Clinical Prognosis in BRAF-Mutated PTC

ABSTRACT

BRAF mutation has recently emerged as a potential prognostic marker for papillary thyroid carcinoma (PTC) due to several studies suggesting that it may condition the development of tumors with aggressive behavior. A study of the phenotypes of thyroid follicular cell lines and transgenic mice characterized by targeted expression of BRAF mutation indicates that, at variance with RET/PTC rearrangement, it induces or facilitates genomic instability and higher invasiveness and eventually deeper tumor de-differentiation and more significant suppression of apoptosis. An analysis of differential gene expression of PTCs harboring BRAF mutation versus PTCs characterized by other genetic alterations shows an important impairment of the expression of genes related to intra-thyroidal iodine metabolism machinery, up-regulation of Glut-1 mRNA, methylation-induced gene silencing of tumor suppressor genes and up-regulation of pro-angiogenetic proteins such as VEGF. Correlation of BRAF mutation with PTC clinico-pathological features yields controversial results, with several studies showing the association with unfavourable clinico-pathological qualities, while others do not confirm the findings. This review will summarize the studies in favor or in contrast with a role of BRAF mutation as a prognostic marker in PTC. We will also indicate what information we still need in order to routinely introduce this indicator in clinical practice. (Arq Bras Endocrinol Metab 2007;51/5:736-747)

Keywords: BRAF mutation; Papillary thyroid carcinoma; Prognostic stratification; Prognostic marker; Iodine metabolism; ¹³¹I ablative treatment

RESUMO

Prognóstico Clínico no CPT com Mutações BRAF. Mutações no BRAF surgiram recentemente como potenciais marcadores prognósticos do carcinoma papilífero de tireóide (CPT) graças a vários estudos que sugerem que ele possa condicionar o desenvolvimento de tumores com comportamento agressivo. Um estudo do fenótipo das células de linhagem folicular de tireóide em camundongos transgênicos caracterizados pela expressão direcionada de mutações BRAF, indicam, à semelhança dos rearranjos RET/PTC, que ele induz ou facilita a instabilidade genômica, a alta invasividade e, por fim, uma profunda desdiferenciação tumoral com supressão mais significativa da apoptose. Uma análise da expressão gênica diferencial do CPT associado com mutações BRAF versus o CPT caracterizado por outras alterações gênicas mostra uma redução importante da expressão dos genes relacionados com a maquinaria do metabólismo do iodo intratiroideano, aumento da regulação do mRNA do Glut-1, silenciamento gênico induzido por metilação dos genes supressores tumorais e aumento da regulação das proteínas pró-angiogênicas, como a VEGF. A correlação da mutação BRAF com os achados clínico-patológicos do CPT mostra resultados controversos, com vários estudos indicando associação com parâmetros clínico-patológicos desfavoráveis e outros não confirmando esses achados. Esta revisão sumaria os estudos a favor ou não do papel da mutação BRAF como um marcador prognóstico no CPT. Indicaremos, também, quais informações são ainda necessárias para a introdução rotineira deste indicador na prática clínica. (Arq Bras Endocrinol Metab 2007;51/5:736-747)

Descritores: Mutação BRAF; Carcinoma papilífero de tireóide; Estratificação prognóstica; Marcador prognóstico; Metabolismo de iodo; Terapia ablativa com ¹³¹I
**BRAF Prognostic Value**

**Puxeddu & Moretti**

**BRAF MUTATIONS IN PTC**

BRAF is a serine/threonine kinase, which, after activation by RAS-GTP, triggers sequential phosphorylation and activation of MEK and ERK (27). Three functional RAF isoforms have been described in humans, A-RAF, B-RAF or BRAF and C-RAF (also termed c-RAF-1). Among these, BRAF, with its gene located on chromosome 7, is the most potent activator of the MAPK pathway (28). Very recently, large-scale genomic screens have detected mutations of BRAF in 66% of malignant melanomas and at a lower frequency in colorectal and ovarian cancers (29).

