BRAF mutation in thyroid cancer

M Xing

Division of Endocrinology and Metabolism, Department of Medicine, Johns Hopkins University School of Medicine, 1830 E. Monument St/Suite 333 Baltimore, MD 21287, USA

(Requests for offprints should be addressed to M Xing; Email: mxing1@jhmi.edu)

Abstract

Genetic alteration is the driving force for thyroid tumorigenesis and progression, based upon which novel approaches to the management of thyroid cancer can be developed. A recent important genetic finding in thyroid cancer is the oncogenic T1799A transversion mutation of BRAF (the gene for the B-type Raf kinase, BRAF). Since the initial report of this mutation in thyroid cancer 2 years ago, rapid advancements have been made. BRAF mutation is the most common genetic alteration in thyroid cancer, occurring in about 45% of sporadic papillary thyroid cancers (PTCs), particularly in the relatively aggressive subtypes, such as the tall-cell PTC. This mutation is mutually exclusive with other common genetic alterations, supporting its independent oncogenic role, as demonstrated by transgenic mouse studies that showed BRAF mutation-initiated development of PTC and its transition to anaplastic thyroid cancer. BRAF mutation is mutually exclusive with RET/PTC rearrangement, and also displays a reciprocal age association with this common genetic alteration in thyroid cancer. The T1799A BRAF mutation occurs exclusively in PTC and PTC-derived anaplastic thyroid cancer and is a specific diagnostic marker for this cancer when identified in cytological and histological specimens. This mutation is associated with a poorer clinicopathological outcome and is a novel independent molecular prognostic marker in the risk evaluation of thyroid cancer. Moreover, preclinical and clinical evaluations of the therapeutic value of novel specific mitogen-activated protein kinase pathway inhibitors in thyroid cancer are anticipated. This newly discovered BRAF mutation may prove to have an important impact on thyroid cancer in the clinic.

Endocrine-Related Cancer (2005) 12 245–262

Introduction

Thyroid cancer is the most common endocrine malignancy. It can be classified histologically into follicular epithelial cell-derived papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), anaplastic thyroid cancer (ATC), and para-follicular C-cell-derived medullary thyroid cancer (MTC), which account for approximately 80, 15, 2, and 3% of all thyroid malignancies, respectively (Hundahl et al. 1998). Thyroid cancer harbors several highly prevalent genetic alterations, some of which are seen only in this cancer. The classical oncogenic genetic alterations commonly seen in thyroid cancer include Ras mutations (Fagin 2002, Bongarzone & Pierotti 2003), RET/PTC rearrangements (Nikiforov 2002, Santoro et al. 2002, Tallini 2002), and PAX8-peroxisome proliferator-activated receptor γ (PPARγ) fusion oncogene (Kroll et al. 2000, McIver et al. 2004). Various activating Ras mutations, widely seen in other cancers as well, occur mainly in FTC and the follicular variant of PTC (Vasko et al. 2003, Zhu et al. 2003). RET/PTC rearrangement represents a recombination of the promoter and N-terminal domain of a partner gene with the C-terminal region of the RET gene, resulting in a chimeric oncogene with a protein product containing a constitutively activated RET tyrosine kinase. At least 10 types of RET/PTC rearrangement have been identified, which differ by their 5’ partner genes, with RET/PTC1, RET/PTC2, and RET/PTC3 being the most common and occurring mainly in PTC and some benign adenomas. The PAX8-PPARγ occurs both in FTC and benign thyroid adenoma (Cheung et al. 2003, Sahin et al. 2005). The recently discovered activating mutation in BRAF (the gene for the B-type Raf kinase, BRAF), the focus of this review, represents
the most common genetic alteration in thyroid cancer. The RET and other mutations responsible for the less common and histologically distinct MTC, which are derived from parafollicular cells, are reviewed elsewhere (Koper & Lamberts 2000, Ichihara et al. 2004, Santoro et al. 2004). Most of the genetic alterations in thyroid cancer exert their oncogenic actions at least partially through the activation of the RET/PTC→Ras→Raf→mitogen-activated protein kinase (MAP kinase)/extracellular-signal-regulated kinase (ERK) kinase (MEK)→MAP kinase/ERK pathway (referred as the MAP kinase pathway hereafter). Activation of this pathway is a common and important mechanism in the genesis and progression of human cancers through upregulating cell division and proliferation. When constitutively activated, the MAP kinase pathway leads to tumorigenesis (Peyssonnaux & Eychene 2001, Hilger et al. 2002).

The discovery of activating mutations of the gene for BRAF has expanded the array of the known genetic alterations that activate the MAP kinase pathway and underscores the importance of this pathway in human cancer (Davies et al. 2002). Among the three forms of Raf kinases, BRAF, with its gene located on chromosome 7, is the most potent activator of the MAK kinase pathway (Sithanandam et al. 1992, Mercer & Pritchard 2003). BRAF-activating missense point mutations in the kinase domain are clustered in exons 11 and 15 of the gene and the T1799A transversion mutation accounts for more than 80% of all the BRAF mutations (Davies et al. 2002). This mutation had been formerly called T1796A, based on the NCBI GenBank nucleotide sequence NM 004333, which missed a codon (three nucleotides) in exon 1 of the BRAF gene. With the correct version of the NCBI GenBank nucleotide sequence NT 007914 available, this BRAF mutation is now designated T1799A (Kumar et al. 2003), the term used in this review. The T1799A mutation results in a V600E (formerly designated V599E) amino acid substitution in the protein product and subsequent constitutive activation of the BRAF kinase. The V600E mutation is thought to mimic phosphorylation in the activation segment of BRAF by inserting a negatively charged residue adjacent to an activating phosphorylation site at Ser-599 (Davies et al. 2002). This is believed to cause the conversion of BRAF to a catalytically active form by disrupting the association of the activation segment with the ATP-binding P loop, which normally holds BRAF in an inactive confirmation (Dhillon & Kolch 2004, Hubbard 2004, Wan et al. 2004). The oncogenic and transforming function of the mutated V600E BRAF has been well demonstrated (Davies et al. 2002).

