, PIK3CB, and PIK3CD for p110α, p110β, and p110δ, respectively). By contrast, there are five variants of the p85 regulatory subunit, designated p85α, p55α, p50α, p85β, or p55γ; the first three regulatory subunits represent splice variants of the same gene (PIK3R1), the other two encoded by different genes (PIK3R2 and PIK3R3, respectively) (48-50).

The α-type (PIK3CA) and β-type (PIK3CB) p110 subunits are widely expressed in different tissues, including thyroid whereas the other type of p110 subunits has a limited expression. The most highly expressed regulatory subunit is p85α. So far a central role in cancer has been demonstrated only for class IA PI3Ks, which transduce signals downstream of oncogenic tyrosine kinase receptors (51, 52). In fact, PIK3CA, encoding the class IA PI3K catalytic subunit p110α, is the only PI3K gene identified with common mutations and gene amplification in human cancer (51). Most of these mutations are gain-of-function mutations that are located in hot spot regions in the helical and kinase domains of the gene encoding p110α (E542K, E545K, and H1047R) (52). These mutations result in a p110α variant that is active independent of the p85 regulatory subunit and leads to increased cell proliferation, invasiveness, resistance to apoptosis, and malignant transformation (51, 52). Several reports indicate that, *in vitro,* cellular transformation activity exerted by mutant PIK3CA is dependent on mTOR, which suggest that the major downstream signal for tumour promoting activity of constitutively active PI3Kα is funnelled through the Akt-

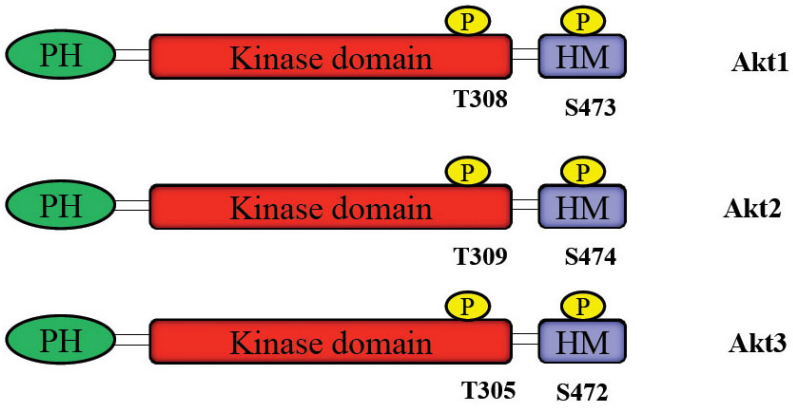

Figure 4. Schematic representation of the three Akt isoforms. In mammalian cells Akt comprises three highly homologous members (>80% protein sequence identity) termed Akt1/PKBα, Akt2/PKBβ and Akt3/PKBγ, encoded by three different genes located on chromosomes 14q32, 19q13 and 1q43, respectively. Activation of Akt is a multi-step process that involves membrane binding and phosphorylation. Akt kinases share the same structural organization, containing an N-terminal pleckstrin homology (PH) domain, a central catalytic domain and a C-terminal regulatory region. The PH domain of Akt can bind specifically to D3-phosphorylated phosphoinositides with high affinity and mediates Akt activation. Full Akt activation is obtained through phosphorylation on two critical aminoacids: one such residue lies within the kinase domain activation loop (Thr 308 in Akt1) and the second lies in the C-terminus (Ser 473 in Akt1).

mTOR axis (53). However, recent studies have also suggested that PI3K might promote cancer also through an Akt-independent pathway (54).

3.2. The phosphatase PTEN

PTEN (phosphatase and tensin homolog deleted on chromosome 10)/MMAC1 (mutated in multiple advanced cancers)/TEP-1 (TGFβ-regulated and epithelial cell-enriched phosphatase) (hereafter referred as to PTEN) is a tumour suppressor gene localized to chromosome 10q23 (55-57). The PTEN protein has been shown to have protein and lipid phosphatase activity (58, 59). The lipid phosphatase activity of PTEN can dephosphorylate the D3 position of PtdIns-3,4-P2 and PtdIns-3,4,5-P3, the lipid products of the PI3K lipid kinase activity (60), thus antagonizing signalling through the PI3K pathway. Indeed, cells lacking PTEN function exhibit a marked increase in the intracellular levels of PtdIns-3,4,5-P3 and Akt activation (61, 62). PTEN represents a pivotal regulator of critical cellular functions such as proliferation and survival. A large body of evidence indicate that PTEN functions as a tumour suppressor (63). Inactivating germ-line mutations in the gene encoding PTEN are the cause of the so-called PTEN hamartoma tumour syndrome (PHTS), a tumour susceptibility syndrome that includes Cowden syndrome (CS), Bannayan-Riley-Ruvalcaba syndrome (BRRS), Proteus syndrome (PS), and Proteus-like syndrome (64). PHTS includes benign and malignant thyroid neoplasias as part of the phenotype. In addition, loss of PTEN function is a frequent finding in sporadic tumours. Several mechanisms have been reported to account for the reduced levels of PTEN observed in cancer cells, including gene mutations and LOH, reduced transcription caused by gene promoter hypermethylation, reduced translation via microRNA (miR21) overexpression or increased protein degradation (65-67).

Targeted disruption of *Pten* in the mice leads to embryonic lethality (68). Mutant embryos show abnormal proliferation but not significant differences in apoptosis. Interestingly, Pten+/- mice, as the Cowden's syndrome patients, develop a variety of neoplasms including thyroid tumours (69-71). The majority of these tumours exhibit loss of the wild type allele, underscoring the importance of loss of PTEN function in tumour formation.

3.3. Ras GTPases

The Ras genes (H-Ras, K-Ras, and N-Ras) encode highly related proteins with GTPase activity that are located at the inner surface of the cell membrane and play a central role in the intracellular transduction of signals arising from cell membrane tyrosine kinase receptors and G-protein-coupled receptors (GPCRs) (72). In its inactive state, Ras is bound to guanosine diphosphate (GDP); upon activation, it releases GDP and binds guanosine triphosphate (GTP), thus transiently activating downstream signalling and terminates signalling by hydrolizing GTP (72). Ras proteins convey signals from tyrosine kinase receptors and GPCRs to a cascade of mitogen-activated kinases (MAPK), which activate the transcription of target genes resulting in cell proliferation and/or to the PI3K/Akt pathway that contribute to cell growth and proliferation, migration and survival (72).

Ras mutations play an important role in malignant transformation and progression, representing the most common type of abnormality of dominant oncogenes in human cancer including thyroid carcinoma (9, 10). Oncogenic activation of Ras proteins (H-, K-, and N-Ras) is due to point mutations affecting the GTP-binding domain (codons 12 or 13) in Exon 1 or the GTPase domain (codon 61) in Exon 2, which lock the protein in the active GTP-bound form. Ras mutants are able to activate both the PI3K-Akt and MAPK signalling cascades and, conversely,

oncogenic transformation by mutant K-Ras requires activation of both MAPK and PI3K-Akt pathways (73). Consistent with this observation is the relative resistance of thyroid cancer cells harbouring Ras mutations to MEK inhibitors at difference of cell lines with B-Raf-V600E mutations (74, 75).

