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A minireview: the role of MAPK/ERK and PI3K/Akt pathways in thyroid follicular cell-derived neoplasm

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1. ABSTRACT

The MAPK/ERK (mitogen - activated protein kinase/extracellular signal-regulated kinase signaling pathway) and PI3K/Akt (lipid kinase phoshoinositide-3-kinase signaling pathway) play an important role in transmission of cell signals through transduction systems (ligands, transmembrane receptors and cytoplasmic secondary messengers) to cell nucleus, where they influence the expression of genes that regulate important cellular processes: cell growth, proliferation and apoptosis. The genes, coding the signaling cascade proteins (*e.g.*, *RET, RAS, BRAF, PI3K, PTEN, AKT*), are mutated or aberrantly expressed in thyroid cancer derived from follicular thyroid cell. Genetic and epigenetic alternations, concerning MAPK/ERK and PI3K/Akt signaling pathways, contribute to their activation and interaction in consequence of malignant follicular cell transformation. This review is focused mainly on genetic alterations in genes, coding signaling pathway proteins. Moreover, it is additionally pointed out that genetic, as well as epigenetic DNA changing *via* aberrant methylation of several tumour suppressor and thyroid-specific genes are associated with tumour aggressiveness, being a jointly responsible mechanism for thyroid tumorigenesis. The understanding of this molecular mechanism provides access to novel molecular therapeutic strategies for inhibiting oncogenic activity of signaling pathways.

2. INTRODUCTION

Thyroid cancer accounts for the majority of the endocrine malignancy in the world and the incidence of this type of cancer is still increasing (1). Follicular cell-derived thyroid carcinoma – most common endocrine malignancy – includes several morphological subtypes which have traditionally been classified in independent groups: well-differentiated thyroid carcinoma (WDTC) represents more than 90% of all thyroid cancers, whereas poorly differentiated thyroid carcinoma (PDTC) and undifferentiated (anaplastic) thyroid carcinoma (UTC; ATC) accounts for approximately 2% to 10% (2-4) (Figure 1).

These subtypes of thyroid cancer are phenothypically distinct and display different potential degree of malignancy, from relatively indolent (PTC, papillary thyroid carcinoma) – with an excellent prognosis – to highly aggressive tumours (UTC) with a poor prognosis, due to lack of effective treatment. PTC, belonging to WDTC, is the most common type and accounts for 60-80% of all thyroid gland malignancies (2). It is confirmed that PDTCs displays intermediate biological and clinical features between WDTC and UTC. Indeed, clinical, epidemiologic, and genetic evidences, provided by many studies, support the hypothesis of gradual progression and dedifferentiation of thyroid cancer derived from

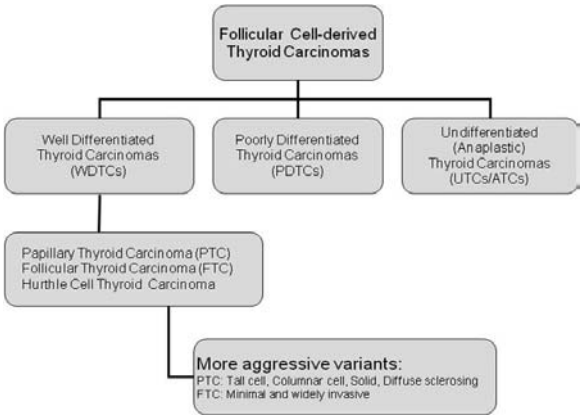

Figure 1. Classification of the follicular cell-derived thyroid carcinoma with emphasize to more aggressive variants of WDTCs.

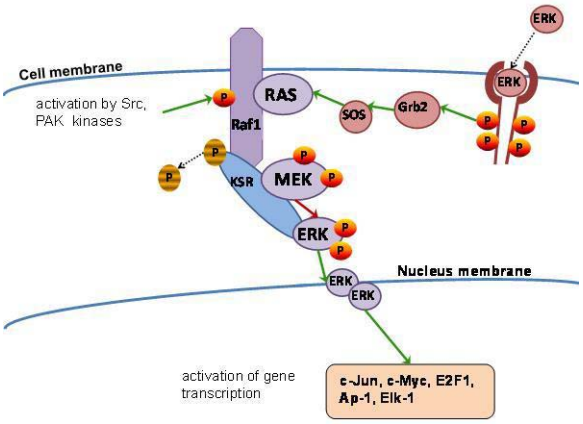

Figure 2. Schematic activation of the MAPK/ERK pathway. Activated tyrosine kinases serve as binding sides for proteins with enzymatic activity (RAS, PI3K) and adapter proteins (Grb2-SOS, Shc).

follicular thyroid cells (5,6). Many studies have supported that PDTCs display a high tendency to recurrence of symptoms, metastasis and progressive dedifferentiation of follicular cells (4,7-9).

Although the results, obtained in many studies, strongly support the progression concept in thyroid tumorigenesis, the knowledge about possible molecular mechanisms, underlying this process, is still insufficient. However, there have been studies, assessing the molecular factors that may play an essential role in dedifferentiation of WDTC (6,10,11). These factors affect tumour proliferation, growth, cellular survival, and may contribute to developing of particular thyroid cancer phenotype. An identification of molecular alterations, involved in thyroid carcinomas of follicular origin, may help implement novel therapeutic strategies beside surgery and adjuvant radioactive iodine treatment. Moreover, the recognized molecular factors may serve as potential therapeutic targets.

3. RAS/RAF/MEK/ERK SIGNALING PATHWAY

RAS/RAF/MEK/ERK (MAPK/ERK; mitogen-activated protein kinase/extracellular signal-regulated kinase) signaling pathway, called "MAPK pathway" is a classical conserved intracellular signaling pathway that transmits signals which regulate cell proliferation, differentiation, apoptosis and survival (12-14). This pathway functions as a family of numerous receptor, non-receptor kinases, GTP-binding proteins and transcription factors (*c-Myc, Ets, CREB ,c-Jun, c-Fos*), which can be activated or inactivated by protein phosphorylation (14,15). Activation of this pathway conducts cells into mutator phenotype by increasing cell proliferation and dedifferentiation and inhibition of apoptosis (15,16). Transduction of oncogenic signals generated by cell surface receptors and cytoplasmic signaling components into the nucleus involving important protein-protein interaction (14) (Figure 2).

Physiologically, in cells which have not undergone any transformation, the activation of the signaling MAPK/ERK cascade starts at the cell membrane receptor upon its stimulation by extracellular mitogenic signals (*e.g.*, growth factors, differentiation factors, tumour-promoting substances) (15). Most of these factors stimulate the activation of extracellular receptor tyrosine kinases (RTKs, *e.g.*, RET, NTRK1) or various non-receptor cytoplasmic serine/threonine-specific kinases (Raf family, MAP, ERK), as well as small GTP-binding Ras proteins. The activation of Ras – a membrane-bound small G protein working upstream of MAPKs – occurs through an exchange of GDP for GTP, which converts Ras into its active form. Furthermore, Ras undergoes a conformational change and becomes active upon stimulation by coupling the Shc/Grb2/SOS (Shc adapter protein/growth factor receptor bound protein 2/son of sevenless) protein complex (see Figure 2). The process of RAS protein recruitment to the cell membrane is connected with post-translational modifications, preferentially in cysteine residue, through farnesylation mediated by farnesyl transferase (17). Ras protein upon activation, induces Raf proteins (A-Raf, B-Raf and C-Raf) which are translocated to the cellular membrane.