The importance of BRAF mutation also in thyroid cancer was revealed by several studies, which showed the frequent occurrence of this genetic alteration in PTC, with a prevalence ranging from 29 to 83% (mean 44%) (26). A unique somatic T1799A transversion in exon 15, resulting in a V600E amino acid substitution (BRAFV600E), has been detected in virtually all cases analyzed so far, with the exception of a K601E mutation found in some follicular variants of PTC (30,31), a G474R mutation, involving exon 11,
found in one follicular variant of PTC (31), a deletion of 3 nucleotides between codons 600 and 601 resulting in the VK600-1E mutation found in 3 PTC metastatic lymph-nodes (32) and a solid variant of PTC (33), an insertion of 3 nucleotides at position 599 resulting in the V599Ins mutation, detected in a classic variant of PTC (34), and a gene rearrangement AKAP9-BRAF described in 4 radiation-associated PTCs (35). All these mutations have been shown, or are thought, to destabilize BRAF kinase inactive conformation and to induce an oncogenic constitutive activation of the enzyme (35-37).

BRAF mutations occur more frequently than other known PTC genetic alterations such as RET (8–33% of PTC) and NTRK1 rearrangements (5–15% of PTC) and RAS point mutations (0–21% of PTC) (26). Interestingly, when these mutations have been simultaneously searched for, they have been shown to be mutually exclusive, accounting together for an initiating genetic event in about 70% of PTCs (34,38-40). These data, indicating self-sufficiency of each genetic alteration in follicular cell transformation, represent genetic evidence for the constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in PTC (38) and for a key role of the MAP kinase cascade activation in the transformation of thyroid follicular cells, as confirmed by in vitro studies on thyroid cell lines (41,42).

The demonstration that thyroid-targeted BRAFV600E transgenic mice develop thyroid tumors with PTC features (43) and the frequent detection of BRAF mutations in papillary microcarcinomas (20–52%) (30,44-47), thought to be early precursors of PTC, have been interpreted as evidence for an initiator role of BRAF mutations in PTC.

BRAF mutations are present not only in PTC, but to a smaller extent also in poorly differentiated and anaplastic thyroid carcinoma, especially when a well-differentiated PTC component containing the same mutation is detectable (44,48-52).

In conclusion, BRAF mutation appears to play a very prominent role in PTC tumor genesis. This perception prompted numerous studies aimed at disclosing the phenotype induced by BRAF mutation in thyroid follicular cells and more generally in papillary thyroid carcinomas.

**IN VITRO STUDIES ON THE BIOLOGICAL EFFECTS OF BRAF CONSTITUTIVE ACTIVATION IN THE THYROID**

The study of the biological effects elicited by BRAF constitutive activation in the thyroid was approached in two ways: either by studying the phenotypic and genomic changes induced by the expression of BRAFV600E in thyroid cell lines and in the thyroids of transgenic mice (table 1), or by analyzing differential gene expression in human PTC samples characterized by BRAF mutation versus normal thyroid tissue or PTC samples harboring different genetic alterations (table 2).

Conditional expression of BRAFV600E in the well-differentiated rat thyroid cell line PCCL3 was shown to induce de-differentiation and to confer little growth advantage because of concomitant activation of DNA synthesis and apoptosis (53). Interestingly, these effects were similar to those elicited by the induction of RET/PTC expression in the same cell model (54). However, BRAFV600E, in contrast to RET/PTC, was also shown to facilitate the acquisition of secondary genetic events through induction of genomic instability (53). Similarly, comparison of the expression profiles of PCCL3 cells characterized by the conditional expression of BRAFV600E, RET/PTC3, or RET/PTC3 with small interfering RNA-mediated knockdown of BRAF, showed the existence of a gene cluster commonly regulated by RET/PTC3 and BRAFV600E and two gene clusters specifically activated by each of the two oncoproteins (55). BRAF-induced gene cluster included matrix metalloproteinase 3 (MMP3), MMP9 and MMP13. Accordingly, conditional expression of BRAFV600E was associated with markedly increased invasion into Matrigel, while conditional expression of RET/PTC did not activate this phenomenon (55). Very recently, the transient expression of BRAFV600E in PCCL3 cells was also shown to sharply impair both Na+/I- symporter (NIS) expression and targeting to the plasma membrane, indicating loss of differentiation and predicting deficit of NIS-mediated 131I uptake (56). Interestingly, this impairment was not totally dependent on the MEK-ERK pathway. Finally, acute BRAFV600E over-expression in thyroid cancer cells was shown to increase nuclear factor κB (NF-κB) DNA-binding activity, resulting in up-regulation of anti-apoptotic proteins c-IAP-1, c-IAP-2, and X-linked inhibitor of apoptosis, up-regulation of MMP1 and MMP9 and cell invasion into Matrigel (57). In summary, these data indicate that the expression of BRAF mutation compared with other genetic alterations confers a more aggressive phenotype to follicular cells, including the acquisition of genomic instability and invasiveness and eventually of deeper loss of differentiation and stronger apoptotic resistance. Accordingly, targeted expression of BRAFV600E in thyroid cells of transgenic mice induced
the development of invasive PTCs, which transitioned to poorly differentiated carcinomas (43), while RET/PTC1 and RET/PTC3 thyroid-targeted transgenic mice developed only differentiated tumors which did not show loss of differentiation unless crossed with p53−/− animals (58-61).