3.4. Tyrosine Kinase Receptors

Activation of tyrosine kinase cascades in thyroid cells regulates cell proliferation and differentiation (76). In 1998, Tanaka *et al* identified at least 21 tyrosine kinases expressed in thyrocytes, including 16 receptor-type and 5 non-receptor type, that in addition to TSH, may play a role in regulating the growth and differentiated functions of thyroid cells (77). In particular, since IGF1, FGF, EGF and the corresponding receptors are expressed by thyroid cells, it is likely that autocrine and/or paracrine loops involving such growth factors regulates growth and differentiation of normal thyrocytes (78, 79). IGF1 and EGF have been shown to stimulate proliferation of rat and dog thyroid cells synergistically with TSH (80, 81); HGF has been reported to have the strongest mitogenic activity on dog thyroid cells (82) (see legend to Figure 2). Moreover, certain subtypes of thyroid carcinomas are characterized by the aberrant expression of activated receptor-type tyrosine kinases such as Ret and NTRK1 proto-oncogenes consequent to chromosomal rearrangements (9, 10) or by overexpression of tyrosine kinase receptors such as MET [the receptor for hepatocyte growth factor (HGF)] or the EGF receptor (EGFR).

The Ret/PTC oncogene is generated by chromosomal rearrangements resulting in the fusion of the Ret tyrosine-kinase domain to the 5'-terminal region of heterologous genes. All RET-fused genes provide putative dimerization domains to the chimeric Ret/PTC genes, allowing dimerization and ligand-independent activation of Ret tyrosine kinase, which is essential for the transformation of thyroid cells. Among human tumours, Ret rearrangements are apparently restricted to the thyroid gland and are specific for PTC (81). Ret/PTC rearrangements are found on average in ~20% of adult patients, though is more common in in patients with a history of radiation exposure (50–80%) (84-86). However, the reported prevalence of RET/PTC from different geographic regions varies widely, with the highest prevalence reported in the pediatric population. In particular, increased prevalence of Ret/PTC rearrangements was observed in children from the areas affected by nuclear disaster at Chernobyl (3, 4).

The NTRK1 (or TRKA) gene located on 1q22 encodes a tyrosine kinase receptor for the nerve growth factor (NGF). Similar to Ret, NTRK1 is activated in thyrocytes by chromosomal rearrangements that fuse the NTRK1 tyrosine kinase domain to the 5'-terminal region of heterologous genes. The recombination events that cause the oncogenic activation of NTRK1 include an inversion fusing NTRK1 to non-muscular tropomyosine (TPM3) gene located at 1q31, a different intra-chromosomal rearrangement that juxtaposes NTRK1 to the 5'-end of a translocated promoter region (TPR) gene localized at 1q25 or to the 5'-sequence of a TRK-fused gene (TFG) localized on chromosome 3 (TRK-T1, TRK-T2 and TRK-T3 oncogenes, respectively) (reviewed in 87). In all cases the resulting chimeric proteins exhibit constitutively tyrosine kinase activity. The TRK oncogenes also appear restricted to PTC, with a frequency of 10% of cases (88).

Conceivably, all these activated tyrosine kinase receptors convey their mitogenic signals through the PI3K pathway. Indeed PI3K signalling has been shown to be required for the stimulation of mitogenesis by Ret/PTC1 or Ret/PTC3 oncogenes (89). Similarly, M*Et al*so converges on Akt activation (90).

4. PERTURBATIONS OF AKT SIGNALING IN THYROID CANCER

Combining all the data from the existing literature, it appears that in thyroid cancer Akt activation, as detemined by S473 phosphorylation, is frequent and is associated with more aggressive disease. Active Akt is observed more frequently in patients with undifferentiated cancer (40-50% of PTC and FTC; ~93% of ATC, respectively) (37, 91-97). Moreover, recent studies have suggested that cellular compartimentalization of activated Akt may be important in determining its cellular effects (37). In particular, it was proposed that nuclear localization of activated Akt1 promotes invasion and migration in thyroid cancer cells (37). In invasive FTC phospho-Akt localizes primarily to the nucleus, whereas in PTC, it localizes to the cytoplasm, except for the cells at the invasive edge or in metastatic regions where it is localized also in cell nuclei (37).

Different mechanisms that may account for increased Akt signalling in thyroid cancer cells have been proposed. These include amplification or activating mutation in two of the genes encoding Akt kinases, genetic abnormalities in the molecules upstream in the PI3K pathway such as mutation and/or amplification of the catalytic subunit of PI3K (PIK3CA) or loss of Pten (See 9, 10 for review). See Figure 4 for a summary of the molecular alterations in the members of the PI3K-Akt pathway in thyroid cancer. Gain-of–function mutations of two different Akt isoforms, namely Akt1 and Akt3, have been reported to occur in human cancer (98, 99). A unique mutation at nucleotide 49 of the gene encoding Akt1 that results in the substitution of a lysine for glutamic acid at the amino acid 17 (Akt1-E17K) within the PH has been recently discovered (98). The E17K substitution allows membrane recruitment of Akt1 independent of PtdIns binding, increases activity of Akt1 kinase, and confers to Akt1 the capability to transform fibroblasts *in vitro* and induce leukaemia in mice (98). Akt1-E17K mutant has been detected in multiple cancers such as breast, colon, ovarian, lung, endometrial, bladder and prostate carcinomas (98, 100-103). More recently, a mutation homologous to the E17K in Akt1 has been identified also in the PH domain of Akt3 in malignant melanoma (99). In thyroid cancer, the presence of a heterozygote E17K mutation in the Akt1 gene was observed at a relatively high frequency (9/55, 16%) in metastatic lesions of advanced cancer but

not in the corresponding primary tumours, which suggested that Akt1 mutations were acquired during tumour progression (94). Akt1 mutations were most common in metastasis of tall cell variant PTC (17%), Hürthle cell carcinoma (33%), and poorly differentiated PTC (19%) (94). Conversely, no mutation in the genes encoding Akt2 and Akt3 has been reported in thyroid cancer so far. Surprisingly, the knock-in of E17K mutant into the AKT1 gene had minimal phenotypic consequences and did not recapitulate the biochemical and growth characteristics observed with somatic cell knock in of PIK3CA mutations, suggesting that mutations in critical genes within the PI3K pathway are not functionally equivalent (104).

In addition to mutations, an increase in the gene copy number of Akt1 in FTC (8%) and ATC (~19%) and of Akt2 in FTC (~22%), respectively, has also been reported (97). It is not yet known whether amplified Akt differs from mutated Akt in its capability of to activate downstream Akt signalling.