Normally the Raf family proteins are activated by series of modifications: dimerization (18), phosphorylation/dephosphorylation on different domains (19), disassociation of the Raf kinase inhibitory protein (RKIP) (20) and, finally, its activity is modulated by chaperone proteins, such as heat-shock protein 90 (Hsp90) (21). The activated Raf kinases phosphorylate and activate two mitogen-activated protein kinases: meiosis-specific serine/threonine protein kinases (MEKs), *i.e.*, MEK1 and MEK2, as well as two immediately downstream extracellular-signal-regulated kinases (ERKs), *i.e.*, ERK1 and ERK2. This cascade of phosphorylation of several serine/threonine-specific kinases leads to alterations in the expression of various genes in the nucleus, *i.e.*, a set of transcription factors, including NF-kbeta, AP-1, c-Myc, Ets-1 and c-Jun, which were initially identified as

Table1. Protein kinase functions in MAPK/ERK signaling pathway and their genetic alterations in neoplastic transformation of thyroid follicular cell

Gene	*Locus*	Protein and its function	Activation	References
RET *NTRK1*	10q11.2 1q23-24	Membrane receptor tyrosine kinases for extracellular growth factors (GDNF, NGF); cell proliferation and differentiation control.	Rearrangements *RET*/PTC, *TRK*	11,24-27
BRAF	7q34	non-receptor signaling transduction protein; cytoplasmic serine/threonine kinases; cell proliferation, differentiation, angiogenesis and adhesion control.	Point mutations, *BRAF/AKAP9* rearrangements	11,28,29
MPK1 *MAP2K*	22q11.2 15q21	Protein mitogen-activated serine/threonine kinases (EC 2.7.11), MAP; extracellular signal transduction proteins.	Gene mutations (?)	Any published

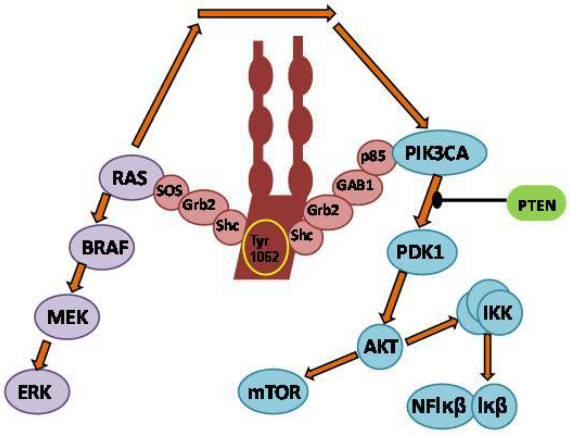

Figure 3. The activation of MAPK/ERK pathway *via* Tyr1062 autophosphorylation and Shc protein binding.

protooncogenes involved in cell proliferation, growth, survival, and tumourigenesis (22,23).

3.1. Aberrant signaling through the RAS/RAF/MEK/ERK pathway in thyroid tumorigenesis

Many recent studies, focused on molecular background of thyroid cancers, derived from follicular epithelial cell, have proven that aberrant signaling through the RAS/RAF/MEK/ERK cascade is a crucial factor for thyroid tumour initiation and development. Many genetic alterations concern the genes which are crucial in MAPKs signaling cascade (the genes encoding for membrane receptor tyrosine kinases and non-receptor tyrosine kinases) (Table 1).

3.1.1. The role of *RET*/PTC rearrangements

The essential role in the constitutive activation of RAS/RAF/MEK/ERK and initiation of thyroid tumorigenesis is played by recombinant RET proteins, consisting of the tyrosine kinase domain of the RET (membrane receptor tyrosine kinase for the glial cell-derived neurotrophic factor; GDNF), and a portion of an unrelated protein. The recombinant RET proteins derive from *RET*/PTC (rearranged in transformation/papillary thyroid carcinoma) rearrangements, that result from paracentric inversion or reciprocal chromosomal translocation of 17 and/or 10 chromosome.

Physiologically, *RET* expression is restricted to a subset of cells derived from embryonic neural crest cells. It is confirmed that, in the thyroid gland, a wild-type *RET* is expressed at high levels in the parafollicular C-cells, whereas in follicular cell activation of *RET,* the oncogene occurs by fusion of TK domain of *RET* gene with 5' terminal region of various genes (*e.g.*, H4, RIalphaTKA, ELE1, GOLGA5). These gene fusions become a source of active promoters for the expression of TK domain of RET protein (25,30). In consequence of gene fusion, dimerization and ligand-independent constitutive RET protein activation take place (30,31). There are, at least, 15 different *RET*/PTC rearrangements, with *RET*/PTC1-3 sequences as the most common in PTC (25,30). *RET*/PTC oncogenic sequences occur in about 3-60% of sporadic cases of PTC, depending on population, thus, they are recognized as genetic markers of this type of cancer (6,25,30,32). In our study, conducted in Polish population, the most common rearrangements of *RET* oncogene (*RET*/PTC1-3) has been found in 21% of studied PTC cases (33). These differences between the prevalence of *RET*/PTC rearrangements are due to population variability, diverse methodology, nonclonal features of *RET*/PTC, as well as genetic heterogeneity of PTC (34). Similarly to many human cancers, autophosphorylation of tyrosine (Tyr) in 1062 position of RET protein was documented is a crucial molecular change during thyroid tumorigenesis (31). *RET*/PTC oncogenic sequence may bind the Shc adapter protein *via* Tyr1062 autophosphorylation that leads to MAPK/ERK pathway activation (35) (Figure 3).

A molecular study, focused on the role of *RET*/PTC in cell activity, confirmed the diversity of biological effects of *RET*/PTC oncogenic activation, concerning the stimulation of cell proliferation, survival and extracellular matrix (ECM) invasion. The biological effects of RET/PTC/RAS/BRAF depend on the integrity of this signaling transduction and phosphotyrosine activation (35,36). In thyroid tumours with *RET*/PTC1 induction, the signal transduction mediated by phosphotyrosine 294, 404, or 451 has been shown to be critical for *RET*-induced transforming activity in *vitro* (36). Moreover, the presence of *RET*/PTC1 or *RET*/PTC3 in both mono- and polyclonal microscopic foci of thyroid nodules - not classified as PTC - may promote the PTC development (37). The special ability of *RET*/PTC oncogenic sequences to initiate the tumorigenesis was confirmed by *in vitro* experiments on thyroid follicular cell culture and *in vivo* studies on transgenic mouse model (36-39). Transgenic animals with confirmed expression of oncogenic sequences *RET*/PTC1 and *RET*/PTC3 were able to develop thyroid tumours (38,39). Regarding *RET*/PTC1, this oncogenic sequence is

