BRAF in papillary thyroid carcinoma

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Abstract. Novel genetic findings about papillary thyroid carcinoma identify BRAF gene as a subject of great interest. Involvement of BRAF gene in pathogenesis of PTC, diagnostic value and the putative prognostic significance of its T1799A mutation are summarized in this article. Furthermore, a particular attention is focused to the role of pre-operative detection of BRAF mutation in the FNAB specimens of thyroid nodules and to the use of this gene as target for an effective cancer therapy.

Keywords: BRAF mutation, papillary thyroid cancer, fine-needle aspiration

1. Introduction

Thyroid cancer is the most prevalent malignant endocrine tumor and papillary thyroid cancer (PTC), derived from follicular epithelial cells, accounts for approximately 80% of all thyroid malignancies [60]. Fine-needle aspiration (FNA) of thyroid nodules is the main tool for the optimal diagnostic and therapeutic management of thyroid nodular pathology. However, cytological examination is often inconclusive because of suboptimal quality material or the limited ability to discriminate between benign and malignant lesions; it provides an indeterminate diagnosis [59] and is subject to inter-observer variability [8,63].

The indeterminate diagnostic class (Thy3), as described by Poller [49], is a cytological finding that limits resort to surgery and suggests further analysis before total thyroidectomy. Cyto-histological correlation studies showed that 3–52% of indeterminate nodules were malignant tumors [42,58,72]. This means that numerous patients with no malignant tumor are subjected to an invasive procedure that exposes them to complications like hypoparathyroidism or laryngeal nerve injury, besides hypothyroidism. A method that can enhance the diagnostic accuracy of cytological examination would be of benefit to a large percentage of patients.

Useful information to refine indeterminate FNAs could be provided by the understanding of genetic alterations involved in thyroid neoplastic transformation and the application of biomolecular assays to cytological material.

2. PTC Pathogenesis

A pivotal role in the pathogenesis of PTC seems to be played by alterations of mitogen-activated protein-kinase (MAPK) pathways (Fig. 1).

Mitogen-activated protein kinase (MAPK) pathways are important within cells for the transduction of extracellular signals to the nucleus to allow appropriate response to external stimuli. The main components of these pathways are tyrosine kinase cell surface receptors, such as Ret, a small G-protein (Ras) and three kinases: Raf protein, MEK and ERK. This pathway permits the signal to move via phosphorylation and activation of the kinases ultimately to ERK, which can then affect transcription factors such as ELK, SAP, and other substrates involved in cell growth, differentiation, and survival. The ERK pathway has been found to be hyperactivated in about 30% of all cancers; three members of this pathway, Ret, Ras and Raf, are known oncogenes [6].

Ras is frequently mutated in many cancers, including cancer of the pancreas, lung, colon, thyroid, liver, and testis [17]. Ret is a receptor tyrosine kinase that, is essential for the development of the sympathetic, parasympathetic and enteric nervous systems, and the kidneys of mice [57]. When it is deregulated Ret can become a potent oncoprotein; germline mutations in Ret cause three related dominantly inherited cancer syndromes: multiple endocrine neoplasia type 2A (MEN2A), MEN2B, and familial thyroid carcinoma (FMTC).

Somatic chromosomal rearrangements of the Ret gene, resulting in a constitutively activated chimeric form of the RET protein (RET/PTC), are genetic hallmarks of PTC [67]. They have been reported to be
present in 5–87% of PTCs depending on the authors and detection methods [5,55]. Different translocations or inversions that cause recombination of the intracellular kinase-encoding Ret domain with heterologous genes are known [25], and their formation in vivo and in vitro induced by irradiation has been demonstrated [44]. A higher rate of this alteration has been found in populations exposed to external radiation [36,61] but also in benign conditions such as adenomas and Hashimoto’s thyroiditis [18].

Another protein involved in the MAPK pathway is RAF, a serine/threonine kinase that acts as an immediate downstream effector of RAS. RAF transmits signals from RAS to the mitogen-activated protein-kinase (MAPK) pathway through mitogen-activated protein/extracellular signal-regulated kinase (ERK) kinase (MEK) and ERK (RAS-BRAF-MEK-ERK) [14]. In mammalian cells, three isoforms of RAF have been identified: ARAF, BRAF and CRAF, each having a different tissue expression [13]. Among these three isoforms, BRAF has a higher affinity for MEK and a greater ability to activate the MAPK pathway [41] (Fig. 1). The BRAF gene is composed of 18 exons spanning a region of 190284 bp mapping to 7q34. It transcribes a 2478 base mRNA.