Constitutive activation of Akt can occur also through mutations of other genes upstream in the pathway. Increased copy number of the PIK3CA gene, that encodes p110α, has been frequently detected in thyroid cancer. Gene amplification at 3q26.3, where the PIK3CA gene is located, is detected in 12-13% of FA, 5-16% of PTC, 24-30% of FTC and 50% of ATC (105-109), though ethnic variation between Middle Eastern, Western or Asian populations exists (96). In addition to PIK3CA, amplification of the gene encoding PI3K p110ß has been reported to occur in 46% of FTC and 38% of ATC (97). Constitutive activation of p110α is also caused by the occurrence of activating mutations in the PIK3CA gene. Several studies have reported the presence of activating mutations within the helical (exon 9) and/or the kinase (exon 20) domains of PIK3CA in primary thyroid cancer and cancer-derived cell lines (91-93 105, 107). PIK3CA mutations are rare in primary well-differentiated PTC (0-3%) (93, 106, 107), more common in well-differentiated FTC (6-13%) (93, 106) and consistently frequent in ATC (12-23%) (93, 106). PIK3CA p110α mutations are particularly common in the metastatic lesions of patients with radioactive-iodine refractory disease, similarly to Akt1 (94). So far, PIK3CA and Akt1 mutations are apparently mutually exclusive, suggesting that they may have equivalent roles (94).

Accordingly, it is not yet known whether PIK3CA mutations or amplification are sufficient to cause thyroid cancer *in vivo*. Mutant PIK3CA alleles are transforming in MCF-10 breast cells *in vitro* and in the chorioallantoic membrane of the chicken *in vivo* (110). However, transgenic mouse models indicated that activated PIK3CA mutant is able to induce fully malignant cancer in the lung but not in the ovary (111, 110). Therefore, further studies will be required to fully characterize the role of this oncogene in thyroid cancer development and progression.

In tumours where ATC coexisted with a better-differentiated tumour component, PIK3CA and Akt1 mutations were exclusively observed in the ATC component (93, 94, 109). This finding suggests an exclusive role for oncogenic PIK3CA and Akt1 mutants in promoting progression from more differentiated to less differentiated or completely undifferentiated cancer (93, 94). Consistently, mutant Akt1 and PIK3CA are found in the metastatic lesions but not in the corresponding primary tumours (94). Conversely, Ras and B-Raf are considered early events in thyroid carcinogenesis that are able to initiate tumours, since Ras mutations are observed both in FA and FTC and B-Raf mutations are generally found in both differentiated PTC and undifferentiated ATC. Accordingly, the generation of murine models has shown that B-Raf and N-Ras can initiate thyroid cancerogenesis *in vivo* (16 and references therein). These results suggest that, at difference with genes involved in the MAPK pathways (i.e. B-Raf) the constitutive activation of PI3K signalling is probably insufficient by itself to initiate the growth of a malignat thyroid cancer, since loss of PTEN results in follicular adenoma; conversely, aberrant PI3K signalling may facilitate progression and dedifferentiation of tumour cells (113).

Mutation analysis of human thyroid cancer indicates also that the MAPK and PI3K-Akt pathways cooperate in the pathogenesis and progression of advanced or metastatic thyroid cancer (93, 94, 106). The great majority of tumours carrying the Akt1-E17K (76%) also displayed concomitant B-Raf mutation, but not Ret/PTC rearrangement or Ras mutations, indicating that simultaneous signalling form Akt and B-Raf efficiently contribute to the development of dedifferentiated cancer (94). Similarly, most tumours (73%) with PIK3CA mutations also showed mutations in Ras and/or in B-Raf genes (93, 96). Likewise, the presence of PIK3CA amplification was observed in approximately half PTC harbouring Ras mutation (96) and in an even higher percentage of PDC (50%) and ATC (40-100%) with concurrent B-Raf mutations (93, 94). Whatever the explanation for coexisting mutations, the putative cooperation of PI3K/Akt signalling and Ras, or B-Raf signalling has already been observed in colon cancer and might have important implications also in the behaviour of thyroid carcinoma (114).

Expectedly, Akt activation is observed in almost all ATC harbouring PIK3CA mutations (>90%) (93). However, because Akt activation can occur regardless of the presence of PIK3CA or Akt1 mutations, other molecular mechanisms that account for Akt activation in undifferentiated thyroid cancer cases, such as PTEN down-regulation or Ras activation, have been proposed. Genetic alterations that inactivate the PTEN gene, including mutations and deletions, are uncommon in sporadic thyroid tumours (90, 106, 107, 115-117). Moreover, allelic losses of the PTEN locus at 10q23.3, though frequent in FA and FTC (up to 25%), are not coupled with mutations in the second allele (118, 119). Conversely, thyroid carcinoma frequently shows decreased expression of PTEN, at both mRNA and protein levels in ~40% of the well-differentiated thyroid carcinomas and lost in most ATC (90, 116-119), in many cases through methylation of the PTEN gene promoter (120, 121). In a limited subset of

thyroid carcinomas, PTEN inactivation has been associated with Akt activation (90).

Additional mechanisms that account for the activation of the PI3K/Akt signalling pathway in thyroid cancer involve aberrant signalling from mutant Ras or tyrosine kinase receptors (See 9, 10 for review). Conceivably, Ret/PTC or NTRK1 rearrangements, Met overexpression and Ras mutations play an important role in activating the PI3K/Akt signalling in thyroid cancer. Ras plays a role in aberrant Akt signalling in thyroid cancer, both directly or indirectly. Activation of the PI3K/Akt pathway by mutant Ras proteins has been shown to occur in FTC, which commonly harbours activating Ras mutations (122). Accordingly, PI3K is a well-characterized direct downstream effector of Ras (123). The p110 catalytic subunit of PI3K possesses a Ras binding domain that mediates binding and direct Ras-dependent activation. Expression of mutated Ras protein in thyroid cells induces activation of PI3K, and this causes an increase in the dependence of thyrocytes on PI3K signalling for survival (124). On the other hand, PI3K is an essential anti-apoptotic effector in the proliferative response of human thyroid cells to mutant Ras (125, 126).

The PI3K-Akt pathway can be efficiently activated also through signalling from aberrant tyrosine kinase receptors. This may be a relevant mechanism particularly in PTC, which most commonly harbours Ret/PTC and NTRK1 rearrangements or Met overexpression (9, 10). Different mechanisms have been proposed for Ret/PTC-dependent Akt activation in thyroid cells. As most tyrosine kinase receptors Ret/PTC can activates PI3K signalling, possibly through phosphorylation of Insulin Receptor Substrate 1 (IRS-1) (127) and XB130 (128), or through RAI(ShcC/N-Shc)-dependent recruitment of GAB1 to phosphorylated tyrosine 1062 (129), which was shown to be essential for Ret-mediated activation of PI3K. Accordingly, PI3K signalling is necessary to Akt activation by Ret/PTC, since PI3K-specific inhibitor LY294002 has been shown to reduce Akt activation in Ret/PTC-transfected rat thyroid cells (92). Alternatively, Ret/PTC can directly phosphorylate and activate Akt1 by direct phosphorylation of Y315, which facilitate Akt phosphorylation of T308 and S473 and activation (130, 131). Signalling of Ret/PTC1 and hepatocyte growth factor receptor (Met) also converges on Akt activation. Met overexpression alone strongly activates Akt signalling in thyroid cells (90). On the other hand, a strong correlation between expression of Met and Akt activation has been demonstrated in PTC (132).