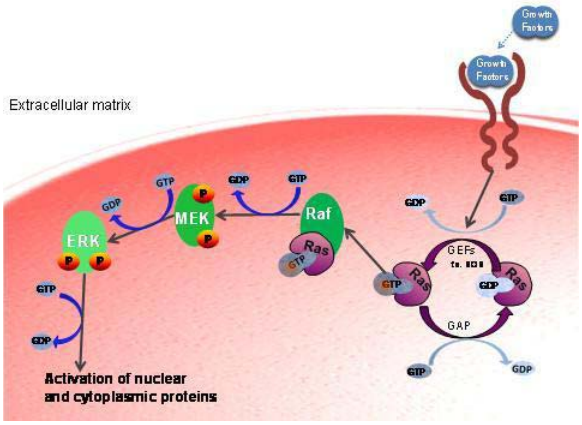

Figure 4. Adapter protein Ras activation by phosphorylation.

characteristic mainly in the classic variant of PTC (40,41), whereas *RET*/PTC3 is often associated with tall cell variant of PTC and is recognised as radiation-related genetic event (25,42). It should be stressed that *RET*/PTC sequence may act as an important functional factor in the thyroid gland. The activation of *RET*/PTC in follicular thyroid cell culture inhibits the transcription process of genes which are important in thyroid gland activity regulation or thyroid-specific transcription factors (*e.g.,* TTF-1, PAX8) *via* interaction with Shc proteins and activation of the RAS/RAF/MEK pathway (35). A recent study has suggested that constitutively active *RET*/PTC in PTC cells may phosphorylate tyrosine 705 of STAT3 (signal transducer and activator of transcription 3) – latent cytoplasmic transcription factor important in the regulation of cell growth and tumorigenesis in many types of human cancer (24). Phosphorylation causes dimerization, translocation and, in consequence, activation of the STAT3 and STAT3-signaling pathway (43). Additionally, it was confirmed that serine/threonine kinase *LKB1* – acting as a tumour suppressor gene – may inhibit *RET*/PTC-dependent activation of oncogenic STAT3 signaling pathway (43). Point mutations in *LKB1* cause a loss of LKB1 kinase activity (44). These molecular alterations are present in many types of sporadic cancers, with particularly high frequency in lung carcinoma (45). Moreover, *LKB1* gene mutations are recognized in rare autosomal dominant disorder in man, *i.e.*, Peutz-Jeghers syndrome (PJS), associated with predisposition to PTC (46). It also should be noted that *RET*/PTC in beta-catenin pathway promotes glycogen synthase kinase 3 beta (GSK3beta) phosphorylation, leading to cell proliferation (47). It appears that in beta-catenin transcriptional activity towards neoplastic transformation, *RET*/PTC1 cooperates – at transcriptional and signaling levels – with *MET* protooncogene (48). According to numerous studies, *MET* is overexpressed in PTC (49,50), associated with more aggressive behaviour, and is constitutively active in human thyrocytes, expressing *RET*/PTC1 (48). Additionally, the study of Siraj *et al.* (50) documented that *MET* may activate Akt protein, i.e., kinase transducing signals from growth factors and oncogenes to downstream targets, that control crucial elements in tumour development.

3.1.2. The role of *TRK* rearrangements

An alternative to *RET*/PTC molecular changes, involved in the biological effects of constitutive activation of MAPK pathway, are oncogenic rearrangements of *NTRK1* gene that encode the receptor for the high-affinity nerve growth factor (NGF) (51). Oncogenic rearrangements of *NTRK1* (*TRK* sequences) – also found in PTC – represent less frequent genetic events. Studies performed in different populations revealed the occurrence of *TRK* rearrangements of 0-50% (reviewed in 52). In our study focused on Polish population, the main types of *TRK* sequences, i.e., *TRK*-T1 and *TRK*(TPM3), are recognised in 12% of studied PTC (33).

Oncogenic *TRK* sequences originate from the fusion of the TK domain of *NTRK1* oncogene with 5'terminal sequence of, at least, three different genes (*e.g.*, tropomyosin 3, *TPM3*; translocated promoter region, *TPR*; TRK fused gene, *TFG*) (53). The oncogenic activity of *TRK* sequence in thyroid follicular cells was confirmed *in vivo* in transgenic mice, where *NTRK1*chimeric protein (TRKT1) expression led to PTC development (54). Recently, it has been confirmed that NTRK1 cell signalling is modulated by the presence of p75 protein which is able to co-expression with NTRK1 receptor and contributes to enhanced neurotrophins binding. Moreover, p75 neurotrophin receptor and NTRK1 co-expression enhances the specificity of other TRKs for their preferred ligands (55,56). The detection of *TRK* rearrangements in PTC and neoexpression of p75 provide some evidence for their participation in the etiopathogenesis of PTC (56). Additionally, it has been evidenced that *TRK* rearrangements may activate the MAPK pathway through a step upstream of Ras (51).

3.1.3. The role of Ras protein and *RAS* mutation

The activation of tyrosine kinase receptor (RET or NTRK1) *via* autophosphorylation of tyrosine residues in the intracellular domain, involving numerous adaptor proteins, leads to activation of membrane Ras proteins (G proteins) (15). Ras represents a family of small guanosine triphosphate (GTP)-binding proteins, which are recognized as essential downstream molecules of many cell receptors in the MAPK/ERK signaling pathway. Physiologically, Ras proteins, under stimulating signals from hormones, growth and differentiation factors, become activated *via* inducing the exchange of GDP with GTP, which converts Ras protein into active protein conformation (Figure 4). The Ras protein with bound GTP can activate a protein kinase cascade MAPK/ERK, which ultimately leads to phosphorylation of transcription factors in the nucleus, which, in turn, alter several gene expressions (22).

It has been confirmed that, after posttranslational modification of Ras proteins, they are recruited to the internal surface of cell membrane and serve as "molecular switching" (Ras-GTP/ Ras-GDP) in cell signal transfers. Moreover, Ras activation is connected with phosphorylation of Shc proteins and binding of adaptor Grb2/SOS (growth factor receptor-bound protein 2/son of sevenless) protein complex (57). The activated Ras protein functions as an adapter that binds to Raf kinases (A-Raf, B-

Raf, C-Raf) – *i.e*., intracellular MAPK effectors – and creates signaling regulation complex Ras-Raf. This active complex is transmitted to cell membrane where, *via* phosphorylation, MAPK/ERK cascade become activated. Finally, B-Raf activates the MEK and ERK kinases and initiates the induction of gene expression in cell nucleus (15) (see Figure 2).

The activation of Ras in thyroid tumours involves point mutations in the *RAS* gene. Typically, the activation of mutations occurs in certain codons of one of three Ras family genes (*N-RAS, K-RAS* and *H-RAS*) – mainly in codon 61 of *N-RAS* gene. This type of point mutation in "initiator" *RAS* gene is particularly prevalent (20-50%) in FTA (follicular thyroid adenoma*)* and FTC (follicular thyroid carcinoma), whereas, less frequently, it occurs in PTC (0-15%) (58,59). Our investigation, focused on the genetic background of PTC in the Polish population, confirmed the presence of point mutations in K-RAS at codons 31 and 61 with frequency of about 8% (60). Interestingly enough, in the follicular variant of PTC, remarkably frequent activating mutations in *RAS* oncogenes were observed which correlated with less prominent features of tumours (61,62). In relation to *RAS* mutation, it was suggested that the follicular variant of PTC might represent a similar to FTC molecular pathway of tumorigenesis.