Since its initial discovery, BRAF mutations have now been reported in numerous types of human cancer with various frequencies (Garnett & Marais 2004), being most prevalent in melanomas and nevi, present in 66 and 82% of these dermatologic lesion types, respectively (Davies et al. 2002, Pollock et al. 2003).

Over the last 2 years, substantial work has also described BRAF mutations in thyroid cancer, with a prevalence second only to that in melanoma. Discovery of this genetic alteration has created the opportunity to develop novel clinical strategies for the management of thyroid cancer. This review summarizes recent achievements in this exciting research area and highlights the clinical implications of this mutation in thyroid cancer.

**High prevalence, specificity and oncogenic role of the T1799A BRAF mutation in PTC**

Numerous studies have consistently shown a high prevalence of BRAF mutation in thyroid cancer, ranging from 29 to 83% (Namba et al. 2003, Kim et al. 2004; more references are listed in Table 1). The BRAF mutation found in thyroid cancer is almost exclusively the T1799A transversion mutation in exon 15. This mutation is a somatic mutation in sporadic thyroid cancers (Kimura et al. 2003, Xu et al. 2003) and was found not to be a germ-line mutation in a large series of familial PTCs (M. Xing, unpublished results). The only other BRAF mutation reported in thyroid tumors was the K601E mutation found in two benign thyroid adenomas (Soares et al. 2003, Lima et al. 2004) and three follicular-variant PTCs (Trovisco et al. 2004). The mutations in exon 11 of the BRAF gene found in other human cancers were not found in thyroid cancer (Cohen et al. 2003, Fukushima et al. 2003, Kimura et al. 2003, Namba et al. 2003, Frattini et al. 2004, Perren et al. 2004, Puxeddu et al. 2004). A rare but interesting genetic alteration that can also cause constitutive activation of BRAF is the recently reported in vivo fusion of the BRAF gene with AKAP9 gene through a paracentric inversion of the long arm of chromosome 7. This results in a recombinant AKAP9-BRAF oncogene, which appears to occur in PTCs induced by radiation exposure and results in the loss of the autoinhibitory regulatory domains of BRAF and hence constitutive activation of the kinase (Ciampi et al. 2005, Fusco et al. 2005).

The present review is focused on the T1799A BRAF mutation, and the term BRAF mutation hereafter specifically refers to the T1799A BRAF mutation. As
shown in Table 1, in all the studies published to date **BRAF** mutation has been found only in PTCs and some apparently PTC-derived ATCs, but not in FTCs, MTCs, or benign thyroid neoplasms (adenoma or hyperplasia). The **BRAF** mutation-positive ATCs were likely derived from **BRAF** mutation-positive PTCs as suggested by the co-existence of PTC and ATC components in the same tumor, which both harbored the **BRAF** mutation (Nikiforova et al. 2003, Begum et al. 2004, Cohen et al. 2004). As summarized in Table 1, the pooled data on sporadic adult thyroid cancer patients from the 29 studies revealed an overall prevalence of **BRAF** mutation of 44% (810/1856) in PTC and 24% (23/94) in ATC. None of the 165 FTCs, 65 MTCs, or 542 benign neoplasms harbored the **BRAF** mutation. This association of PTCs with the **BRAF** mutation, demonstrated consistently in various studies with patients from different geographical and ethnic backgrounds, strongly supports a unique role of **BRAF** mutation in the pathogenesis of PTC. **BRAF** mutation is the most prevalent among the known common oncogenic genetic alterations in thyroid cancer, including the **ras** mutations, **RET/PTC** rearrangements, and **PAX8-PPARγ** rearrangements. The high frequency and specificity of **BRAF** mutation suggest that this mutation may play a fundamental role in the initiation of PTC tumorigenesis. This idea was supported by the presence of **BRAF** mutation in micro PTC (Nikiforova et al. 2003, Sedliarou et al. 2004, Trovisco et al. 2004). The presence of **BRAF** mutation in both the differentiated PTC components and the undifferentiated components in ATC tumors suggest a role for **BRAF** mutation in disease progression (from well-differentiated PTC to undifferentiated ATC; Nikiforova et al. 2003, Begum et al. 2004, Cohen et al. 2004). Consistent with this concept, a study by Sedliarou et al. (2004) showed that when well-differentiated tumors contained less-differentiated