5. SIGNALLING DOWNSTREAM AKT IN THYROID CANCER

A recent search of the literature has allowed the identification of over 100 non-redundant Akt substrates, for ~20 of which there have been multiple independent published reports (Figure 3) (44). Expectedly, it appears that each function downstream Akt –proliferation, survival, metabolism- is mediated by multiple targets and is frequently dependent on the cell context. It is worth noting that Akt can either cause the activation (*i.e.*, Mdm2, IκKα/β) or the inactivation (*i.e.*, p21, p27, Bad, procaspase-9, FOXO3a, and GSK3α/β) of specific substrates. In most cases Akt-dependent phosphorylation usually results in the change of stability, activity and/or localization of the substrate proteins, which contribute to increase cell proliferation, motility, protein synthesis and gluconeogenesis as well as inhibit apoptosis (44). One common mechanism whereby Akt-mediated phosphorylation results in substrate inhibition is the regulation of substrates' cellular localization as in the case of Bad, FOXOs, p27. Akt substrates' localization is mediated through the interaction with 14-3-3 proteins, cytoplasmic anchors that bind specifically to phosphoproteins and retain them in the cytoplasm (133) away from their targets (Figure 3).

5.1. The mTOR pathway

Among the numerous downstream effectors of Akt, activation of the mammalian target of rapamycin (mTOR) kinase [also known as FK506 binding protein 12-rapamycin associated protein (FRAP)1] has recently taken center stage, thanks to the increasing evidence that, in different cell types, it is directly responsible for growth and proliferation associated with PI3K activation (134, 135). mTOR is a serine/threonine protein kinase in the PI3K cascade that regulates protein synthesis and plays an important role in multiple biological processes such as cell growth and survival (136-138). By regulating ribosomal biogenesis and protein translation, mTOR controls cell growth and can restrict cell cycle progression in the presence of suboptimal growth conditions (134-137).

In mammalian cells, mTOR exists in two functionally distinct complexes, namely mTORC1 and mTORC2 (Figure 3). MTORC1 is a critical regulator of translation initiation and ribosome biogenesis and plays an evolutionarily conserved role in cell growth control (reviewed in 134). mTORC1 is composed of mTOR, Raptor, mLST8, and PRAS40 (proline-rich Akt substrate 40kDa), and is sensitive to inhibition by the macrolide antibiotic rapamycin. Conversely, mTORC2 is composed of mTOR, Rictor, mLST8 and Sin1. mTORC2 was originally reported to be insensitive to rapamycin, though recent studies indicate that it may be indirectly inhibited by long-term treatment with rapamycin (134).

mTORC1 activates p70 ribosomal protein S6 kinase (S6K1) and, at the same time, releases eukaryotic translation initiation factor 4E (eIF-4E) by phosphorylating inhibitory eIF-4E binding proteins 1-3 (4E-BPs) (134). In turn, activated S6K1 phosphorylates the ribosomal protein S6 to increase translation of mRNAs with 5'-terminal oligopolypyrimidine tracts whereas mTOR-dependent phosphorylation of 4E-BPs releases the initiation factor eIF4E to promote cap-dependent translation. This results in increased synthesis of growth-related proteins such as cyclin D1, Myc, and vascular endothelial growth factor (VEGF) (135-139).

The dynamic interplay between Akt and mTOR is remarkably complex (Figure 3). On one hand, Akt

activates the downstream mTOR kinase by inhibiting the complex formed by the tumour suppressor proteins TSC-1 and TSC-2 (also known as hamartin and tuberin) through direct phosphorylation of TSC-2 at S939 and T1462 (140). TSC-2 forms a heterodimeric complex with TSC-1 that inhibits the GTPase Rheb, a selective activator of mTORC1. Akt phosphorylation of TSC2 suppresses the GTPase-activating activity exerted by the TSC1/TSC2 complex on Rheb, thus resulting in the activation of mTORC1 (134, 140). Akt can activate mTORC1 also through a TSC-2-independent mechanism. Akt directly phosphorylates PRAS40, a protein that associates with mTORC1, thus relieving PRAS40 inhibitory effect on mTORC1 (134). However, the other cellular mTOR-containing complex, mTORC2, acts upstream Akt since it has been identified as the kinase that phosphorylates Akt at S473, thus contributing to its activation (134).

Mechanisms whereby mTOR promotes tumorigenesis include Akt activation either by mTORC2 or mTORC1-dependent increase of the synthesis of growth-related proteins. So far, activating mutations in the gene encoding mTOR have not been identified. However, many human cancers including thyroid carcinomas are characterized by aberrant activation of mTOR, possibly through deregulation of upstream components that regulate mTOR (141-143). Accordingly, the enhanced sensitivity of cancer cells exhibiting oncogenic activation of the PI3K-Akt pathway to mTORC1 inhibitors illustrates the importance of mTORC1 activation downstream Akt (139-141). However, the existence of a negative-feedback inhibition exerted by mTORC1 on PI3K-Akt signalling in cells with activated mTOR may limit the therapeutic use of mTOR inhibitors (134).

mTOR has been shown to be a key effector of TSH-mediated proliferative signals in thyroid follicular cells. Brewer *et al.* showed that the *in vivo* proliferative response to chronic TSHR stimulation of mice treated with sodium perchlorate and methimazole relies on the activation of the mTOR/S6K1 axis, and that mTOR inhibition abrogates the hyperplastic responses to increased TSH levels (144). In this model, goitre development is apparently independent of Akt activity, underlying the existence of an Akt-independent pathway leading to mTOR activation upon TSH stimulation. This consistent with recent works showing that Akt-independent activation of mTOR is recognized as a mechanism of control of cell proliferation in luteal cells (145), B lymphocytes and endometrial stromal cells (146, 147). mTOR regulates also the function of normal thyrocytes both *in vitro* and *in vivo*, since it participates in the control of thyroid iodide uptake by regulating NIS protein expression (148). Interestingly, the finding that mTOR inhibition avoids the activation of both S6K1 and Akt suggests the involvement of mTORC1 and mTORC2 in NIS regulation.