Moreover, an *in vitro* study, conducted on thyrocyte cell line, has documented that K- or N- *RAS* protooncogene activation leads to a decreased expression of thyroid-specific genes (e.g., *Tg, TSH*) with a simultaneous increase of the proliferation index of follicular thyroid cells in the thyroid gland (63,64). Regarding the fact that *RAS* mutations have been recognized in benign, as well as in malignant thyroid neoplasms, it is approved that the activation of *RAS* oncogene appears in early steps of tumorigenesis in the thyroid gland (65,66). However, it has been documented that *RAS* activation – mainly *via* mutations at codon 61 – is involved in the progression and poor outcome of thyroid tumours (67). Moreover, it is considered that *RAS* oncogene activation is not a sufficient molecular event which leads to initiation of follicular cell transformation, as well as it has been approved to be an intermediate molecular event in cancer dedifferentiation and progression. In order to recapitulate the importance of Ras signalling molecules in thyroid cancer, it should be emphasised that the activated form of Ras protein (Ras-GTP) functions as an adapter protein that binds Raf kinases and causes their translocation to the cell membrane, where the next step of RAS/RAF/MEK/ERK pathway activation – *i.e*., Raf activation - takes place (68).

3.1.4. The role of B-Raf kinase and *BRAF* mutation

The active form of Ras (Ras-GTP) as an adapter protein may bind B-Raf with high affinity. B-Raf belongs to the family of Raf proteins (A-Raf, B-Raf, C-Raf), which are serine/threonine-specific kinases that integrate and regulate upstream flowing signals trough the MAP/ERK pathway (15). Among the three mammal Raf proteins, B-Raf kinase is an essential signalling protein with the highest kinase activity. It is known as the most important activator of MEK (22,68). Regarding A-Raf and C-Raf kinases, they play irrelevant role in molecular pathogenesis of thyroid gland, due to rare genetic alteration in genes coding these proteins (69).

The study of Davies *et al* (70) has documented the constitutive activation of B-Raf protein *via* point mutations of *BRAF* gene as a common genetic event in several types of human tumours, confirming the key role of this Raf kinase in tumorigenesis. Similarly, *in vitro* studies in benign thyroid cell models, have demonstrated a particularly important role for B-Raf kinase in the regulation of thyroid-specific differentiation and proliferative capacity (71). The activating mutations in *BRAF* gene – responsible for B-Raf kinase activity – are the most prevalent (36-69%), defined genetic abnormalities in thyroid cancers (72-75). Among all the mutations in *BRAF* gene, T1799A – causing a substitution of single glutamic acid for valine in 600 position of B-Raf protein (V600E) – has been recognized as the most important and frequently occurring (13-69%) in different populations (29,72-78). V600E mutation is characteristic mainly for PTC (about 45% cases), but also for PDTC and ATC derived from PTC (72,74-76,79,80). This fact confirms the hypothesis that some ATCs arise from PTCs and B-Raf kinases may be functionally important in thyroid dedifferentiation, although the mutations in *BRAF* may not be recognized as an independent factor inducing this process. The experiments on *BRAF* V600E positive transgenic mouse model have confirmed that this type of mutation possesses an oncogenic activity for thyroid cancer development. Transgenic mouse develops invasive and poorly differentiated thyroid cancer with progressive local invasion (81). Regarding human thyroid tumours, it should be stressed that the relationship between the *BRAF* V600E mutation and aggressive tumour behaviour, as well as the predictive nature of *BRAF* mutations, are controversial. In several studies, the presence of *BRAF* V600E mutation was associated with a more aggressive clinical course, an increase of tumour size and invasion *via* underexpression of matrix metalloproteinases (MMPs) (72,82-85). Moreover, the frequent occurrence of V600E mutation has been emphasised in more aggressive histopathological variants of PTC, i.e., tall cell or columnar cell, with the loss of responsiveness to radioiodine (84). On the other hand, this observation has not been confirmed by others in different populations (86-88). Finally, it has been proposed that a detection of *BRAF* V600E might be a useful marker for screening patients with unfavourable outcomes and in making decision to undertake a more aggressive initial therapy. This aspect is specially important for the early diagnosis of PTC in fine-needle aspiration samples. Another frequent mutation, recognized in *BRAF* gene and characteristic for thyroid cancer, is K601del (T1799-1801TGAdel), associated with the lymph node metastasis of PTC (79) and K601E (A1802G) recognized to be common in FTA (follicular thyroid adenoma) and in follicular variant of PTC (72,74,89). The mechanism of B-Raf protein activation has recently been investigated (90). It has been confirmed, that *BRAF* mutants acting as oncogenes and, particularly, as mutations in exon 15 of *BRAF* gene, leading to substitutions at 600 and 601 position

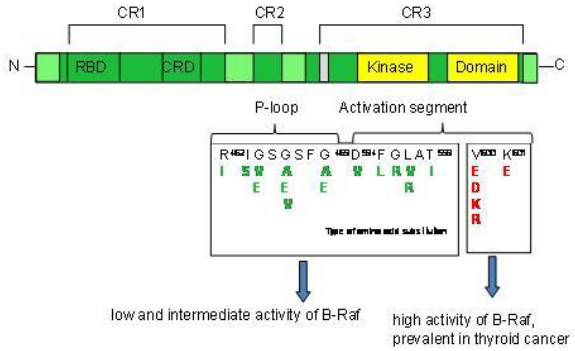

Figure 5. The schematic structure of B-Raf protein with marked exemplary *BRAF* mutations, recognized as intermediate activators of kinase B-Raf activity, in P-loop and activation segments.

(V600E and K6001del//E) of B-Raf protein, have been recognized as the most constitutively activating mutations in thyroid cancer (72,74,79). It is suggested that V600E substitution, within the activation segment of the kinase domain, mimics the phosphorylation, due to the proximity of glutamine acid COOH residue, and leads to phosphorylation at 600 position in amino acid chain of B-Raf kinase domain (29). This new form of interaction keeps the B-Raf protein in a catalytically competent conformation, following continuous MEK phosphorylation (90). Biochemical analysis of V600E mutation, performed in many types of human cancers, has revealed that this molecular event disrupts the interaction between P-loop and activation segment of B-Raf kinase, thus disturbing their conformation (90,91). Moreover, a functional analysis of *BRAF* mutations – including V600E and K6001del/E – indicates that most of them are clustered in the activation segment of kinase domain, that significantly increases kinase activity and may be accepted as a strong kinase activator. After all, several BRAF mutations are recognised as intermediate activators of kinase activity but, probably, they are not essential activating mutations in *BRAF* gene (90) (Figure 5).

Recently, another important factor – *BRAF/AKAP9* rearrangements, increasing the BRAF signalling – has been recognised in a small subset of PTCs as a novel mechanism of MAPK/ERK pathway activation (28). Moreover, the same research group has discovered an additional mechanism of BRAF kinase activation, involving numerous gains on chromosome 7 (there *BRAF* gene is located) or gene amplification in the classic variant as well as in the oncocytic variant of PTC (Hurthle cells) (92).