<table>
<thead>
<tr>
<th>Report</th>
<th>PTC</th>
<th>FTC</th>
<th>ATC</th>
<th>MTC</th>
<th>Benign neoplasm</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28/78 (36)</td>
<td>0/10 (0)</td>
<td>–</td>
<td>–</td>
<td>0/26 (0)</td>
<td>Kimura et al. 2003</td>
</tr>
<tr>
<td>2</td>
<td>24/35 (69)</td>
<td>0/16 (0)</td>
<td>–</td>
<td>0/3 (0)</td>
<td>0/20 (0)</td>
<td>Cohen et al. 2003</td>
</tr>
<tr>
<td>3</td>
<td>21/56 (38)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/24 (0)</td>
<td>Xu et al. 2003</td>
</tr>
<tr>
<td>4</td>
<td>23/50 (46)</td>
<td>0/18 (0)</td>
<td>–</td>
<td>–</td>
<td>0/72 (0)</td>
<td>Soares et al. 2003</td>
</tr>
<tr>
<td>5</td>
<td>40/76 (53)</td>
<td>0/8 (0)</td>
<td>0/7 (0)</td>
<td>0/9 (0)</td>
<td>–</td>
<td>Fukushima et al. 2003</td>
</tr>
<tr>
<td>6</td>
<td>49/170 (29)</td>
<td>0/11 (0)</td>
<td>2/6 (33)</td>
<td>–</td>
<td>0/20 (0)</td>
<td>Namba et al. 2003</td>
</tr>
<tr>
<td>7</td>
<td>45/119 (38)</td>
<td>0/32 (0)</td>
<td>3/29 (10)</td>
<td>0/13 (0)</td>
<td>0/111 (0)</td>
<td>Nikiforova et al. 2003</td>
</tr>
<tr>
<td>8</td>
<td>18/30 (60)</td>
<td>0/12 (0)</td>
<td>–</td>
<td>–</td>
<td>0/9 (0)</td>
<td>Xing et al. 2004a</td>
</tr>
<tr>
<td>9</td>
<td>14/28 (50)</td>
<td>0/14 (0)</td>
<td>2/10 (20)</td>
<td>0/14 (0)</td>
<td>0/54 (0)</td>
<td>Xing et al. 2004b</td>
</tr>
<tr>
<td>10</td>
<td>8/16 (0)</td>
<td>0/6</td>
<td>–</td>
<td>–</td>
<td>0/21 (0)</td>
<td>Xing et al. 2004c</td>
</tr>
<tr>
<td>11</td>
<td>45/124 (36)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Trovisco et al. 2004</td>
</tr>
<tr>
<td>12</td>
<td>–</td>
<td>–</td>
<td>8/16 (50)</td>
<td>–</td>
<td>–</td>
<td>Begum et al. 2004</td>
</tr>
<tr>
<td>13</td>
<td>58/70 (83)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Kim et al. 2004</td>
</tr>
<tr>
<td>14</td>
<td>30/82 (37)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Nikiforova et al. 2004</td>
</tr>
<tr>
<td>15</td>
<td>36/95 (38)</td>
<td>0/2 (0)</td>
<td>2/2 (100)</td>
<td>0/1 (0)</td>
<td>0/32 (0)</td>
<td>Cohen et al. 2004</td>
</tr>
<tr>
<td>16</td>
<td>19/60 (32)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Frattini et al. 2004</td>
</tr>
<tr>
<td>17</td>
<td>18/56 (32)</td>
<td>0/5 (0)</td>
<td>0/4 (0)</td>
<td>–</td>
<td>0/1 (0)</td>
<td>Fugazzola et al. 2004</td>
</tr>
<tr>
<td>18</td>
<td>24/60 (40)</td>
<td>0/5 (0)</td>
<td>0/1 (0)</td>
<td>–</td>
<td>0/6 (0)</td>
<td>Puxeddu et al. 2004</td>
</tr>
<tr>
<td>19</td>
<td>–</td>
<td>–</td>
<td>6/17 (35)</td>
<td>–</td>
<td>–</td>
<td>Soares et al. 2004</td>
</tr>
<tr>
<td>20</td>
<td>97/232 (42)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Penko et al. 2004</td>
</tr>
<tr>
<td>21</td>
<td>26/69 (38)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/27 (0)</td>
<td>Salvatore et al. 2004</td>
</tr>
<tr>
<td>22</td>
<td>13/46 (28)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Sedliarou et al. 2004</td>
</tr>
<tr>
<td>23</td>
<td>55/91 (60)</td>
<td>0/3 (0)</td>
<td>–</td>
<td>–</td>
<td>0/24 (0)</td>
<td>Vasil'ev et al. 2004</td>
</tr>
<tr>
<td>24</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/40 (0)</td>
<td>Krohn et al. 2004</td>
</tr>
<tr>
<td>25</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0/10 (0)</td>
<td>Kimura et al. 2004</td>
</tr>
<tr>
<td>26</td>
<td>7/15 (47)</td>
<td>0/7 (0)</td>
<td>–</td>
<td>0/24 (0)</td>
<td>–</td>
<td>Perren et al. 2004</td>
</tr>
<tr>
<td>27</td>
<td>37/72 (51)</td>
<td>0/8 (0)</td>
<td>0/2 (0)</td>
<td>0/1 (0)</td>
<td>0/45 (0)</td>
<td>Hayashida et al. 2004</td>
</tr>
<tr>
<td>28</td>
<td>38/61 (62)</td>
<td>0/8 (0)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Porra et al. 2005</td>
</tr>
<tr>
<td>29</td>
<td>37/65 (57)</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>M Xing et al. unpublished results</td>
</tr>
<tr>
<td>Overall</td>
<td>810/1856 (44)</td>
<td>0/165 (0)</td>
<td>23/94 (24)</td>
<td>0/65 (0)</td>
<td>0/542 (0)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1 Frequency of the T1799A transversion **BRAF** mutation in sporadic adult thyroid tumors
components, the prevalence of \textit{BRAF} mutation was increased significantly. The \textit{BRAF} mutation is not the only driving force for the formation of ATC, as many ATC tumors do not harbor this mutation; this latter only driving force for the formation of ATC, as many increased significantly. The \textit{BRAF} components, the prevalence of \textit{T1799A} mutation in the common subtypes of PTC