Microarray studies conducted on human PTC samples, genotyped for BRAF<sub>V600E</sub> point mutation, RET/PTC1 and RET/PTC3 rearrangement and point mutations of KRAS, HRAS and NRAS allowed detection of distinct expression profiles for the BRAF, RET/PTC and RAS gene mutation groups (62). Differences were so specific that by using the 20 most significantly regulated genes in each subset, these simple classifiers were able to classify correctly the mutational status of all the tumors. These findings clearly indicate that mutational status is the principal determinant of gene expression variation within these tumors, predicting differences in phenotype and therapy-responsiveness between PTC genetic subsets. Accordingly, this study allowed the detection of a significant reduction of the TPO gene expression specifically in PTCs harboring the BRAF mutation, which was also confirmed at the protein level by immunohistochemistry. A similar result was also reported by another group that confirmed lower total TPO mRNA expression in PTCs bearing BRAF mutations in contrast with those not characterized by the genetic alteration, although
in this case the difference was not statistically significant (63). Because thyroperoxidase plays a pivotal role in iodine organization during thyroid hormone synthesis, these results suggest that \textit{BRAF} mutant tumors should display a lower iodine retention capacity.

Similarly, another study showed a statistically significant relationship between the methylation-associated silencing of the \textit{SLC5A8} gene, coding for an apical iodine transporter (AIT), important to ensure a passive transfer of the halogen across the apical membrane of the follicular cell, and the T1799A point mutation of the \textit{BRAF} gene in conventional PTCs (64). Moreover, a recent immunohistochemical study reported a significantly low NIS expression and impaired targeting to the membranes in \textit{BRAF} mutation-positive samples (56). Because NIS plays a pivotal role in thyroid iodine uptake, this finding suggests impairment of iodine transport from the extra-cellular compartment to the cytoplasm of the follicular cell in \textit{BRAF} mutant tumors.

Very recently, our group examined the expression of a panel of thyroid differentiation markers, including NIS, AIT-B, thyroglobulin (Tg), TPO, TSH receptor (TSH-R), the transcription factor PAX8, and glucose transporter type 1 (Glut1), in 38 PTCs harboring \textit{BRAF} mutation, and 28 PTCs with wild-type \textit{BRAF} (Durante C, Puxeddu E, Filetti S and Russo D, unpublished observation). Whereas mRNA levels of all thyroid-specific genes examined, except TSH-R, were reduced in PTCs versus normal thyroid tissues, \textit{BRAF} mutation-positive tumors presented significantly lower mRNA expression levels of NIS, AIT-B, Tg, and TPO than \textit{BRAF}-wild type ones. On the contrary, the two groups presented similar levels of TSH-R and PAX8 mRNA expression. Moreover, Glut-1 transcript levels were increased in all PTCs, and