Additionally, it has lately been documented that LKB1 - an upstream activator of AMPK (AMP-activated protein kinase) – is preferentially associated with B-RAF V600E activation *via* phosphorylation on multiple sites (mainly in Ser325 and Ser428) of LKB1 by ERK in melanoma cell (93). The activity of LKB1-AMPK protein kinase signaling pathway – involved in the regulation of cell proliferation in mammalian cancer – is negatively regulated by the oncogenic B-RAF V600E mutants, probably through its downstream MEK-ERK kinase signaling cascade (93). It may be possible that suppression of LKB1 function by B-RAF V600E plays an important role in B-RAF V600E-driven tumorigenesis in thyroid cancer, but this hypothesis should be confirmed in experimental studies.

3.2. *RET*/PTC, *RAS*, *BRAF* in RAS/RAF/MEK/ERK pathway and their influence on gene expression profiles in thyroid neoplasms

According to many studies, focused on the mechanisms of activation of MAPK/ERK pathway, it has been accepted that B-Raf (as well as other Raf kinases) are downstream signal transducing molecules for RET/PTC. Moreover, in human PTC activating mutations in *BRAF* and *RAS* genes, similarly as in *TRK* and *RET*/PTC rearrangements, are largely mutually exclusive. Therefore, it was suggested that the RAS/RAF/MEK/ERK pathway may represent a linear cascade whose activation promotes thyroid tumorigenesis (89,90). To test this hypothesis, many *in vitro* and *in vivo* studies have been conducted to determine the expression profile of MAPK/ERK cascade genes and the phenomenon of their overlapping as well as their role in the thyroid transformation (10,73,94,). The results of these studies demonstrate that *BRAF*, *RAS* and *RET*/PTC alterations are mutually exclusive (73,89,95) and independently sufficient for cell signaling activation. On the other hand, it is suggested that *RET*/PTC and *BRAF* or *RAS* mutations can coexist, although very rarely, especially *RET*/PTC cooperates with *BRAF* alteration (96). It is possible, that none of these molecular events individually could confer any advantage for clonal tumour proliferation and could thus become established in subsequent cell generations. However, one certain thing is that the presence of *RET*/PTC induces dedifferentiation of thyroid tumours. Regarding Ras, this protein influences on apoptosis of thyroid cell with *RET*/PTC alterations, as evidenced in a rat thyroid cell model (97).

Interestingly, the activation of Ras, Raf, and RET proteins – through the similar molecular mechanisms – can determine different biological features of thyroid tumours (11). It should be emphasised that PTCs with *RET*, *RAS* or *BRAF* alterations have been confirmed to determine the characteristic clinical and histopathological features of thyroid tumours. Thus, *RAS* mutation is involved mainly in the follicular variant of PTC and determines lung metastases (58,67), while the presence of *BRAF* mutation predisposes to extrathyroid invasion and dedifferentiation at more advantageous tumour stage (82,83).

Recently it has been recognized that, in human PTCs, the presence of *RET*/PTC, *RAS*, *BRAF* genotypes determines different gene expression profiles in thyroid tumours (10,85,94). In vivo, *RET*, *TRK* or *RAS* differently influence on dedifferentiation and thyroid-specific genes (e.g., Tg or TSH) activation. It was confirmed, that *RET*/PTC3 significantly reduced Tg mRNA level or active form of TSH, while *TRK*-T1 sequences, induced *in vivo* in follicular thyroid cell, influenced on Tg or TSH only at the minimal level (64). Moreover, Denning *et al.* (98) documented that *RET*/PTC1 oncogenic sequences altered

the immunoprofile of thyroid follicular cell and influenced on thyroid cancer development.

Gene expression analysis, based on DNA oligonucleotide microarrays data, has classified the tumours into three distinct groups, depending on their mutational genotype (94). Mesa *et al.* (85), based on transfected rat thyroid clonal cell population (PCCL3), established functional categories of genes that may help explain some differences and similarities in thyroid cancer features. Their study confirmed the existence of functional gene clusters – defined as a functional group of genes – that were preferentially activated by *RET* or *BRAF* expression. It is worth noting, that in thyroid cells with constitutively expressed oncogenic *BRAF,* the expression of MMP – important in tumour invasion – is much greater than in cells harbouring *RET*/PTC3 oncogenic sequences. This fact may explain the predisposition of tumours with oncogenic *BRAF* expression to the more aggressive outcome and extrathyroid invasion and dedifferentiation from PTC to ATC (82,83). Moreover, it has been confirmed that *RET/*PTC3*, HRAS* and *BRAF* oncogenes upregulate a large group of overlapping genes, involving CXC chemokines and their receptors, which are recognised as stimulators of mitogenic and invasive capacity of thyroid cancer cells (10). Finally, it has been documented that a large group (about 24%) of *RET*/PTC3 regulated genes are simultaneously dependent on *BRAF* gene expression. Additionally, a gene cluster coding for the mitochondrial electron transport chain pathway – influencing cell variability – was down-regulated in this group of cells with *RET*/PTC3 and BRAF expression (85).

4. PI3K/AKT SIGNALING PATHWAY AND ITS ROLE IN MALIGNANT TRANSFORMATION

PI3Ks (phosphatidylinositol 3-kinases) have been classified into three major subfamilies – classes IA and B, classes II and III – based on the their structure and the substrate specificity (99). The most extensively investigated PI3Ks are class IA PI3Ks which are involved in a number of cellular functions, including cell growth, proliferation, and survival (100). It has been confirmed that only Class I (A and B) PI3Ks are involved in cancer development (101).

Class IA PI3Ks can be activated by receptor tyrosine kinases (RTKs), including: EGFR (epidermal growth factor receptor), PDGFR (platelet-derived growth factor receptor), FGFR (fibroblast growth factor receptor), IGF-1R (insulin-like growth factor 1 receptor), VEGFR (vascular endothelial growth factor receptor), interleukin receptors, interferon receptors, and integrin receptors. Additionally, intracellular proteins such as protein kinase C (PKC), SHP1, Rac, Rho, and Src can also activate PI3K (102).

Physiologically, after ligand-induced activation of specific receptors, PI3K can be activated through binding to the receptor p85 regulatory subunit of PI3K, followed by recruitment of the catalytic subunit of PI3K, p110, to this complex.

Alternatively, PI3K can be activated as a result of Ras activation (GTP- binding protein) which is able to induce the membrane translocation and activation of p110 subunit of PI3K. Upon activation, PI3K phosphorylates phosphatidylinositol-3,4-diphosphate (PIP2) into phosphatidylinositol-3,4,5-triphosphate (PIP3). The latter substrate, PIP3, acts as a second messenger, which binds PDK1 (phosphoinositide-dependent kinase 1) through pleckstrin homology (PH) domain and then activation of PDK1 occurs. The activated form of PDK1 phosphorylates Akt (cellular homolog of murine thymoma virus Akt8 oncoprotein) at Thr308 position activating its serine-threonine kinase activity. Akt is also recruited to the lipid-rich plasma membrane by its PH domain (99, 103) (Figure 6).

Akt (also known as protein kinase B, PKB) is the primary mediator of PI3K-initiated signaling. Akt has three isoforms: Akt1, Akt2, and Akt3 (PKBalpha, PKBbeta, and PKBgamma), which are encoded by three different genes. Upon activation, Akt phosphorylates a number of downstream targets for regulating various cellular functions, such as cell survival, cell cycle progression, migration, proliferation, metabolism, tumor growth, and angiogenesis (104,105,106).