<table>
<thead>
<tr>
<th>Report</th>
<th>Conventional PTC</th>
<th>Follicular-variant PTC</th>
<th>Tall-cell PTC</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28/53 (53)</td>
<td>2/30 (7)</td>
<td>6/6 (100)</td>
<td>Nikiforova et al. 2003</td>
</tr>
<tr>
<td>2</td>
<td>28/42 (67)</td>
<td>6/51 (12)</td>
<td>–</td>
<td>Cohen et al. 2004</td>
</tr>
<tr>
<td>3</td>
<td>58/70 (83)</td>
<td>–</td>
<td>–</td>
<td>Kim et al. 2004</td>
</tr>
<tr>
<td>4</td>
<td>28/53 (53)</td>
<td>0/32 (0)</td>
<td>1/3 (33)</td>
<td>Trovisco et al. 2004</td>
</tr>
<tr>
<td>5</td>
<td>–</td>
<td>–</td>
<td>11/14 (79)</td>
<td>Frattini et al. 2004</td>
</tr>
<tr>
<td>6</td>
<td>18/47 (38)</td>
<td>0/6 (0)</td>
<td>–</td>
<td>Fugazzola et al. 2004</td>
</tr>
<tr>
<td>7</td>
<td>19/35 (54)</td>
<td>–</td>
<td>–</td>
<td>Puxeddu et al. 2004</td>
</tr>
<tr>
<td>8</td>
<td>16/35 (45)</td>
<td>3/22 (14)</td>
<td>5/9 (55)</td>
<td>Salvatore et al. 2004</td>
</tr>
<tr>
<td>9</td>
<td>36/52 (69)</td>
<td>2/9 (22)</td>
<td>–</td>
<td>Porrà et al. 2005</td>
</tr>
<tr>
<td>10</td>
<td>15/24 (63)</td>
<td>8/25 (32)</td>
<td>14/16 (88)</td>
<td>M. Xing et al. unpublished results</td>
</tr>
<tr>
<td>Overall</td>
<td>246/411 (60)</td>
<td>21/175 (12)</td>
<td>37/48 (77)</td>
<td></td>
</tr>
</tbody>
</table>

Different subtype compositions of PTC, when analyzed without subtype stratification in various reports, may partially explain the wide variation in the prevalence of \textit{BRAF} mutation reported by different authors. It should be pointed out that different observers may sometimes define the histological types of thyroid cancer differently (Franc 2003, Lloyd et al. 2004), which may affect the accuracy in reporting the tumor-subtype pattern of \textit{BRAF} mutation. However, the distribution pattern of \textit{BRAF} mutation among the three most common subtypes of PTC – conventional PTC, follicular-variant PTC, and tall-cell PTC – most likely represents a true phenomenon as these histological types of PTC can usually be defined with relative ease and the \textit{BRAF} mutation pattern described here has been consistently revealed in all the studies that reported PTC subtypes in the analysis of \textit{BRAF} mutation (Table 2). Therefore, \textit{BRAF} mutation appears to play a major role in the tumorigenesis of tall-cell PTC and conventional PTC. This may explain some of the common features seen in these two subtypes of PTC, such as their high tendency to undergo lymph node metastasis. As tall-cell PTC and conventional PTC are more aggressive than follicular-variant PTC, and as tall-cell PTC is known to be particularly aggressive (Merino & Monteagudo 1997, Akslen & LiVolsi 2000, Prendiville et al. 2000), the order of tall-cell variant $>$ conventional variant $>$ follicular-variant PTC in the prevalence of \textit{BRAF} mutation is consistent with the idea that \textit{BRAF} mutation is a driving force behind thyroid cancer’s
aggressivity. This will become more evident in the discussion regarding the prognostic value of \textit{BRAF} mutation.

\textbf{Mutual exclusivity between \textit{BRAF} mutation and other common genetic alterations in thyroid cancer}

Mutual exclusivity between \textit{BRAF} mutation and \textit{ras} mutation was seen in several types of human cancer, including, for example, colorectal cancer (Rajagopalan \textit{et al}. 2002), melanoma (Omholt \textit{et al}. 2003), and ovarian cancer (Singer \textit{et al}. 2003). Mutual exclusivity between these two mutations was also seen in thyroid cancer (Fukushima \textit{et al}. 2003, Kimura \textit{et al}. 2003, Soares \textit{et al}. 2003, Frattini \textit{et al}. 2004). These and other studies (Kumagai \textit{et al}. 2004, Lima \textit{et al}. 2004, Nikiforova \textit{et al}. 2004, Vasil'ev \textit{et al}. 2004) similarly showed mutual exclusivity between \textit{BRAF} mutation and \textit{RET}/\textit{PTC} rearrangements in thyroid cancer. In fact, no study showed more than one type of these three common genetic alterations in the same case of thyroid cancer, except one study showing the overlap of \textit{BRAF} mutation with \textit{RET}/\textit{PTC} (Xu \textit{et al}. 2003). In this study, however, immunohistochemical staining was used to define the presence of \textit{RET}/\textit{PTC} using C-terminal-specific antibodies. The results may therefore be non-specific as the antibodies used may not reliably discriminate between the rearranged and the wild-type RET proteins. Expression of the wild-type RET or \textit{RET} proto-oncogene was previously demonstrated in PTC, particularly in PTC that lack the major \textit{RET}/\textit{PTC} rearrangements (Bunone \textit{et al}. 2000). In general, the data on \textit{BRAF} mutation, \textit{ras} mutation, and \textit{RET}/\textit{PTC} rearrangements in thyroid cancer support the idea that each of the three genetic alterations alone is sufficient to cause thyroid tumorigenesis. The mutual exclusivity among these common genetic alterations in thyroid tumor may not be surprising, though, as the signaling pathways of these activating genetic alterations share the common MAP kinase pathway, albeit at different steps. A single oncogenic alteration along this pathway is likely sufficient to drive thyroid cell transformation and tumorigenesis. The genetic data supporting \textit{BRAF} mutation as an independent oncogenic event for PTC tumorigenesis is consistent with the results from the transgenic mouse studies mentioned above (Knauf \textit{et al}. 2004).