### Table 2. Differential gene expression correlated to \textit{BRAF} mutation in PTC tissue samples.

<table>
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<tr>
<th>Gene</th>
<th>Function</th>
<th>Expression changes detected in \textit{BRAF}^{V600E} PTC</th>
<th>References</th>
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<td>TPO</td>
<td>Thyroperoxidase&lt;br&gt;Thyroid hormone biosynthesis: Iodine organization into tyrosines of thyroglobulin&lt;br&gt;Coupling of tyrosil residues</td>
<td>mRNA down-regulation&lt;br&gt;Reduced protein expression (IHC)</td>
<td>Giordano TJ et al. (62) Di Cristofaro J et al. (63)</td>
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<td>SLC5A8/AIT</td>
<td>Apical iodine Trasporter&lt;br&gt;Iodine transfer across the apical membrane of the follicular cell</td>
<td>Methylation-associated silencing&lt;br&gt;mRNA down-regulation</td>
<td>Porra V et al. (64) Durante C &amp; Puxeddu E et al. (unpublished observation)</td>
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<tr>
<td>NIS</td>
<td>Na(^+/)I- symporter&lt;br&gt;Iodine transfer from the extracellular compartment inside thyroid follicular cells</td>
<td>Reduced protein expression (IHC) and impaired targeting to the membranes (IHC)</td>
<td>Riesco-Eizaguirre G et al. (56)</td>
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<tr>
<td>Tg</td>
<td>Thyroglobulin&lt;br&gt;Thyroid hormone storage protein and main constituent of colloid</td>
<td>mRNA down-regulation</td>
<td>Durante C &amp; Puxeddu E et al. (unpublished observation)</td>
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<tr>
<td>Glut1</td>
<td>Glucose transporter 1&lt;br&gt;Glucose transport from the extra-cellular compartment inside thyroid follicular cells</td>
<td>mRNA up-regulation</td>
<td>Durante C &amp; Puxeddu E et al. (unpublished observation)</td>
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<tr>
<td>VEGF</td>
<td>Vascular-endothelial growth factor&lt;br&gt;Important growth factor involved in blood vessel formation in normal and tumoral tissues</td>
<td>Increased protein expression (IHC)</td>
<td>Jo YS et al. (68)</td>
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<tr>
<td>TIMP3</td>
<td>Inhibitor of metalloproteinase-3&lt;br&gt;Inhibits matrix metalloproteinases activation</td>
<td>Methylation-associated silencing</td>
<td>Hu S et al. (69)</td>
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<tr>
<td>DAPK</td>
<td>Death-associated protein kinase&lt;br&gt;Involved in apoptosis activation</td>
<td>Methylation-associated silencing</td>
<td>Hu S et al. (69)</td>
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IHC: Immunohistochemistry.
additional significant increases were noted in BRAF-mutation positive tumors compared to the BRAF-wild type counterpart.

In the first instance, the gene expression results reported so far account for a significant impairment of intra-thyroidal iodine metabolism in BRAF mutant tumors, suggesting that this subset of PTC is characterized by a lower responsiveness to radioactive therapy due to defects in iodine uptake and retention. Secondly, the up-regulation of the glucose transporter Glut-1, indicative of an increased glucose metabolism, predicts visualization of BRAF mutant tumors and their metastases by F-18-fluorodeoxyglucose (18-FDG) positron emission tomography (PET). Intriguingly, these findings recapitulate the typical clinical features of less differentiated and more aggressive PTCs, which lose iodine responsiveness but gain 18-FDG metabolism capacity (65-67).

Finally, two further studies still in favor of the more aggressive biology of BRAF mutant PTCs deserve to be mentioned. The first reports a significant up-regulation of vascular endothelial growth factor (VEGF) in BRAF mutation positive PTCs compared with BRAF-wild type PTCs, and its strong positive correlation to larger tumor size and extra-thyroidal invasion and more advanced tumor stage (68). The latter analyzed promoter methylation-induced gene silencing of several recently identified tumor suppressor genes, including those for tissue inhibitor of metalloproteinase-3 (TIMP3), the above-mentioned SLC5A8/AIT, death-associated protein kinase (DAPK) and retinoic acid receptor β2 (RARβ2), in BRAF mutation-positive and -negative PTC tumor samples (69). Methylation of TIMP3, SLC5A8/AIT and DAPK was significantly associated with BRAF mutation and with several aggressive features of PTC, including extra-thyroidal invasion, lymph node metastasis, multifocality and advanced tumor stage. The authors concluded that their data suggest that aberrant methylation and consequent silencing of the studied tumor suppressor genes may be an important step in BRAF mutation-promoted tumorigenesis and aggressiveness of PTC.

In conclusion, all the presented data clearly indicate that expression of BRAF mutation is associated with induction of a more aggressive phenotype of transformed follicular cells when compared to other genetic alterations. Thus, it is conceivable that also at the clinical level the presence of BRAF mutation might be strongly associated with unfavorable clinicopathological features and outcomes. Surprisingly, this was not always the case in the series analyzed so far.