It is worth noting that Akt kinase can cause inactivation of specific substrates or may activate other proteins. For instance, Akt can enhance cell survival through the inhibition of pro-apoptotic proteins such as forkhead (FOXO) family of transcription factors and Bcl2-antagonist of cell death (BAD) and can block the apoptosis through the induction of survival proteins such as Bcl2, IkappaB kinase (IKK), and human double minute 2 (HDM2) (101,106).

It has been demonstrated that the PI3K/Akt signaling pathway play a key role in regulating cell cycle progression and proliferation. By blocking FOXO-mediated transcription of cell-cycle inhibitors, including p27Kip1, or directly by phosphorylating and thus inactivating p27Kip1, Akt promotes the G_1/S phase transition. Akt induces cell proliferation by inhibiting the TSC1-TSC2 complex. Akt can also indirectly stabilize the cell-cycle protein c-Myc and cyclin D1 by inhibiting GSK3 (glycogen synthase kinase 3) (106,107).

It has been shown that PI3K/Akt signaling is important for regulating HIF-1alpha and VEGF expression which are the confirmed mediators transmitting signals for tumor growth and angiogenesis. Moreover, it has been documented that Akt – the major target of PI3K – transmits oncogenic and angiogenic signals in malignant transformation (106).

The regulation of cell survival and cell cycle progression by the PI3K/Akt pathway implicates a crucial role of this pathway in carcinogenesis and cancer development. Indeed, the relationship between dysregulated PI3K activity and the onset of cancer is well-documented.

PI3K mutations – leading to increased PI3K signaling – have frequently been observed in many human cancers, including cancer of the thyroid gland

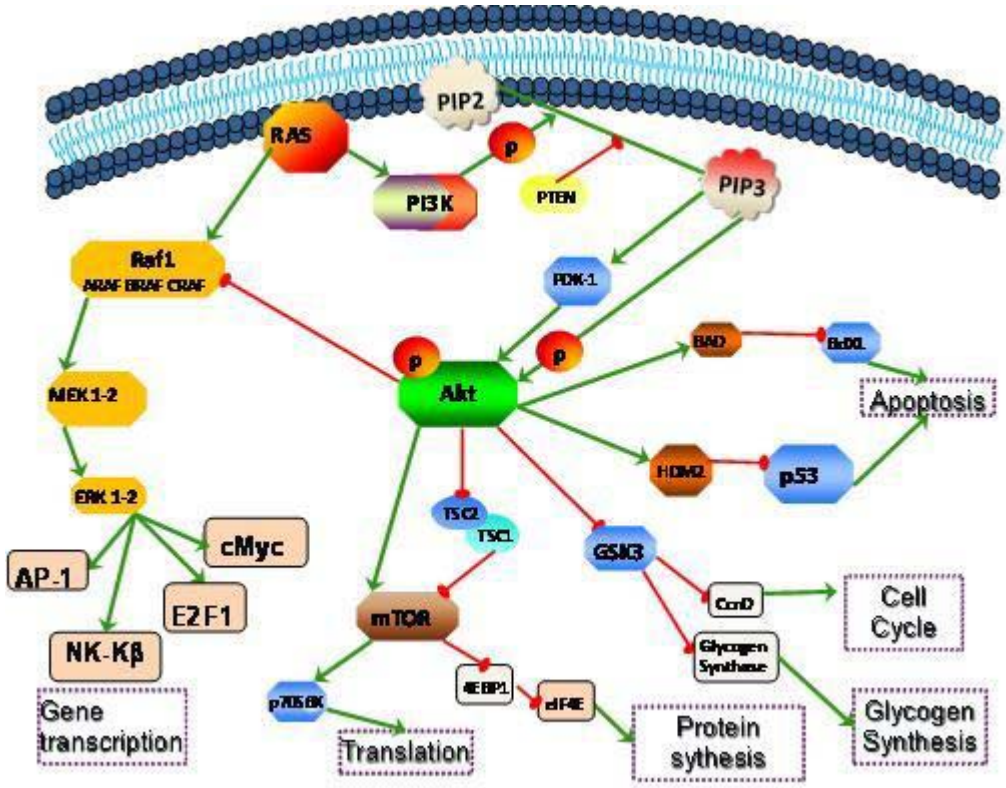

Figure 6. Schematic activation of the PI3K/Akt pathway.

(103,106,108-114).Those mutations involve genes encoding PI3K subunits, i.e., *PIK3CA* and *PIK3CB* (encoding the p110 catalytic subunits) and *PIK3R1* (encoding p85alpha regulatory subunit). *PIK3CA* alterations include activating somatic mutations, largely found in hot-spot regions of this gene, as well as frequent gene amplification, that lead to constitutively active form of the enzyme, similarly to the effect of somatic mutations in *PIK3R1* gene (108,115-117). *AKT* gene mutations and amplifications are also observed in a number of human cancers, including thyroid tumours (118-121).

The tumor suppressor gene, *PTEN* (phosphatase and tensin homologue deleted on chromosome 10), encodes the protein acting as the antagonist of PI3K. PTEN is a dual function lipid and protein phosphatase. Its lipid phosphatase function removes the 3′ phosphate of PIP3, leads to degradation of it and hence, it terminates the signaling downstream of activated PI3K. *PTEN* gene is frequently mutated or lost in many human cancer; the decreased *PTEN* expression is correlated with the progression of solid cancers (107,122-124).

4.1. Aberrant signaling of PI3K/Akt pathway in thyroid tumorigenesis

There are clinical correlations and experimental *in vitro* and *in vivo* data that PI3K/AKT signaling pathway plays the key regulatory role, both in thyroid tumor formation and progression. It has been demonstrated that PI3K signaling pathway plays a central role in proliferation, survival, invasion and motility in thyroid tumorigenesis. Several recent studies have investigated the role of genetic alterations in this signaling pathway in thyroid cancers (114,118,125,126). It has been shown that inhibition of PI3K and AKT isoforms reduce thyroid cancer cell cycle progression at G_2/M phase transition and can induce apoptosis (127). Studies in animal models have indicated a role for AKT in thyroid metastasis process (118,128).

The results of the study conducted by Hou *et al.* (125) indicate that 31% of benign follicular adenomas, 55% of FTCs, 58% of ATCs and 24% of PTCs harbored one of PI3K abnormalities, namely *PIK3CA* mutation or *PIK3CA* amplification or *PTEN* mutation. This supports a role for PI3K signaling in thyroid tumorigenesis, particularly for follicular neoplasias, and in thyroid cancer progression toward ATC.

In case of sporadic PTC, activation of the PI3K/Akt signaling pathway appears to be most prominent in tumor progression and activation of the RAS/RAF/MEK/ERK pathway is of primary importance in PTC development. In general, the data obtained from the studies on thyroid carcinomas suggest that genetically activated PI3K signaling represents a later-stage event in PTC, as enhanced activation of *Akt* dominates in the invasive forms of PTCs (105,118). In sporadic follicular thyroid cancers, the mutually exclusive nature of loss of *PTEN* expression and activating mutations and amplifications of the *PIK3CA* gene supports the ability of constitutive PI3K signaling to cause FTC (103).