Like various genetic alterations, loss of expression of the pro-apoptotic tumor suppressor Ras-associated factor 1 (RASSF1) through an epigenetic alteration, gene methylation, is another important mechanism in the tumorigenesis of many human cancers (Pfeifer \textit{et al}. 2002). The three splice variants (A, B, C) of RASSF1 all possess a Ras-association domain (Dammann \textit{et al}. 2000). Ras has been shown to be able to use RASSF1 as a direct effector in the downstream signaling (Vos \textit{et al}. 2000). Therefore, RASSF1 may function through a Ras-like signaling pathway. Promoter methylation of \textit{RASSF1A} was frequently found in thyroid tumors (Schagdarsurengin \textit{et al}. 2002, Xing \textit{et al}. 2004) and this methylation silenced the expression of \textit{RASSF1A} gene in thyroid tumor cells (Schagdarsurengin \textit{et al}. 2002). Therefore, aberrant methylation of \textit{RASSF1A} may represent another important oncogenic mechanism in thyroid tumorigenesis. Intriguingly, aberrant methylation of \textit{RASSF1A} was recently found to be mutually exclusive with \textit{BRAF} mutation in PTC (Xing \textit{et al}. 2004). High-level \textit{RASSF1A} methylation occurred mostly in FTC (Xing \textit{et al}. 2004), similar to \textit{ras} mutations that also occur frequently in FTC (Vasko \textit{et al}. 2003). Among different PTC subtypes, \textit{ras} mutations were highly prevalent in follicular-variant PTC, while \textit{RET}/\textit{PTC} rearrangements, like \textit{BRAF} mutation, were more prevalent in conventional PTC (Zhu \textit{et al}. 2003) and tall cell-variant PTC (Basolo \textit{et al}. 2002). Therefore, it appears that PTC subtype-predislections may partially account for the mutual exclusivity of these genetic and epigenetic alterations recently reported in thyroid cancer. In most of these studies, analysis of all PTC for genetic alterations was conducted without stratification of histological subtypes. To be certain about the mutual exclusivity of these common genetic alterations and their respective roles in thyroid tumorigenesis in each specific subtype of PTC, it would be necessary to examine all of these genetic and epigenetic alterations simultaneously in each of the specific subtypes of PTC.

\textit{BRAF} mutation and \textit{RET}/\textit{PTC} rearrangements may act at steps that are different but close in their shared oncogenic pathway, resulting in conventional PTC, whereas \textit{ras} mutations and \textit{RASSF1A} methylation may act at different but related steps along their shared oncogenic pathway resulting in FTC and follicular-variant PTC. Although thyroid tumorigenesis caused by these genetic and epigenetic alterations may all involve the MAP kinase pathway, each of these genetic and epigenetic alterations, particularly those that act in this pathway at a step proximal to Raf kinase, may involve additional signaling pathways. For example, the phosphoinositide 3-kinase/Akt pathway, which is known to also play an important role in thyroid tumorigenesis, can be activated by Ras (Gire \textit{et al}. 2000, Cheng & Meinkoth 2001) or \textit{RET}/\textit{PTC} (Kim \textit{et al}. 2003, Miyagi \textit{et al}. 2004). This may partially
explain the distinct characteristics of different subtypes of thyroid cancer that harbor different genetic and epigenetic alterations.

**Reciprocal age-association of BRAF mutation and RET/PTC rearrangements**

It is well known that RET/PTC is particularly common in the pediatric PTC that occurred in the victims of the Chernobyl nuclear accident (Ito et al. 1994, Fugazzola et al. 1995, Klugbauer et al. 1995, Nikiforov et al. 1997). A similarly high prevalence of RET/PTC has also been found in non-radiation-exposed sporadic pediatric PTC (Nikiforov et al. 1997, Fenton et al. 2000, Penko et al. 2004). As BRAF mutation and RET/PTC are together responsible for the majority of conventional PTC, the most common subtype of PTC, and are mutually exclusive in adult sporadic PTC, their relationship in pediatric PTCs, particularly in those that occurred as a result of the Chernobyl nuclear accident, has drawn much interest (Kumagai et al. 2004, Lima et al. 2004, Nikiforova et al. 2004, Xing et al. 2004b). As summarized in Table 3, and consistent with previous reports (Ito et al. 1994, Fugazzola et al. 1995, Klugbauer et al. 1995, Nikiforov et al. 1997), these recent studies uniformly showed a high prevalence of RET/PTC in both radiation-exposed and sporadic pediatric populations. As may be expected from the mutual exclusivity of RET/PTC and BRAF mutation observed in sporadic adult PTC and from the known high frequency of RET/PTC in radiation-exposed PTC, the initial study on a small series of PTC from Chernobyl victims showed a low prevalence of BRAF mutation (Xing et al. 2004b). In several subsequent larger studies on Chernobyl victims, the prevalence of BRAF mutation in PTC was found consistently to be low in this special population, ranging from 0 to 12% (Kumagai et al. 2004, Lima et al. 2004, Nikiforova et al. 2004). As in sporadic adult PTC patients, mutual exclusivity of BRAF mutation and RET/PTC was also demonstrated consistently in this Chernobyl population. It would be interesting to know, in a large series, how frequent the recently discovered radiation-sensitive recombinant AKAP9-BRAF oncogene (Ciampi et al. 2005, Fusco et al. 2005) would truly be and whether it, like the BRAF mutation, is mutually exclusive with RET/PTC in Chernobyl- or radiation-related PTCs.