MTOR has been shown to be a key effector of the proliferative signals funnelled through PI3K in thyroid cells. Recent studies indicated that the *in vivo* proliferative response to chronic PI3K activation relies profoundly on the activation of the mTOR/S6K1 axis (149). Conditional loss of Pten in the mouse thyroid follicular cells stimulates autonomous growth leading to the development of adenoma, possibly driven by increased cyclins D1 and D3 (149). MTOR inhibition in Pten mutant mice restores virtually normal proliferation rates, despite the presence of still elevated Akt activity (149). These data extend independent findings obtained in the TRβPV/PV knock-in mouse model of thyroid follicular carcinoma (148). TRβPV/PV mice harbour a mutant thyroid hormone receptor beta gene that promotes thyroid cancer and distant metastasis similar to human FTC (150). In this strain, inhibition of PI3K signaling reduces tumour cell proliferation by down-regulating TOR activity and cyclin D1 levels. In addition, mTOR inhibition potently suppresses proliferation of human thyroid cancer cells harbouring genetic alterations in the PI3K/Akt pathway (151-153).

5.2. Apoptosis-related pathways

Akt enhances cell survival by regulating expression and function of several pro-apoptotic genes (Figure 3) (44). The Bcl-2 family of proteins is a major intracellular regulator of apoptotic signalling, with at least 20 members in mammalian cells (154). Proapototic members including Noxa, Puma, Bim and Bad are transcriptionally or post-translationally activated by extracellular pro-apoptotic signals and/or by intracellular damage and are inhibited by intracellular pro-survival proteins such as Akt (154, 155). Similarly, Akt regulates expression and/or function of anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w and Mcl-1 that protect cells from a wide range of apoptotic stimuli, including chemotherapeutic drugs and irradiation (154, 155).

Akt directly phosphorylates and inhibits Bad (156, 157). Survival factors stimulate Akt-mediated phosphorylation of Bad on S136, and this creates a binding site for 14-3-3 proteins, which triggers release of Bad from the mitochondrion (157). In the unphosphorylated state, Bad is targeted to the mitochondria where it forms a complex with Bcl-2 or BclXL, inhibiting their anti-apoptotic activity. Conversely, when phosphorylated, Bad associates with 14-3-3 proteins in the cytoplasm allowing Bcl-2 or BclXL binding to Bax and Bak, multidomain Bcl-2 family members that function as an obligate gateway for the activation of apoptosis via the mitochondrial and endoplasmic reticular pathways presumably as pore-forming complexes, to release holocytochrome c in the cytoplasm.

Akt suppresses apoptosis also by inhibiting the activity of pro-apoptotic transcription factors, such as FOXO and p53. The FOXO (Forkhead box-containing protein, O subfamily) transcription factors belong to the winged helix/forkhead family and are the closest homolog of Daf-16 in C. Elegans. Mammalian cells express three FOXO isoforms: FOXO1 (FKHR), FOXO3a (FKHRL1) and FOXO4 (AFX) (reviewed in 158 and 159). These transcription factors are negatively regulated through Akt-dependent phosphorylation (160-163). Akt phosphorylates FOXO1 on T24, S256, and S319, and FOXO3a and FOXO4 on equivalent sites (161, 162). Akt phosphorylation of FOXO proteins occurs in the nucleus.

14-3-3 proteins displace phosphorylated FOXO transcription factors from the promoters of target genes and trigger their export from the nucleus and consequent degradation. Through this mechanism, Akt blocks FOXO-mediated transcription of target genes that promote apoptosis and cell-cycle arrest (164). In cancer cell lines lacking functional PTEN, FOXO1 and FOXO3a are constitutively phosphorylated, cytoplasmic and unable to activate transcription. Reconstitution of PTEN expression restores nuclear localization of FOXO1 and restores its ability to activate FOXO responsive elements in the promoters (163). FOXOs can induce apoptosis through the up-regulation of FasL and of the pro-apoptotic Bcl-2 interacting mediator (Bim1) (161, 166). Thyroid cells carrying an activated Akt allele become resistant to programmed cell death induced by starvation because Akt inhibits the induction of proapoptotic genes (Fas, Fas ligand, Bad) in starved cells (167). Notably, activated Akt was found to correlate with phosphorylated Bad in a subset of thyroid cancer specimens (95).

A third Akt target that promotes survival is Mdm2 (or Hdm2 in human), an E3 ubiquitin ligase that catalyzes p53 degradation. Akt phosphorylates Mdm2 on S166 and S186, and this promotes translocation of Mdm2 to the nucleus, where it negatively regulates p53 function (168, 169). Two transcriptional targets of p53 that are essential for p53-induced apoptosis are Puma and Noxa (170). However, the relative importance of downregulation of these p53 targets to Akt-mediated cell survival in thyroid cancer cells has not been thoroughly examined.

In addition to the inhibition of pro-apoptotic factors, Akt can also induce the expression of anti-apoptotic genes through the activation of the transcription factor Nuclear Factor kappa B (NF-κB) (171-174). Akt activate the IκB kinases (IκKs), which, in turn, phoshorylate IκB, an inhibitory protein that sequesters NF-κB in the cytoplasm, targeting it for degradation by the proteosome (175-177). This allows NF-κB to translocate to the nucleus and activate transcription of a variety of anti-apoptotic genes including the cellular inhibitors of apoptosis (c-IAP1, c-IAP2 and XIAP), TNF receptor-associated factors (TRAF1 and TRAF2), Gadd45b, the Bcl-2 homologue A1/Bfl-1, IEX-IL and Bcl-xL (178, 179). Much of the pro-survival ability of Akt is mediated through NF-κB activation, being one of the major culprits of resistance to chemotherapy (180-182). Several studies have demonstrated that Akt mediates NF-κB activation by tumour necrosis factor (TNFα) and PDGF (173, 183). Although it is likely that there are multiple levels of cross-talk between the PI3K-Akt and NF-κB pathways, one mechanism has been attributed to direct phosphorylation of the amino acid residue T23 on IκB kinase α (IκKα) by Akt, thereby leading to activation of this kinase upstream NF-κB and release of IκB-mediated inhibition of NF-κB (173). Activation of IκKα by Akt may occur also through phosphorylation of NF-κB subunit p65 at S534 (184). Whatever the mechanism, the inhibition of endogenous Akt by overexpression of PTEN results in decreased NF-κB transcriptional activity and sensitization of cells to TNF-induced apoptosis (185). Therefore, the findings suggest that loss of PTEN or activation of Akt in thyroid cancer cells might contribute to carcinogenesis by activation of the NF-κB pathway. Similarly, Ret/PTC and B-Raf-V600E oncoproteins induce degradation of IκBα and activation of NF-κB signaling in thyroid cancer cells (186, 187).