In the last four years we have seen the publication of at least sixteen studies which correlated BRAF mutation with PTC clinical features predictive of recurrence/persistence or death, including age, gender, tumor size, extra-thyroidal invasion, lymph node metastases, distant metastases, multifocality, and stage and/or with pathological characteristics, including histologic variants of PTC (30,44-48,56,68,70-77). Six of these studies also correlated BRAF mutation with disease recurrence (47,56,70,75-77), while two specifically analyzed PTC microcarcinoma series (46,47). Moreover, during the same period the literature was enriched with three studies specifically analyzing BRAF mutation prevalence in PTC lymph node metastases (32,78,79), with three studies exploring BRAF mutation prevalence in anaplastic thyroid carcinomas (50-52), and with five studies searching for the prevalence of BRAF mutation in radiation-induced or sporadic PTC of young subjects (80-84).

Table 3 summarizes the findings of the sixteen studies correlating BRAF mutation with PTC clinicopathological features.

In detail, four studies found a statistically significant association of BRAF mutation with an older age at the time of diagnosis of PTC (30,44,73,77). Of note, three of these studies included one hundred patients or more. In agreement, very recently at least five papers have reported a low prevalence of BRAF mutation in PTCs diagnosed in childhood (80-84). The inter-studies discrepancy may be related to heterogeneity in age distribution of patients in the different cohorts. In detail, inclusion of pediatric PTC or of a young adult population, whose tumors are less frequently positive for BRAF mutation, or of older patients, whose PTC are eventually more frequently positive for the genetic alteration, may contribute to increasing the divergences of ages between the BRAF mutation positive and negative groups allowing the studies to reach statistically significant differences. Although older age is an unfavorable prognostic factor (85), a relationship between BRAF mutation, older age, and higher clinical aggressiveness still needs to be established.

Two studies correlated BRAF mutation with male gender (71,75). The inconsistency of the observation, not detected by the other 13 studies that addressed this issue, and the elusive biological mechanism regulating such a gender preference, imply a possible patient selection bias.
Tumor size was detected as being significantly increased in \(\text{BRAF}\) mutation-positive PTCs in two studies (68,75) and significantly reduced in a third one (76). In all the other studies no statistically significant difference was detected between \(\text{BRAF}\) mutation-positive and -negative tumors. The conclusion may be that \(\text{BRAF}\) mutation does not significantly influence tumor growth. However, considering other findings possibly correlating \(\text{BRAF}\) mutation with higher aggressiveness or invasiveness, the conclusion may be broader: \(\text{BRAF}\) mutation does not favor tumor growth, but under the same growth conditions may induce earlier activation of tumor invasiveness.

In agreement with the latter statement, three studies detected a significant correlation between \(\text{BRAF}\) mutation and extrathyroidal invasion (44, Table 3. Findings of the studies correlating \(\text{BRAF}\) mutation with PTC clinicopathological features.

<table>
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<th></th>
<th>Sedliarou et al. (microPTC)</th>
<th>Fugazzola et al.**</th>
<th>Xu et al.</th>
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<tr>
<td>(\text{BRAF}^{\text{V600E}}) cases/total</td>
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<tbody>
<tr>
<td>(\text{BRAF}^{\text{V600E}}) cases/total</td>
<td>38/104</td>
<td>49/105</td>
<td>45/124</td>
<td>38/126</td>
<td>102/161</td>
<td>149/203</td>
<td>107/219</td>
<td>99/260</td>
<td>105/219</td>
<td>107/219</td>
<td>107/219</td>
</tr>
<tr>
<td>Age</td>
<td>&lt; 0.0001</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
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<tr>
<td>Male gender</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>Tumor size</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Extrathyroidal invasion</td>
<td>0.03</td>
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<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>Lymph node metastases</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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</tr>
<tr>
<td>Distant metastasis</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>Multifocality</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
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<td>Stage</td>
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<td>NS</td>
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</tr>
</tbody>
</table>

* In favour of BRAFWT subgroup; **The patients from the two studies were pooled with others in the study by Fugazzola & Puxeddu et al; *** I+II vs III+IV NS: not statistically significant; —: not calculated
which enrolled 260 patients (77). Of note, four of these studies had the largest case collection of the series. The remaining studies were unable to detect this association, including an Italian multicenter one, which enrolled 260 patients (77).

A significant correlation between BRAF mutation and lymph node metastases was pinpointed only by one study (76). Another study, conducted on a small microcarcinoma cohort, found a correlation close to statistical significance (47). However, at least three other recently published papers support the possibility that BRAF mutations, either clonally present in the primary tumor or de novo formed in the metastatic cells, play a pivotal role in papillary thyroid carcinoma cells seeding at the level of lymph nodes (32,78,79). In detail, two of these studies showed a significantly higher prevalence of lymph node metastases in BRAF mutation-positive tumors (78,79), while all three produced proof of possible de novo BRAF mutation formation in PTC lymph node metastatic cells.