Interestingly, the study by Lima et al. (2004) on Chernobyl victims showed that the average age of the children at the time of radiation exposure was much higher for the group with BRAF mutation than the group with RET/PTC. In the study by Kumagai et al. (2004), when the Chernobyl radiation-exposed children were divided into two age groups, none (0%) of the 15 cases in the group at or younger than 15 years harbored the BRAF mutation, whereas eight (24%) of the 33 cases in the group older than 15 years harbored this mutation. Several of these studies (Kumagai et al. 2004, Lima et al. 2004, Penko et al. 2004) also showed the mutual exclusivity between RET/PTC and BRAF mutation and a low prevalence of the latter (ranging from 0 to 6%) in non-radiation-exposed sporadic PTC in the pediatric population. From these recent studies, the overall prevalence of BRAF mutation for radiation-exposed and non-exposed pediatric PTC is 6 and 4%, respectively, and the overall prevalence of RET/PTC rearrangements for radiation-exposed and sporadic pediatric PTC is 53 and 52%, respectively (Table 3). The adult PTC patients included in some of these studies (Nikiforova et al. 2004, Xing et al. 2004b) showed uniformly a low prevalence of RET/PTC and a high prevalence of BRAF mutation regardless of their history of radiation exposure. Although the prevalence of RET/PTC rearrangements is generally found to be low in adults.

Table 3 Prevalence of BRAF mutation and RET/PTC rearrangements in radiation-exposed and non-exposed children

<table>
<thead>
<tr>
<th>Report</th>
<th>Radiation-exposed</th>
<th>Non-exposed</th>
<th>Radiation-exposed</th>
<th>Non-exposed</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>15/33 (45)</td>
<td>32/55 (58)</td>
<td>14/34 (41)</td>
<td>3/6 (50)</td>
<td>Xing et al. 2004ab</td>
</tr>
<tr>
<td>3</td>
<td>4/34 (12)</td>
<td>1/17 (6)</td>
<td>3/6 (50)</td>
<td>3/6 (50)</td>
<td>Xing et al. 2004ab</td>
</tr>
<tr>
<td>4</td>
<td>0/15 (0)</td>
<td>1/31 (3)</td>
<td>17/48 (35)</td>
<td>3/6 (50)</td>
<td>Xing et al. 2004ab</td>
</tr>
<tr>
<td>5</td>
<td>1/5 (20)</td>
<td>0/7 (0)</td>
<td>1/5 (20)</td>
<td>0/7 (0)</td>
<td>Xing et al. 2004ab</td>
</tr>
<tr>
<td>Overall</td>
<td>7/109 (6)</td>
<td>2/55 (4)</td>
<td>92/175 (53)</td>
<td>29/56 (52)</td>
<td>Xing et al. 2004ab</td>
</tr>
</tbody>
</table>
and high in children, and children are more susceptible to the effects of radiation, conflicting data do exist. For instance, a study by Elisei et al. (2001) on different groups of thyroid tumor patients with various ethnic and demographic backgrounds showed no association of the occurrence of RET/PTC with age at the time of radiation exposure, albeit with relatively low numbers of study subjects in the cancer groups. This study also showed no difference in the occurrence of RET/PTC in radiation-exposed and non-exposed adult patients.

Therefore, studies in general demonstrate a reciprocal age-association of BRAF mutation and RET/PTC in PTC. Beyond inciting factors, such as radiation, age is apparently an important factor in determining the dominance of the two genetic alterations in PTC. BRAF mutation tends to occur in adults and is a major somatic genetic alteration that drives the formation of PTC in this population, whereas RET/PTC tends to occur in children and is a major somatic genetic alteration that drives the formation of PTC in this population. It appears that young age itself, in addition to radiation, is an important predisposing factor for the development of RET/PTC and subsequent PTC. The concept that RET/PTC is an initiator of the formation of PTC in nuclear-accident victims is somewhat challenged by a recent study of Unger et al. (2004) on Chernobyl-associated PTC. In this study, using an interphase in situ hybridization technique, the authors found RET/PTC rearrangements in some cells of PTC tumors but not in other cells of the same tumor. This raises the possibility that these PTCs might have arisen from different clones or that RET/PTC is a late subclonal event, and thereby challenges the general belief that RET/PTC plays an initiating role in the development of radiation-associated PTC. However, the possibility of inaccurate scoring of, and therefore missing, tumor cells harboring RET/PTC rearrangement due to a technical limitation in this study has been raised (Fagin 2004). Ionizing radiation could induce the formation of RET/PTC in both transplanted human thyroid tissues in mice (Mizuno et al. 1997) and in cultured thyroid tumor cells (Ito et al. 1993). A high prevalence of RET/PTC was also observed in PTC that developed in patients who had external radiation treatment during childhood (Bounacer et al. 1997). The transgenic mouse model demonstrated clearly the ability of RET/PTC1, 2 and 3 to initiate the development of PTC (Jhiang et al. 1996, 1998, Santoro et al. 1996, Powell et al. 1998). Therefore, radiation must have played an important role in the development of RET/PTC and PTC in Chernobyl nuclear accident victims. However, it has long been known that childhood radiation exposure is associated with a higher incidence of thyroid cancer (Duffy & Fitzgerald 1950, Wood et al. 1969, Shore et al. 1985). Radiiodine exposure in fallouts from a thermonuclear test (Conard et al. 1970) and the Chernobyl accident (Kazakov et al. 1992) was followed by a significantly increased incidence of thyroid cancer and, as studied and revealed in the latter case, RET/PTC primarily in child victims. The finding that young age is a risk factor for the development of RET/PTC-positive PTC even in non-radiation-exposed children additionally supports the possibility that young age itself predisposes to RET/PTC development through an unidentified mechanism. It is possible that young age may predispose RET/PTC-harboring PTC to more rapid growth and progression so PTC harboring this genetic alteration may tend to be caught clinically early in life. It would be consistent with this idea to confirm, in a large series of tumors, that the tumor size of RET/PTC-positive PTC in the pediatric population is larger than that of RET/PTC-positive PTC in the adult population.