3. PTC Histologic sub-types

Many histological PTC variants have been described other than the conventional or classic papillary carcinoma, like follicular variant (FVPTC), tall cell variant and the rarer Warthin-like variant, microcarcinoma, oncocytic variant, diffuse sclerosing PTC, columnar variant, hyalinizing trabecular and mucoepidermoid thyroid tumors [4,53] which feed a continuous debate regarding the optimal classification, prognosis and treatment [11,35,40]. Moreover FVPTC, which is the most diffuse variant after the conventional PTC, appeared to be a heterogeneous disease composed of two distinct groups: an infiltrative/diffuse subvariant, which resembles classic papillary carcinoma in its metastatic lymph node pattern and invasive growth, and an encapsulated from, which behave more like FTC [39]. It should pointed out that an accurate and shared definition of the sub-types is needed to compare results reported by different authors and to define the correlation between phenotype and genetic events involved in PTC pathogenesis.

4. BRAF Mutations

BRAF somatic mutations have been reported in several cancers. They mainly cluster to exons 11 and 15
which are included in the kinase domain [14,51]. A spectrum of BRAF mutations is reported in Fig. 2.

In particular, the BRAF mutation in exon 15, T1799A, resulting in a valine-to-glutamate substitution (V600E), seems to mimic phosphorylation in the activation segment and to cause constitutive activation of the kinase thus conferring transforming and oncogenic functions [14].

In the past few years the T1799A mutation has been investigated in many studies and has been shown to be particularly frequent (from 29 to 83%) in thyroid cancer [2,9,19,26,37,56,69]. The mutation is the most prevalent of the known common oncogenic mutations in thyroid cancer and has been found in histologically proven PTCs (in micro PTCs, as well), in probably PTC-derived anaplastic thyroid cancer (ATC), but never in follicular thyroid cancer (FTC), medullary thyroid cancer (MTC), in benign thyroid neoplasms or in thyroiditis [69]. This means that the detection of a BRAF T1799A mutation in the setting of papillary thyroid cancer has a diagnostic specificity of 100% (Table 1).

Its role in initiation and progression of PTC has been clarified by its ability to induce this cancer in transgenic mice in which expression of the T1799A mutation in thyroid cells was targeted [32]. BRAF T1799A induction of in vitro expression in thyroid cell lines conferred a growth advantage activating DNA synthesis. It was not sufficient to transform the normal cells because of the concomitant induction of apoptosis. However, T1799A also induced chromosomal instability, as evidenced by micronuclei and mitotic bridge formation that may facilitate the acquisition of transforming secondary genetic events [43]. Further studies showed that the T1799A mutation is mutually exclusive with other genetic alterations sharing the MAP ki-
nase pathway as RAS mutation [21,46,55] while only two cases, to the date, showed a co-presence of BRAF mutation and RET/PTC rearrangements [15,16]. The T1799A BRAF mutation can be therefore considered an independent oncogenic event for PTC tumorigenesis.

The probability to detect somatic BRAF T1799A mutation in thyroid cancer is age-related and independent from previous radiation-exposure, unlike RET/PTC. An analysis of data published about this BRAF mutation in PTC in different populations highlighted an overall prevalence of 6 and 4% in radiation-exposed and non-exposed pediatric PTC, respectively [69]. Data regarding RET/PTC rearrangements showed an overall prevalence of 53 and 52% in radiation-exposed and sporadic pediatric PTCs, respectively, and has been found in over 60% of post-Chernobyl PTCs [68].

The BRAF mutation is a frequent event in sporadic adult PTCs but is rare in cancers in subjects exposed to radiation during their childhood (0 to 6%) [12,34,38]. If the link between radiation and chromosomal rearrangements as RET/PTC, specially in children, is clear, the association of BRAF mutation and age requires further investigations.

PTCs harboring this mutation may be characterized by a slower growth than cancers with RET/PTC which present a later clinical onset. Currently this is only a hypothesis that need to be further investigated for supportive evidence.

BRAF T1799A mutation prevalence showed a characteristic pattern in the different PTC subtypes, with a higher rate in classic PTC (45–65%), tall cell variant (33–88%), Warthin-like PTC (75%), oncocytic variant with conventional pattern (55%), while a lower prevalence was detected in FVPTC (14%) [64,65,71]. In a study of BRAF mutational status in histologic subtypes, an alternative BRAF mutation, A1801G (K601E), was detected in 7% of FVPTCs, in only a case of follicular adenoma but never in conventional PTC, follicular carcinoma and nodular goiter [65]. A1801G BRAF mutation is mutually exclusive both with T1799A mutation and with other mutation frequently detected in FVPTC like PAX8-PPARγ rearrangement and RAS mutations [3]. These data confirm the close relationship between genetic alteration of BRAF gene and PTC, and furthermore, between two morphological types of papillary thyroid carcinoma (PTC with papillary or mixed follicular/papillary architecture and with a follicular growth pattern) and two specific BRAF gene mutations (T1799A and A1801G, respectively).