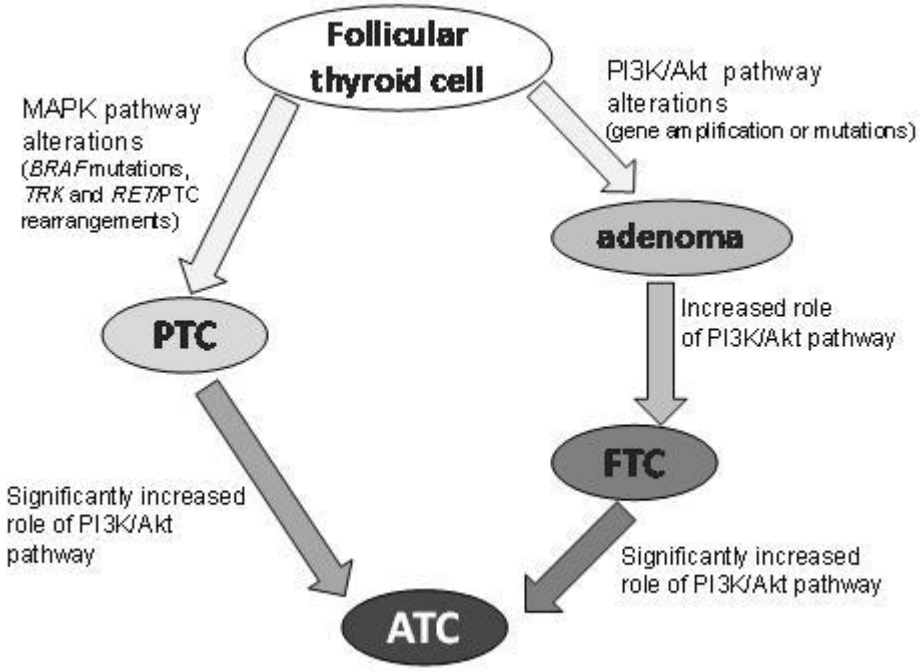

Figure 7. Involvement of the MAPK/ERK and PI3K/Akt pathways in the follicle cell-derived thyroid tumorigenesis.

The coexistence of genetic alterations in the PI3K/Akt pathway, including PIK3CA amplification and mutations, *PTEN* mutations and methylation and *RAS* mutation is most dominant in ATC, being smaller in FTC, and the lowest in PTC and benign lesions (125). In general, the studies confirm that the increased activities of the PI3K/Akt pathway are most often seen in aggressive thyroid cancers, such as ATC, which represents the final step in the multistage genetic model of thyroid follicular cell-derived tumorigenesis (114,129-131). That clearly suggests a role for PI3K signaling in progression from differentiated to undifferentiated thyroid tumours.

A schematic model for the role of PI3K/Akt pathway in thyroid tumorigenesis and progression is presented in Figure 7, considering also the involvement of the MAPK/ERK pathway. It is accepted that oncogenic activation of the PI3K/Akt pathway drives the transformation of follicular thyroid adenoma (FTA) to FTC and then to ATC. Regarding PTC, it develops *de novo* after activation of the MAPK/ERK pathway - by such oncogenic alterations as *RET*/PTC rearrangements or *BRAF* mutation - and its transformation towards ATC may be facilitated by the PI3K/Akt pathway (125,132).

The role of molecular alterations of particular effectors in PI3K/Akt pathway in thyroid tumorigenesis is presented in the Table 2.

4.1.1. The role of *PIK3CA* alterations

PIK3CA gene – which encodes for the catalytic subunit of p110alpha of PI3K – has been found to be amplified or mutated (mainly in exon 9 and 20) in thyroid carcinomas (116,125,126,132). It should be stressed that mutations identified in this gene are relatively common in ATC (114,125), whereas they represent rather rare mechanism of PI3K/Akt activation in PTC (116,126).

Worth of noting is the fact of high frequency of *PI3KCA* gene amplification that predominate over mutations in this gene (114,131). *PIK3CA* gene amplification, has been correlated with increased Akt phosphorylation and its activity in thyroid tumour progression. Wang et al (132) have found a strong tendency toward association of *PIK3CA* amplification and some high-risk clinicopathological characters of thyroid carcinoma.

PI3KCA gene amplification is found in PTC, FTC and ATC. However, there are some differences in frequency of *PIK3CA* amplification in PTC, as reported by authors, ranging from 5-14% to more than 50%, depending, probably, on population (126,132). In most studies, however, a higher frequency of *PIK3CA* gain copy number (defined as 4 or more copies) is observed in FTC (24-28%) than in PTC (114,116,125,132).

Interestingly enough, *PIK3CA* amplification has also been reported in the benign adenomas, thus suggesting that this alteration could be an initiating event in thyroid tumorigenesis (116,125,132).

PIK3CB is another catalytic subunit of PI3K. Copy number gains of this gene have been found in FTC and ATC (131).

Table 2. Molecular alterations in PI3K/Akt pathway in thyroid tumorigenesis

Gene	*Locus*	Biological function	Molecular alteration in PI3K/Akt pathway	References
PIK3CA	3q26.3	encoding p110, the catalytic subunit of PI3K	Activating somatic mutations in hot-spot regions gene (helical and kinase domain), genomic copy number gain, amplification	114,116,125, 126,131,132
PIK3CB	3q22.3			
PIK3R1	5q13.1	encoding p85 alpha, the regulatory subunit of PI3K		
AKT1	14q32.32	encoding AKT, the primary mediator of PI3K	Increased mRNA expression and protein activity	118,126
AKT2	19q13.1-q13.2			
PTEN	10q23.3	encoding phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase; dual function of lipid and protein phosphatase; dephosphorylation of PIP3	Somatic deletion and mutation, promoter methylation, LOH	114,118,125, 130,132-134, 136-140

4.1.2. The role of *AKT* alterations

Akt protein activity, as mentioned earlier, is enhanced by increased PI3K activity or reduced PTEN activity. The other mechanisms include Akt overexpression or *AKT* mutations, genetic changes in kinases or phosphatases that regulate Akt action, mutations in Ras, as well as mutations and/or overexpression of a variety of tyrosine kinase receptors. Increased Akt phosphorylation was found to be correlated with thyroid tumour progression.

It has been demonstrated that the inhibition of Akt isoforms reduce thyroid cancer cell cycle progression at G_2/M phase transition and can induce apoptosis (127). Studies on animal models have indicated some role for Akt also in thyroid metastasis process (118,128).

In human sporadic thyroid cancer – as well as in thyroid cancer cell lines – increased expression of *AKT* as well as increased levels of total Akt activity in comparison with normal tissue specimens has been found. Among the three isoforms of Akt, Akt 2 has appeared to be the predominant form in the malignant tissue (118). In some cases the malignant transformation has been related to *AKT2* gene amplification, although generally the increase of Akt proteins has been more impressive. This fact suggests a more important role for post-transcriptional and post-translational alterations. Interestingly, Akt levels have been increased in all FTC and only in a subset of PTC samples (118).