In contrast to the association of young age with RET/PTC, the studies on BRAF mutation in adult and pediatric populations summarized above clearly show that old age is a predisposing factor for the development of BRAF mutation and PTC harboring this mutation. The prevalence of BRAF mutation in PTC was similarly high in radiation-exposed and non-exposed adult patients (Xing et al. 2004b). In an adult population, Nikiforova et al. (2003) further showed a significant association of BRAF mutation with older age. The study on adult patients by Xu et al. (2003) also showed a clear tendency of association of BRAF mutation with older age, although no statistical significance was reached. Other studies on adult patients did not reveal a specific age predilection of BRAF mutation. In most of these studies, however, the number of study subjects was small or the age range of the study subjects was not sufficiently wide and evenly distributed to reveal a clear association between age and the BRAF mutation. The fundamental basis for this link between older age and the development of BRAF mutation remains unclear. It also remains uncertain whether BRAF mutation-harboring PTC is more slowly growing than RET/PTC-harboring PTC so that the former tends to be caught clinically later in life. If proven to be the case, this could at least partially explain the reciprocal age distribution of BRAF mutation and RET/PTC rearrangements, at least in the non-radiation-exposed population. Regardless of the underlying mechanism, there appears to be an age window below which RET/PTC tends to occur or to be identified and above which BRAF mutation tends to
occurs or to be identified. The data currently available suggest that in most patients, this age window is likely to occur around the late teenage years, but the definition of the precise age range will need a large series of patients with a wide and evenly distributed age range. Knowing this age window may help predict the type of genetic alteration that a patient’s thyroid cancer may harbor.

The diagnostic value of \textit{BRAF} mutation in thyroid cancer

Thyroid nodules are common, and are palpable in approximately 5% of normal adults (Vander et al. 1968) and visualized by sonography in one-third or more of normal adults (Brander et al. 1991, Bruneton et al. 1994). As about 5–8% of palpable thyroid nodules are cancerous, a major task of the initial evaluation of thyroid nodules is to rule out malignancy (Werk et al. 1984, Belfiore et al. 1989). Thyroid fine-needle aspiration biopsy (FNAB) with cytological analysis is a widely used initial diagnostic measure in thyroid nodule evaluation (Hegedus 2004). However, at least 20% of biopsies yield indeterminate cytological findings that cannot distinguish between thyroid cancer and benign tumors with certainty, leaving uncertain the optimal management for these patients (Gharib et al. 1984, Sclabas et al. 2003). As the T1799A \textit{BRAF} mutation occurs exclusively in PTC with a high prevalence, but not in benign thyroid neoplasms (Table 1), it is a specific diagnostic marker for thyroid cancer. Several studies have been conducted to evaluate the diagnostic applicability of \textit{BRAF} mutation detection on FNAB specimens (Baloch et al. 2004, Cohen et al. 2004, Hayashida et al. 2004, Salvatore et al. 2004, Xing et al. 2004c). Most of these studies were retrospective, in which \textit{BRAF} mutation was analyzed on FNAB specimens retrieved from existing cytological slides and in which the \textit{BRAF} mutation status was correlated with the pre-established histopathological diagnoses of the tumors. The study by Xing \textit{et al.} (2004c) was a prospective one, in which FNAB was performed, \textit{BRAF} mutation analyzed preoperatively, and the results then correlated prospectively with the postoperative histological diagnosis of the biopsied thyroid nodule. Regardless of the detection methods used, all these studies demonstrated excellent accuracy and simplicity of \textit{BRAF} mutation detection on FNAB specimens. For \textit{BRAF} mutation-positive PTC, the diagnostic specificity and sensitivity of \textit{BRAF} mutation detection on FNAB specimens were 100% in these studies. Consistent with the studies on primary tumors, in FNAB specimens, \textit{BRAF} mutation was found only in histologically-proven PTC, but not in FTC and benign thyroid tumors (Table 4). The overall prevalence of \textit{BRAF} mutation in PTC in these FNAB studies was 44%, similar to the generally reported prevalence of this mutation (Table 1). It is therefore expected that, as demonstrated by these FNAB studies (Table 4), nearly half of patients with PTC can be diagnosed solely based on \textit{BRAF} mutation analysis on FNAB specimens. If the diagnostic reliability of this \textit{BRAF} mutation approach is confirmed in more studies, PTC diagnosed solely based on \textit{BRAF} mutation detection will probably not need further diagnostic cytology studies. \textit{BRAF} mutation detection is robust and low in cost (Xing \textit{et al.} 2004c), particularly if it can be done in a centrally coordinated laboratory with appropriate methods. In view of the high prevalence of both \textit{BRAF} mutation and PTC, the elimination of the need for cytology examination in nearly half of the patients with PTC undergoing FNAB evaluation could be substantially cost-saving. Moreover, \textit{BRAF} mutation detection may allow for more specific diagnosis of PTC as inter-observer variations in interpreting the cytology patterns of