Only one study found a weak statistically significant correlation between BRAF mutation and distant metastases (p = 0.033) (48). In part this lack of correlation may be related to the low number of patients presenting with distant metastases in each study. Thus, the findings obtained so far should be considered inconclusive.

A correlation between BRAF mutation and more advanced stage of the disease was clearly detectable in four studies (44,56,48,76) and was close to statistical significance in a fifth (73). Four of these studies included about one hundred or more patients. However, two of the three studies including more than two hundred patients, one from Korea (75) and one from Italy (77), were unable to confirm the correlation, although in the case of the Asian study this could be due to the selection of only low-risk cases.

Among the six studies that analyzed the correlation between BRAF mutation and recurrence (47,56,70,75-77), three documented this association (56,75,76). Three of the six studies included less than 70 cases (47,56,70). Among the three studies including a higher number of patients, only the North American (76) and the Korean (75) were characterized by the finding, while the multicenter Italian study (77) was not able to show any statistically significant difference in recurrence rate between BRAF mutation positive and negative tumors. Interestingly, as mentioned above, the Korean study included only low-risk patients with conventional PTC. However, only in the North American study did the detected statistically significant correlation between BRAF mutation and recurrence at univariate analysis remain significant on multivariate analysis, after adjusting for conventional clinico-pathological predictors of recurrence. Moreover, in this study BRAF mutation was also an independent predictor of recurrence in patients with stage I/II disease. Median follow-up periods were respectively 15 months for the North American study, 88 months for the Korean study and 72 months for the Italian study. Thus, the high recurrence rate of BRAF mutation-positive PTC in the North American study may be ascribed to a high rate of early relapses of persistent disease. In this study, a sub-analysis of twenty recurrent PTC patients showed that recurrent disease was more extensive and needed more aggressive treatment (surgical and external radiation therapies) in the BRAF mutation-positive patients than in the BRAF mutation-negative ones. In addition to radioiodine treatment, nine of 13 (69%) recurrent patients with BRAF mutation needed at least one additional surgery and/or external radiation therapy, whereas only one of seven (14%) recurrent patients without the mutation needed additional surgery and no radiotherapy. Moreover, seven of thirteen (54%) patients in the BRAF mutation-positive group versus none of seven (0%) in the BRAF mutation-negative group lacked 131I avidity in their foci of recurrent tumor. Interestingly, these findings are confirmed by the third recent study from Spain, which detected a correlation between BRAF mutation and recurrence on a smaller group of patients over a follow-up period of 36 months (56). Also in this study, the frequencies of recurrences with negative 131I whole body scan were higher in the BRAF mutation-positive group (6/9, 66%) than in the BRAF-mutation negative group (1/3, 33%), predicting a worse outcome and indicating a less differentiated state in the BRAF mutation-positive PTC. All these clinical findings are consistent with the above-mentioned gene expression data (see previous section), which consistently indicate a depression of the intra-thyroidal iodine metabolism machinery in BRAF mutation-positive PTC.

Nine of the ten studies which analyzed correlation of BRAF mutation with PTC histological variants found a significant association between the presence of the genetic alteration and a conventional PTC histology (papillary or mixed follicular-papillary growth pattern) and/or between the absence of the genetic alteration and a pure follicular variant histology (30,44,46,56,70,72,73,76,77). Three studies, characterized by the inclusion of a significant number of PTCs of the tall cell variant, also found a significant
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association between BRAF mutation and the tall cell variant histology (44,56,76). Thus, it clearly appears that mutation of BRAF drives the development of PTCs with a pure papillary or tall cell papillary phenotype. The tall cell variant of PTC is considered by many authors more aggressive and less differentiated than the other histological variants of the tumor (17,18). The specific association of a BRAF<sup>K601E</sup> and BRAF<sup>G474R</sup> mutations with the follicular variant of PTC recently described by one group (30) has not been confirmed by any other study so far.

BRAF mutation was also detected in a significant subset of undifferentiated thyroid carcinomas (44,48-52) and in one study in 2 out of 16 poorly differentiated thyroid tumors (44). In most of these studies a noteworthy part of the BRAF mutation-positive undifferentiated or poorly differentiated thyroid carcinomas had a recognizable papillary well-differentiated background component. Where both components were tested simultaneously for the mutation, a 100% concordance of the BRAF profile was shown.