4.1.3. The role of *PTEN* alterations

PTEN gene, which acts as a tumor suppressor gene through the action of its phosphatase protein product, is involved in thyroid tumorigenesis.

The role of *PTEN* in thyroid tumorigenesis was firstly evidenced in the Cowden disease, a dominant genetic syndrome whose characteristics include carcinomas of the thyroid gland, i.e., mostly FTC. Inactivating mutations of *PTEN* have been defined as the cause of this syndrome (133) and molecular alterations of *PTEN*, i.e., point mutations and somatic deletions, were recognized as an important step in the development of thyroid gland cancers (134).

The study, conducted on a mouse model, has shown that complete loss of *Pten* in the thyroid gland was sufficient to induce thyroid neoplasia with increased PI3K signaling (135). In human, several studies have been conducted assessing genetic changes in *PTEN*, i.e., mutations and deletions as well as aberrant expression level, in sporadic benign and malignant thyroid cancers (114,118,136-138). Although, it appeared that inactivating mutations and deletions in *PTEN* gene are relatively high in ATC (125) and they are infrequent in PTC (132).

Despite lack of mutations, decreased expression of *PTEN*, both at mRNA and protein level, is found in thyroid tumours. In addition to LOH (loss of heterozygosity) in one allele, an epigenetic inactivation of the other allele of *PTEN via* its promoter methylation can explain its silencing in thyroid cancer (137,139,140). Hou *et al.* (130) have found significant correlation between *PTEN* methylation and genetic alterations in the PI3K/Akt pathway – especially *PIK3CA* amplification and mutations, as well as *RAS* mutations.

5. INTERACTIONS BETWEEN RAS/RAF/MEK/ERK ALTERATIONS AND PI3K/AKT PATHWAY ACTIVATION

As described earlier in this review, increased mitogenic signaling through classical tyrosine kinase-activated pathways (RAS/RAF/MEK/ERK or MAPK/ERK) has proven to play a major role in thyroid cancer, especially in PTC initiation as well as in progression to ATC (141). The MAPK/ERK cascade is intimately linked with the PI3K/Akt signaling. The results of numerous studies indicate a cross-talk between the two pathways. Both PI3K/Akt and MAPK may result in the phosphorylation of many downstream targets and play a role in the regulation of cell survival and proliferation (103).

Mutations occurring in *BRAF* or *RAS* genes, as well as in *RTK* genes, can dually activate the PI3K/Akt and MAPK pathways. Several studies have been conducted looking for coexistence of genetic alterations involving the above mentioned genes and other important genes in the PI3K/Akt signaling pathway.

A strong association between *RAS* mutations and Akt activation has been confirmed and the obtained results suggest that PI3K/Akt activation by *RAS* mutations is an early genetic event in thyroid tumours development (126,129). Especially, that the concomitant occurrence of *PIK3CA* copy gains and *RAS* mutations was also found in benign thyroid carcinomas (125).

Furthermore, the results of the research work conducted by J. Abubaker *et al*. (126), showed that many PTC samples with *BRAF* mutations had coexisting PIK3CA amplifications, and the authors suggested the existence of a synergism between *BRAF* mutations and PIK3CA amplifications in PTC tumorigenesis. The overlapping of *BRAF* mutation and PI3K/Akt pathway–related genetic alterations was also found in ATC (114,125).

On the other hand, Raf activity has been found to be negatively regulated in some cell types (103) (see Figure 6).

The increasing accumulation of genetic alterations in the PI3K/Akt and MAPK suggests that both pathways play a key role in the tumorigenesis and aggressiveness of thyroid tumours (125,131). This has been further supported by Liu et al (131) who looked for genetic alterations in various RTKs genes – that could activate both the PI3K/Akt and MAPK pathways – in FTC and ATC. They found frequent copy gains of RTK genes (but no mutations), including *EGFR*, *PDGFR*alpha, *PDGFR*beta, and *VEGFR* genes. RTK gene copy gains were more commonly associated with Akt phosphorylation than with ERK phosphorylation, suggesting that genetically altered RTKs preferentially use the PI3K/Akt pathway to promote ATC and FTC tumorigenesis and invasiveness (131).

The coexistence of genetic alteration in MAPK/ERK and PI3K/Akt effectors – relatively frequent in various thyroid tumors, particularly in FTC and ATC (125,132) – confirms the hypothesis of accumulation of molecular changes during dedifferentiation of the thyroid gland.

6. ACKNOWLEDGEMENTS

Both authors equally contributed to this article.

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Abbreviations: MAPK/ERK: mitogen - activated protein kinase/extracellular signal-regulated kinase signaling pathway; PI3K/Akt: lipid kinase phoshoinositide-3-kinase signaling pathway; WDTC: well-differentiated thyroid carcinoma; PDTC: poorly differentiated thyroid carcinoma; UTC: undifferentiated thyroid carcinoma; ATC: anaplastic thyroid carcinoma; PTC: papillary thyroid carcinoma; MAPK/ERK: mitogen-activated protein kinase/extracellular signal-regulated kinase; RTK: receptor tyrosine kinase; Shc/Grb2/SOS: Shc adapter protein/growth factor receptor bound protein 2/son of sevenless; RKIP: Raf kinase inhibitory protein; MEK: meiosis-specific serine/threonine protein kinase; ERK: extracellular-signal-regulated kinase; GDNF: glial cell-derived neurotrophic factor; MPK1: MAP kinase 1 gene; MAP2K: mitogen-activated protein kinase kinase gene; *RET*/PTC: rearranged in transformation/papillary thyroid carcinoma; Tyr: tyrosine; ECM: extracellular matrix; STAT3: signal transducer and activator of transcription 3; GSK3beta: glycogen synthase kinase 3 beta; NGF: nerve growth factor; TPM3: tropomyosin 3; TPR: translocated promoter region; TFG: TRK fused gene; Grb2/SOS: growth factor receptor-bound protein 2/son of sevenless; FTA: follicular thyroid adenoma; FTC: follicular thyroid carcinoma; MMP: matrix metalloproteinase; AMPK: AMP-activated protein kinase; PI3K: phosphatidylinositol 3-kinase; EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptor; FGFR: fibroblast growth factor receptor; IGF-1R: insulin-like growth factor 1 receptor; VEGFR: vascular endothelial growth factor receptor; PKC: protein kinase C; PIP2: phosphatidylinositol-3,4-diphosphate; PIP3: phosphatidylinositol-3,4,5-triphosphate; PDK1: phosphoinositide-dependent kinase 1; PH domain: pleckstrin homology domain; Akt: cellular homolog of murine thymoma virus Akt8 oncoprotein; PKB: protein kinase B; FOXO: family of transcription factors; BAD: Bcl2-antagonist of cell death; IKK: IkappaB kinase; HDM2: human double minute 2; p27Kip1: cyclin-dependent kinase inhibitor; HIF-1alpha: hypoxia-inducible factor 1 alpha; VEGF: vascular endothelial growth factor; PTEN: phosphatase and tensin homologue deleted on chromosome 10; LOH: loss of heterozygosity;

Key words: Activating Mutations, Gene Amplification, Epigenetic Alteration, Signaling Pathways, MAPK/ERK, PI3K/Akt, thyroid Carcinoma, Review

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