Several studies have suggested that NF-κB is strongly activated in thyroid cancer specimens, especially in ATC. Thus, NF-κB inhibition may represent an attractive therapeutic target for the treatment of advanced thyroid cancer (188). p65 expression and activity are significantly increased in thyroid cancer cell lines (189, 190) and in tumour specimens (191, 192). By contrast, inhibition of NF-κB activity in human ATC cell lines leads to reduced invasion associated with differential expression of MMP-13 and MMP-9 (193), increased susceptibility to chemotherapeutic drug-induced apoptosis and inhibition of tumours growth in nude mice. Importantly, the combined treatment of thyroid cancer cells with NF-κB inhibitors [i.e. Dehydroxymethylepoxyquinomicin (DHMEQ), R-Roscovitine] and taxanes strongly sinergize *in vitro* and show a significantly greater inhibitory effect on tumour growth in nude mice (194, 195). Another NF-κB inhibitor, triptolide, functions as an effective apoptotic inducer in a p53-independent, but NF-κB-dependent mechanism, thus providing a promising agent for tumour types with p53 mutation/deletion (196).

5.3. Proliferation-related pathways

Although best known for promoting cell survival and growth, Akt represents also a main regulator of cell proliferation through the phosphorylation of multiple downstream targets impinging on cell-cycle regulation such as Cdk inhibitors and G1 cyclins (Figure 3) (44).

Expression of constitutively active Akt promotes proliferation and survival of thyroid cells without affecting the expression of the differentiated phenotype (92, 167). Thyroid cells carrying an activated Akt allele proliferate in the absence of TSH and insulin (167, 197).

In thyroid cells Akt increased the levels of the G1 cyclins (i.e. cyclins D3 and E) (167) and induced cytoplasmic displacement of p27 (92). By contrast, pharmacological inhibition of PI3K (i.e. with LY294002, wortmannin) is sufficient to inhibit proliferation of human thyroid cancer cells, and this apparently occurs through regulation of the subcellular localization of p27 (37, 92). Transient expression of PTEN inhibits Akt activity in thyroid cancer cell lines and induces cell cycle arrest or cell death in cell contest-dependent manner: PTEN induced G1 cell cycle arrest in PTC-derived cells and/or both G1 arrest and cell death in FTC-, PDC- and ATC-derived cells (91, 198). More recently the direct Akt inhibitor perifosine was shown to potently inhibit the proliferation of cells that harboured PI3K/Akt-activating genetic alterations but to have modest responses in cells that harboured no genetic alterations (151).

p27 inactivation is a critical step in growth regulation exerted by PI3K-Akt in thyroid cancer cells (91).

The PI3K/Akt pathway contributes to inactivation of p27 in thyroid cancer through several mechanisms. Akt can induce phosphorylation-dependent cytoplasmic sequestration of p27 (92), inhibition of p27 gene transcription by targeting the FOXO transcription factors or degradation of p27 through up-regulation of the E3 ubiquitine ligase Skp2. Work from different labs has demonstrated that Akt phosphorylates the p27 cyclin-dependent kinase inhibitor at T157 (199, 200) and T198 (201, 202). By phosphorylating p27 at T157 and T198, Akt induces binding of p27 to 14.3.3, prevents interaction with importin-α, impairs nuclear import, and overcomes p27-induced growth (203, 204). However, although impaired import of p27 into cell nuclei may affect its ability to inhibit cell cycle progression, several experimental evidences supports the idea that p27 exerts additional cytoplasmic functions that foster carcinogenesis (205). Cytoplasmic p27 may suppress apoptosis or regulate migration (206), or increase the number of tissue stem cells and/or progenitors (207), thus allowing cancer cells to dysregulate multiple cellular functions with one hit.

Different mechanisms have been proposed to explain PI3K- and Akt-dependent down-regulation of p27 in thyroid cancer. Akt may inhibit p27 expression in thyroid cancer cells through phosphorylation and cytoplasmatic displacement of FOXO3 (208). Accumulation of FOXO3a correlated with increased phospho-Akt staining and with reduced transcription from the p27 gene in thyroid cancer but not in normal thyroid tissue (208). Alternatively, increased turnover of p27 protein in thyroid cancer has also been reported to occur following increased PI3K-dependent expression of Skp2 (209). The increase in Skp2 levels is induced by expression of oncogenic Ret/PTC and Ras proteins (208), and results in PI3K-dependent p27 loss in human normal thyrocytes (210). Accordingly, pharmacological inhibition of endogenous or transfected Ret/PTC restored p27 expression in rat and human thyroid cells (211). Finally, the recent finding that double mutant mice carrying an activated NTRK1 oncogene in a background of a p27 null allele [TRK-T1/p27(-/-)] displayed a higher incidence of PTC, with a shorter latency period and increased proliferation index, compared with p27 wild-type mice [TRK-T1/p27(+/+)], demonstrated the critical role of this Cdk inhibitor in the contest of tyrosine kinase receptor-driven thyroid carcinogenesis (212).

In addition to the inactivation of Cdk inhibitors, Akt can stimulate cell cycle progression through up-regulation of G1 cyclins. Akt phosphorylates the GSK3 isoforms α and β on a highly conserved N-terminal regulatory site (S21 for GSK3α, S9 for GSK3β) (213). Akt-dependent phosphorylation of GSK3 inactivates the kinase and relieves the constraint imposed by GSK3 on the synthesis and the stability of proteins involved in cell-cycle entry. In particular, GSK3 phosphorylates cyclin D and cyclin E, which play a central role in the G1-to-S-phase cell cycle transition, targeting them for nuclear export and proteasomal degradation (214, 215). Phosphorylation and inhibition of GSK3 by Akt enhance the stability of cyclin D, cyclin E and Myc, promoting cell-cycle entry. Accordingly, cyclins D and E and Myc are frequently overexpressed in the different types of thyroid carcinoma, though it is not known whether this is correlated to increased signalling through the PI3K pathway.

Finally, Akt may also have a role in the deregulation of mitotic checkpoint. Constitutively active Akt is able to promote progression into mitosis, even in the presence of DNA damage (216, 217). One mechanism explaining this observation is that Akt directly phosphorylates the DNA damage checkpoint kinase Chk1 on S280 (218). S280 phosphorylation blocks checkpoint function by stimulating Chk1 translocation to the cytosol, where it is sequestered from the DNA damage-sensing kinases ATM and ATR (219). Accordingly, expression of activated Ras in rat thyroid cells, which activate Akt signalling, induces chromosomal instability, as a consequence of defects in the processing of DNA damage (220).