These findings demonstrate that progression from papillary thyroid carcinoma to poorly differentiated and anaplastic carcinoma may be favored in many tumors by constitutive activation of BRAF. Moreover, together with the evidence that BRAF mutation also occurs in microcarcinomas (30,44-47), they suggest that this mutation, being an early event and probably insufficient alone for the fully aggressive phenotype, may predispose the tumor cell to acquisition of additional genetic alterations, which in turn activate more aggressive pathways and lead to de-differentiation. As seen in the previous section, biological evidence from thyroid cell line (53,56) and transgenic mice experiments (43) support these conclusions. It is noteworthy that a recent study showed that one subset of anaplastic thyroid carcinomas are derived from BRAF mutation positive PTC due to the gain of a p53 mutation (52). RET/PTC rearrangement seems to behave differently and to define a subset of papillary thyroid carcinomas lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotype (52,86).

**CONCLUSIONS AND FUTURE DIRECTIONS**

The perception coming from the described biological and clinical studies is that BRAF mutation is an adverse prognostic indicator for PTC. However, its routine clinical application for prognostic stratification is hampered by the discrepant data coming from the correlation studies summarized in the previous section and by the paucity of robust supporting clinical evidence. Indeed, only the study by Xing et al. (76) is characterized by the identification of a statistically significant correlation, which consistently holds at multivariate analysis.

These discrepancies might be due either to the heterogeneity of the histological variants of PTC or to the age group analyzed or to the small number of studied cases or to genetic differences of the considered populations. However, another explanation should also be taken into account: the possibility that the conflict of the data is related to an important difference in the extent of disease at the moment the patients entered the studies, namely the moment of initial treatment. Geographical differences in the timelines of thyroid cancer diagnosis may play an important role in this. Indeed, countries characterized by an easily accessible and free health care system, by awareness of thyroid disease due to diffusion of endemic goiter, and by intense use of thyroid ultrasonography may have a higher number of incidental early thyroid carcinoma diagnoses. In this context PTCs may not have fully expressed their aggressive potential and may receive a more complete initial surgical treatment so that the clinical measurement of the higher biological aggressiveness of BRAF mutation-positive cancers may not be possible, at least in the short term (maybe in the long term it will). The situation might be different in countries where the most frequently detected tumors are those which are recognized because they are clinically evident. These cases may represent cancers which have developed their aggressive potential and that may be less amenable to radical surgery, and, if characterized by BRAF mutation, less responsive to radioiodine.

Well-designed prospective multicenter studies are needed in order to definitively clarify the role of BRAF mutation as a useful clinical indicator of PTC aggressiveness. Preliminary evaluation of the necessary statistical power and data analysis separating clinically relevant tumors from incidental ones will be important issues. If these studies confirm BRAF effectiveness in prognostic stratification of PTC patients, it is conceivable that in the future preoperative genotyping of thyroid fine needle aspirates (FNA) could alter the surgical approach and radioiodine treatment modalities, perhaps by mandating more aggressive lymph node dissection and by inducing the use of higher doses of <sup>131</sup>I in BRAF mutation-positive PTC. Moreover, a BRAF genotype may condition a more intense follow-up and eventually the use of particular image modalities, such as 18-FDG positron emission tomography, to search for tumor persistence or recurrences.
In conclusion, BRAF mutation might become a novel informative prognostic marker, which can readily be detected in FNA cytological material and has the potential to improve risk stratification and recurrence prediction in patients affected by PTC, thus leading to better informed decisions about initial and long-term management. However, appropriately designed studies aimed at definitively verifying this possibility still need to be initiated.

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We intended to cite all the significant studies in this field, but apologize for the possible, unintended omission of any relevant references.

REFERENCES

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52. Quirós RM, Ding HG, Gattoo P, Prinz RA, Xu X. Evidence that one subset of anaplastic thyroid carcinomas are derived from papillary carcinomas due to BRAF and p53 mutations. Cancer 2005;103:2261-8.


84. Mazzaferri EL, Kioos RT. Clinical review 128: Current approaches to primary therapy for papillary and follicular thyroid cancer. \textit{J Clin Endocrinol Metab} 2001;86:1447-63.


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