Other BRAF mutations in thyroid cancer have been occasionally reported regarding a fusion with the AKAP9 gene through a paracentric inversion of the long arm of chromosome 7 [7,22], an in-frame insertion of three bases at codon 599 resulting in the insertion of an additional valine [2], and a triplet deletion in a case of PTC displaying a predominantly solid growth pattern (VK600-1E) [66].

5. BRAF T1799A mutation analysis

A limit to the molecular detection of the BRAF mutation on thyroid FNA specimens could be “contamination” by normal thyrocytes. Different methods allow detection of the BRAF T1799A mutation and each has its advantages and limitations.

Direct sequencing and dHPLC can detect novel unknown mutations in the gene, but, they require minimum 20% of mutant allele. This involves an enrichment or selection of cancer cells present in the specimen, using for example laser microdissection microscopy.

Real-time allele-specific PCR [24], colorimetric detection based on shifted termination assay [9,70], and mutant-allele-specific PCR amplification (MASA) [56] permitting detection of 1% mutated allele in a DNA sample are alternative methods for identifying the BRAF T1799A mutation.

Finally, an evolution of the gap ligase chain-reaction technique was used to detect point mutations in the presence of up to 10000-fold excess of wild-type allele DNA in a study concerning detection of BRAF mutation in primary biliary tract cancers [23].

6. Diagnostic value

Because of the remarkable prevalence and the 100% specificity of the BRAF T1799A mutation in papillary thyroid carcinoma, this genetic alteration is considered a powerful marker which supplements and completes thyroid FNA cytology. Therefore, many studies have investigated the diagnostic applicability of BRAF mutation detection on FNA specimens [2,26,56,69].

An analysis of the data published until 2005 showed an overall 44% prevalence of the BRAF T1799A mutation in FNAs from histologically confirmed PTCs whereas no mutation was detected in FTC or benign neoplasms. Moreover in 12/69 (17%) cytologically indeterminate cases with insufficient material, detection
of the BRAF mutation led to a diagnosis of PTC that was confirmed by post-operative histology [69].

Finally, it has been showed that detection of the BRAF mutation could be useful to verify inter-observer variations, with 13% of cytologically benign FNA specimens and 7% of FNAs thought to be thyroiditis that were rediagnosed as PTC [1].

In summary, when one considers the findings also from the more recent studies, the prevalence of BRAF mutated PTCs approximates ≈ 50% [2,56].

Hence, failure to detect the BRAF mutation in a FNA specimen of a single patient has no diagnostic value, while, detection of the BRAF T1799A mutation establishes a clear diagnosis of PTC.

7. Prognostic value

Several studies have focused on the prognostic value of the BRAF mutation often providing discordant results (Table 2) [29,45,47,50]. A multicenter study on a series of 219 PTCs showed a significant association between the BRAF mutation and the presence of extrathyroidal invasion, lymph-node metastasis, advanced tumor stage and cancer recurrence at a median follow-up of 15 months [71]. These findings were not confirmed by an Italian multicenter study involving 260 sporadic PTCs with different histological variants in which an association was found between the BRAF mutation and older age at diagnosis (p = 0.001) and the classic variant of PTC (p = 0.0001) but not with gender, tumor nodal metastasis and stage at diagnosis, at a median follow-up of 72 months [20].

Another study examined 203 conventional PTC patients and reported an association of BRAF T1799A with male gender (p = 0.006) and tumor size (P = 0.005) at a median follow-up of 7 years. Statistically, only univariate analysis evidenced an association of the BRAF mutation with tumor recurrence, whereas multivariate analysis adjusted for clinicopathological prognostic factors such as age, gender, tumor size, extrathyroid extension, multifocality and lymph node metastasis failed to show any statistical association, indicating that BRAF T1799A was not an independent predictor [30]. Considering the association between the different PTC histologic subtypes and BRAF mutations prevalence, part of the controversy among these studies may be due to the differences in sample size, particularly regarding the PTC subtypes, and in statistical analysis approach.