6. THERAPEUTIC STRATEGIES OF TARGETING AKT SIGNALLING IN THYROID CANCER

The major therapeutic challenge for thyroid cancer therapy is represented by the high rate of mortality of patients with PDC and ATC due to surgical inoperability at diagnosis or subsequent insensitivity to radioiodine treatment. Whereas most patients affected by differentiated thyroid cancer are successfully treated with thyroidectomy, radioiodine treatment and/or external beam radiotherapy, cytotoxic systemic chemotherapy for advanced, metastatic thyroid carcinomas has limited effectiveness, with response rates typically in the range of 25% or less (221). Therefore, the development of novel, molecularly-based compounds will improve disease outcomes especially in patients with aggressive thyroid cancers. As noted above, aberrant activation of the PI3K/Akt pathway is an essential step for the initiation and maintenance of thyroid cancer. Almost every single node of this pathway can be subject to pathway-activating genetic alterations, which result from the distinct and often mutually exclusive events that include (i) translocation of receptor tyrosine kinases (for example Ret/PTC or NTRK-1) leading to constitutive recruitment and activation of PI3K and downstream effectors; (ii) amplification of PI3K; (iii) presence of activating mutations in the PIK3CA gene encoding the p110α catalytic subunit; (iv) amplification of the downstream kinase Akt; (v) presence of activating mutations in the Akt1 gene (vi) loss or inactivating mutations of the tumor suppressor gene PTEN, or (vi) constitutive recruitment and activation by mutant forms of the Ras oncogenes (10). See Figure 5 for a summary of molecular alterations observed in thyroid carcinoma.

To date, most of the compounds tested in treating thyroid cancer are competitive inhibitors of tyrosine kinases (reviewed in 221). The demonstrated oncogenic roles of mutant Ret/PTC, Ras and B-Raf and the contribution of VEGF angiogenic growth factor receptors to development of thyroid cancer, have lead to different clinical trials with small molecule inhibitors (222, 223). These compounds include sunitinib, sorafenib, motesanib

and axitinib and, consistent with the oncogene addiction hypothesis, have been reported to induce either tumor stabilization or partial remission in a certain percentage of patients with advanced thyroid carcinomas (224-226). These orally administered drugs partially inhibit multiple kinases that include Ret/PTC, B-Raf, VEGF receptors, cMet, and PDGF receptors, at nanomolar concentrations and thus affect multiple downstream signaling pathways including the MAPK and PI3K pathways (222). However, if Akt is activated by loss of PTEN or mutations in PIK3CA or Akt1 itself, the inhibition of upstream receptor activity may be ineffectual. In these cases, patients may benefit of PI3K or Akt inhibitors. In particular, both PI3K and Akt may represent potentially relevant therapeutic targets for advanced thyroid cancer (227, 228). However, since PI3K-Akt signaling plays a critical role in essential processes such as insulin signaling, neuron function, and endothelial activity that may be disrupted with systemic administration of a PI3K inhibitor, a careful analysis of safety parameters in clinical trials will have to be taken into account, especially in patients with thyroid cancer, many of whom have excellent quality of life.

The available data indicate that genotype-based targeting of the PI3K/Akt pathway using Akt and mTOR inhibitors may offer an effective therapeutic strategy for thyroid cancer (151-153). Available Akt inhibitors may be of three types: ATP competitive inhibitors that target the kinase domain, the PtdIns analogs that block binding of PH domain to PtdIns and allosteric inhibitors that stabilize Akt in a "close comformation" that is not capable to be activated by PDK1 (227). Several compounds that inhibit all Akt isoforms have been recently developed and are now in phase I clinical trials (reviewed in 225). A series of potent, ATP competitive Akt inhibitors (IC50=20 nM for Akt versus IC50=1900 nM for PKA) were developed by exploiting the X-ray crystal structure of (−)-balanol, a potent inhibitor of AGC-kinases, in complex with PKA (228-232). Promising Akt inhibitors have also been obtained from indazole–pyridine-based derivatives such as A-443654 (Ki=160 pM for Akt1) (228-231). These compounds are reversible, ATP competitive inhibitors, which decrease the phosphorylation of Akt downstream targets in cells (for example, GSK3α/β, FOXO3, TSC2 and mTOR) and *in vivo* in a dose-dependent manner. In xenograft experiments, A-443654 showed antitumor activity both as a single agent and in combination regimens. However, in these preclinical experiments, treatment had to be discontinued because of multiple side effects in treated animals, raising concerns that the therapeutic application of Akt inhibitors can be limited by inherent metabolic toxicity (232).

Recently, compounds with improved potency, selectivity and safety have been reported (233, 234). For example, GSK690693, an aminofurazan derivative, is an ATP-competitive, pan-Akt kinase inhibitor (IC50=2 nM for Akt1) that has recently entered phase I clinical trials (234). The compound was well tolerated and showed significant antitumour activity *in vivo*. At the moment, the first phase I study in humans to investigate the safety, tolerability, pharmacokinetics and pharmacodynamics of GSK690693 is ongoing.

The allosteric Akt inhibitor perifosine (octadecyl-(1,1-dimethyl-piperidinio-4-yl)-phosphate) (235) has been shown to inhibit *in vitro* proliferation and the *in vivo* growth of xenografted thyroid cancer cells that carried PI3K/Akt-activating genetic alterations (151-153). However, other studies have shown that cells that harbor p85 mutations, PTEN loss or HER2 amplification also show increased sensitivity to perifosine, which suggests that there is not yet available a good biomarker that predict sensitivity to Akt inhibitors (235). In addition, a number of phase II trials with perifosine as single agent have provided disappointing results in patients with different types of carcinoma, melanoma or sarcoma (236 and references therein). An alternative strategy that has been implemented is the use of perifosine in combination with chemotherapy or radiation therapy, with the aim to overcome the development of Akt-dependent drug resistance. However, definitive results from different phase I and II trials have not been disclosed yet.

PI3K, the most important upstream activator of Akt, also represents a therapeutic target for thyroid cancer. Two well-known PI3K inhibitors are the fungal metabolite wortmanin or LY294002. Wortmannin is an irreversible inhibitor (IC50 ≈ 2 nM) that forms a covalent bond with a conserved lysine residue in the catalytic site (237); LY294002 is a classical reversible, ATP-competitive PI3K inhibitor (IC50=1.40 μM) (238). Wortmanin or LY294002 suppress proliferation of thyroid cancer cells *in vitro* (91, 92), though these inhibitors show limited use *in vivo*. More recently, PI3K inhibitors with improved pharmaceutical properties and therapeutic indexes such as PWT-458 (239) and PX-866 (240, 241) have been developed for preclinical studies (224, 242). PX-866 has shown antitumor activity in multiple xenograft models with PIK3CA mutations or PTEN loss, though it was not active in cells harbouring simultaneous PIK3CA and Ras mutations (243).

In parallel high-throughput screenings have led to the discovery and development of novel selective PI3K inhibitors such as NVP-BE-Z235, an imidazo[4,5-c]quinoline derivative that inhibits both PI3K (IC50=4 nM against p110α) and mTOR (IC50=21 nM) (242). This compound potently inhibits proliferation of cancer cell lines with PIK3CA mutations or PTEN loss, though, also in this case, activity was not restricted to cells that harbour these alterations (243). Similarly, lung cancer initiated by mutant K-Ras was resistant to NVP-BE-Z235 (243, 244). Therefore, the picture that emerges from the available preclinical studies is that the presence of PIK3CA mutations or the loss of PTEN confers sensitivity to PI3K inhibitors (though also non-mutant cells may be sensitive) and that the presence of K-Ras mutations predicts resistance. Moreover, it remains to be seen whether p110α mutation-specific inhibitors for cancer therapeutics can be identified.