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<tr>
<td>Nikiforova et al. 2003</td>
<td>Extrathyroidal invasion 16/38 m vs 13/66 wt (p = 0.03)</td>
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<tr>
<td>Namba et al. 2003</td>
<td>Stages of tumor and distant metastasis 7/38 m vs 5/88 wt (p = 0.03)</td>
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<td>Kim et al. 2004</td>
<td>Neck lymph node metastasis 39/58 m vs 4/12 wt (p = 0.05)</td>
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<td>Fugazzola et al. 2004</td>
<td>No association with prognostic factors</td>
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<tr>
<td>Puxeddu et al. 2004</td>
<td>No association with prognostic factors</td>
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<tr>
<td>Xing et al. 2005</td>
<td>Extrathyroidal invasion (p &lt; 0.001)</td>
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<tr>
<td>Fugazzola et al. 2006</td>
<td>Age of diagnosis (p = 0.001)</td>
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<td>Kim et al. 2006</td>
<td>Gender (p = 0.006)</td>
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Other investigations focused on the role of the BRAF T1799A mutation in the biological events linked to the prognosis of thyroid cancer. A recent study investigated the role of the BRAF T1799A mutation and the MAPK pathway in thyroid cell dedifferentiation, particularly in Na\(^+\)/I\(^-\) symporter (NIS) impairment. The loss of NIS function disrupts the normal uptake of I\(^-\) on the thyreocytes. Evaluation of a set of 60 PTC samples to assess for NIS immunoreactivity and BRAF mutation showed a significantly low NIS expression and impaired membrane targeting in BRAF-positive samples (3.5% vs 30%; \(p = 0.005\)). These results suggested that BRAF T1799A correlates with a high risk of recurrence and less differentiated tumors due to the loss of NIS-mediated radioiodine uptake [52].

Other studies have suggested that BRAF gene mutation may have a place in the prognosis of the disease. Vascular endothelial growth factor (VEGF) expression, which was closely correlated with tumor size, extra-thyroidal invasion and stage, was upregulated in PTCs carrying BRAF T1799A mutation (OR = 2.5, CI = 1.1–5.6, \(p = 0.03\)) [27].

These findings suggest that detection of the BRAF mutation on FNA specimens is not only useful for diagnostic purposes, but may also yield prognostic information permitting proper risk evaluation and leading to improved clinical management of PTC.

8. BRAF as therapeutic target

Thyroid cancer is usually curable with the standard surgical treatments, often followed by adjuvant radioiodine therapy. However, an effective novel treatment is strongly needed for inoperable cases and/or neoplasias with impaired active transport of I\(^-\) in thyroid follicular cells.

Targeted cancer therapies attempt to disrupt pathways that are inappropriately regulated in cancer cells. Among solid cancers, thyroid carcinomas represent a promising paradigm for targeted therapy because of the adequate knowledge of many oncogenic events involved in initiation and progression of tumorigenesis, particularly regarding the MAPK pathway.

Obviously BRAF is the major candidate for a role as the target of PTC treatment because of its prevalence, specificity and association with undifferentiated and anaplastic forms.

Bis-aryl urea (BAY43-9006) is a potent and effective RAF inhibitor \textit{in vitro} and in mouse xenografts and it is presently being tested in clinical trials for other forms of cancer [33]. This compound is a CRAF inhibitor although its inhibitory power has been demonstrated in wild-type and T1799A mutated BRAF [28]. BAY43-9006 treatment blocks kinase signaling downstream to RAF kinase, inhibits BRAF-stimulated DNA synthesis and cell proliferation, induces apoptosis in melanoma cells harboring the BRAF mutation, and delays the growth of melanoma tumor xenograft in mice.

Recent investigations have studied different molecules. The effects of AAL-81 and LBT-613, two inhibitors of RAF kinase activity, have been examined on RAF-MAPK/extracellular signal-regulated kinase (ERK) kinase (MEK)-ERK activation in thyroid PCCL3 cells after conditional induction of BRAF T1799A expression [48]. These two molecules were shown to potently block RAS and RAF-dependent MEK phosphorylation in poorly differentiated human thyroid cancer cell lines with either RET/PTC1 or BRAF T1799A.

Inhibition of the pathway involved in thyroid cancer has been also tested using small inhibitory duplex RNA which causes BRAF knockdown in human anaplastic thyroid carcinoma cell lines carrying BRAF T1799A [54]. The results showed that BRAF knockdown inhibited the mitogen-activated protein kinase signaling cascade and the growth of cell lines carrying the BRAF T1799A mutation.

9. Conclusions

The BRAF mutation is an important discovery that promises to significantly enhance our knowledge of the molecular mechanisms by which thyroid cancer initiates and progresses. The perfect positive diagnostic value of this mutation in PTC makes it a powerful tool for improving the accuracy of preoperative fine-needle aspiration biopsy cytology. Since BRAF T1799A does not have a high prevalence in papillary carcinomas, a test for its detection together with additional sensitive and specific molecular assays may increase the proportion of PTCs that can be identified by molecular analysis. The development of increasingly more sensitive detection techniques may lead to the performance of BRAF T1799A tests in blood, thus surmounting the need for fine-needle aspiration and cytological examination. Finally, clinical trials testing the different BRAF-inhibitors may provide new prospects for effective targeted cancer therapy.
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