As with PI3K, also the direct activator of Akt kinases, PDK1, has recently been proved to be a therapeutic target to block this oncogenic pathway. One of the most potent, but nonselective PDK1 kinase inhibitor reported so far is the staurosporine-based compound UCN

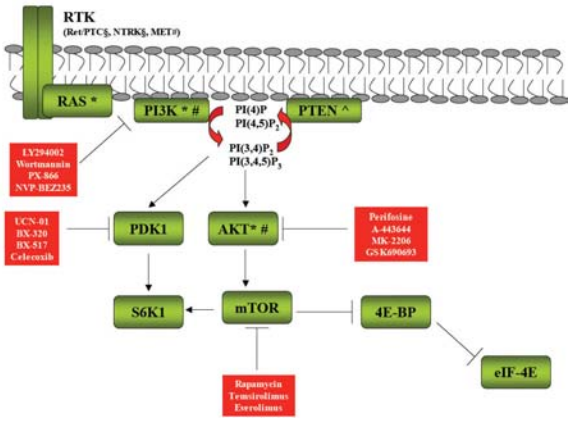

Figure 5. The PI3K-Akt pathway in cancer. Molecular alterations reported in the members of the PI3K-Akt pathway.* Activating mutation; # gene amplification; § gene rearrangements (trasnlocation/inversion); ^ inactivating mutations, LOH, promoter methylation. In the red boxes are indicated the novel compounds developed to inhibit the different members of the PI3K pathway.

01 (IC50=6–33 nM) (245). Originally developed as an inhibitor of protein kinase C, UCN-01 inhibits a broad array of kinases, including Akt and other members of the AGC family of enzymes such as PDK1 (IC50=491 nM for Akt) (246). Inhibition of of tumour growth through UCN-01-dependent inhibition of PDK1 has been observed in murine and human tumor xenografts (245). However, no significant antitumor activity has been reported when UCN-01 was tested in advanced cancer patients in phase I/II clinical trials, both as single agent and in combination with conventional chemotherapeutic drugs (247). In addition, toxic side effects as pulmonary toxicity, nausea/vomiting, lactic acidosis, insulin resistance after treatment with UCN-01 were reported. The aminopyrimidine derivate BX-320, an ATP-competitive inhibitor has also been reported to inhibit PDK1 kinase activity (IC50=39 nM) with good selectivity (248). BX-320 blocks the growth in soft agar of a wide range of tumor cell lines (IC50=0.093–1.32 μM), and is efficacious in a metastasis mouse model when administered orally. More recently, the identification and the optimization of an indoline-based compound series of PDK1 inhibitors have been reported (BX-517; IC50=6 nM) (249, 250). Finally, inhibitory activity against PDK1 has also been reported for compounds originally designed to antagonize different therapeutic targets (i.e. celecoxib, a cyclooxygenase-2 inhibitor) in a variety of cancer cells through inhibition of PDK1 (251). Celecoxib itself is currently being investigated in phase II/III clinical studies as a single agent or in combination therapy.

Another attractive target in the PI3K-Akt pathway is mTOR, one of the most relevant downstream effector of Akt in thyroid cancer. Rapamycin, a well known mTOR inhibitor, has been shown to inhibit cell proliferation of thyroid cancer cells *in vitro* (252) as well as to inhibit proliferation of thyroid cells in thyroid targeted *Pten*-null mice *in vivo* (144). The mTOR inhibitors CCI-779 (temsirolimus) and RAD-001 (everolimus) are potent inhibitors of mTORC1 complex and thus suppress ribosomal protein and cap-dependent translation (252). mTOR inhibitors have been tested as single agents in phase II studies in a variety of cancer types with promising results in breast carcinoma, glioblastoma, renal cell carcinoma and lymphoma (236 and references therein). At the moment both everolimus and tensirolimus have been approved in the Unites States as front-line therapy of resistant renal cell carcinoma (236).

Finally, the available data from different preclinical studies provide compelling evidence that the combination of PI3K and MEK inhibitors would be a rational approach in patients showing simultaneous activation of these pathways. The combined treatment with MEK/MAPK and PI3K inhibitors are particularly efficient in inhibiting K-Ras driven lung cancer in mouse (253) and in xenografted human basal-like breast cancer cells (254). Importantly, a collaborative effort has been launched to study the antitumor effects of the combination of MK-2206 (an allosteric Akt inhibitor) and AZD6244 (a MEK inhibitor), two early-stage compounds from Merk and AstraZeneca, respectively (227 and references therein).

Because PI3K activation in advanced thyroid cancer can occur in combination with increased signalling through the MAPK pathway, there has been a growing interest in defining a combination regimen that inhibits both pathways for patients with aggressive thyroid cancers. In particular this approach may be particularly relevant for tumours harbouring genetic alterations of both pathways (B-Raf and PIK3CA or B-Raf and Akt1, respectively). Accordingly, preclinical studies with compound [K-rasG12D/pten(-/-)] mice showed that combined pharmacological inhibition of PI3K (LY294002) and MEK/MAPK (UO-126) completely inhibited the growth of double mutant cancer cell lines, providing a compelling rationale for the simultaneous targeting of these pathways in thyroid cancer (73). See Figure 5 for a summary of the compounds targeted to the different members of the PI3K-Akt pathway.

7. CONCLUSIONS

In conclusion, genetic alterations that activate the PI3K-Akt pathway, including rearrangement or amplification of tyrosine kinase receptors, Ras mutations, PTEN loss and mutations and amplification of PK3CA and Akt1, are common in thyroid cancer. Importantly these genetic alterations are preferentially observed in aggressive cancers such as PDC and ATC, which account for most of the incurable cases of thyroid cancer. Therefore it appears that targeting PI3K-Akt pathway in patients with advanced thyroid cancer may represent a rationale approach to improve survival. For this reason, several specific PI3K, Akt or mTOR inhibitors have been developed and many of these inhibitors currently under clinical evaluation represent a promising approach for the treatment of thyroid cancer patients. However, evidence continues to accumulate that the different Akt isoforms have diverse and sometimes even opposing functions in different cell settings. Therefore, to develop effective new compounds

that limit relevant Akt signalling in thyroid cancer cells, it will be important to gain a more complete picture of the precise roles of the PI3K/Akt pathway and, in particular, of the different kinase isoforms in the specific contest of PDC and ATC. Moreover, the complex regulation of the PI3K-Akt pathway poses practical issues concerning the design of clinical trials, potential toxicities and criteria for patient selection. Further studies are needed to develop more effective single agents or to identify rational combinations of therapeutic targets that have synergistic effectiveness without enhanced toxicities.

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