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|c|c|}
\hline
\textbf{Report} & \textbf{Histological diagnosis of the nodule} & \textbf{Cytologically indeterminate} & \textbf{Reference} \\
& \textbf{PTC} & \textbf{FTC} & \textbf{Benign} & \textbf{Cancer} & \textbf{Benign} & \textbf{Total} \\
\hline
1 & 22/54 (41) & 0/2 (0) & 0/32 (0) & 5/32 (16) & 0/23 (0) & 5/55 (9) & Cohen \textit{et al.} 2004 \\
2 & 26/69 (38) & – & 0/27 (0) & 4/15 (27) & 0/19 & 4/34 (12) & Salvatore \textit{et al.} 2004 \\
3 & 8/16 (50) & 0/6 (0) & 0/21 (0) & 2/14 (14) & 0/12 (0) & 2/26 (8) & Xing \textit{et al.} 2004c \\
5 & 30/58 (51) & – & – & 1/8 (13) & – & 1/8 (13) & Hayashida \textit{et al.} 2004 \\
\hline
Overall & 86/197 (44) & 0/8 (0) & 0/80 & 12/69 (17) & 0/54 (0) & 14/168 (8) & \textbf{This report is an abstract without complete information at this time, and their data cannot be included fully for discussion in this review.} \\
\hline
\end{tabular}
\caption{\textit{BRAF} mutation in thyroid fine-Needle aspiration biopsy (FNAB) specimens}
\end{table}
FNAB specimens do exist (Greaves et al. 2000, Al-Shaikh et al. 2001). In fact, this point is well illustrated by the study of Baloch et al. (2004), in which 13% (seven of 53) of FNAB specimens cytologically read as benign and 7% (one of 14) of FNAB specimens read as thyroiditis were positive for BRAF mutation and the diagnoses were able to be corrected to PTC by mutation analysis.

Nevertheless, BRAF mutation detection alone on FNAB specimens is unlikely to solve the diagnostic dilemma of indeterminate cytology on FNAB. As summarized in Table 4, 17% of thyroid cancers with indeterminate cytology can be diagnosed by BRAF mutation analysis. When all the cases with indeterminate cytology were evaluated as a whole, only a small portion (8%) of the patients could be diagnosed with BRAF mutation detection. This is because the majority of thyroid tumors with indeterminate cytology are benign thyroid neoplasms harboring no BRAF mutation and only about 15% of thyroid tumors with indeterminate cytology prove to be PTC (Selabas et al. 2003). Given the overall prevalence of BRAF mutation of around 45% in PTC (Table 1), 15% as PTC of the cytologically indeterminate cases can be translated into about 7% that will be positive for BRAF mutation, consistent with the BRAF mutation rate found on indeterminate cytological specimens in the several recent reports (Table 4). Moreover, many of the thyroid cancers with indeterminate cytology, particularly those with follicular neoplasm patterns, are FTC and follicular-variant PTC, with the former harboring no BRAF mutation and the latter carrying the mutation at a very low prevalence (Tables 1 and 2). Obviously, a positive BRAF mutation has a perfect positive predictive value and can establish the diagnosis of PTC, but a negative result in a specific patient will not be of any diagnostic value. It remains to be demonstrated definitively how effective BRAF mutation analysis on thyroid FNAB can truly be in addressing the diagnostic dilemma of indeterminate cytology. Nearly 300,000 new thyroid nodules are detected annually in the United States (Castro & Gharib 2000). If all of these thyroid nodules are to be evaluated with FNAB, approximately 90,000 (assuming a 30% rate of indeterminate cytology) of them may yield indeterminate cytological findings. With a diagnostic sensitivity of 8% (Table 4) for BRAF mutation detection on cytologically indeterminate FNAB, about 7200 patients per year in the United States could be helped with a definitive diagnosis of PTC by this technique and the optimal management of these patients could be pursued. Practically, it may be worth testing BRAF mutation on readily retrievable FNAB specimens from cytology slides when conservative follow-up of a cytologically indeterminate thyroid nodule is clinically debatable in a patient. The combination of BRAF mutation with additional sensitive and specific molecular markers will likely be the next step in increasing the FNAB diagnostic sensitivity. This approach was tested recently by combined use of BRAF mutation with RET/PTC (Salvatore et al. 2004), a process which did indeed improve the diagnostic sensitivity. However, the diagnostic specificity of this approach needs to be further investigated on large studies as RET/PTC is sometimes found in benign thyroid tumors (Nikiforov 2002, Santoro et al. 2002, Tallini 2002). Combined use of BRAF mutation with Ras mutation in conjunction with FNAB to diagnose thyroid cancer is also being investigated (Baloch et al. 2004), but a similar diagnostic specificity limitation also potentially exists as Ras mutations are also frequently seen in benign thyroid neoplasms (Tallini 2002, Vasko et al. 2003).

As cancer cells can dislodge into the bloodstream, efforts have been made to establish sensitive methods to detect BRAF mutation that could potentially be used on serum DNA samples. The technique of single-stranded DNA conformation polymorphism was recently used to detect BRAF mutation in plasma DNA from thyroid cancer patients, but apparently failed to provide sufficient sensitivity (Vdovichenko et al. 2004). Real-time allele-specific amplification for detection of the BRAF mutation was tested, which allowed detection of 1% mutated allele in a DNA sample (Jarry et al. 2004), a sensitivity that is unlikely to be sufficient for detection of mutated BRAF allele in blood samples. Lilleberg et al. (2004) recently reported the use of mutant allele-specific PCR amplification followed by detection with a denaturing HPLC platform that uses post-separation fluorescence technology to detect mutated alleles that represent <0.1% of the total analyzed DNA. With this method, the authors were able to scan for BRAF mutation as well as various ras mutations in plasma DNA from patients with colon cancer with 100% sensitivity. It remains to be tested whether this method can also be applied to thyroid cancer patients. The gap ligase chain-reaction technique was demonstrated to be a more sensitive method and could detect point mutations in the presence of up to 10,000-fold excess of wild-type allele DNA (Abravaya et al. 1995). A modified version of this method specifically for BRAF point mutation was developed recently and, with its high sensitivity, was used to rule out BRAF mutation in primary biliary tract cancers (Goldenberg et al. 2004). It would be interesting to see whether this stable and sensitive
method could reliably detect \textit{BRAF} mutation in the serum DNA of thyroid cancer patients or other \textit{BRAF} mutation-positive cancer patients. Using an even more sensitive sequence-specific real-time PCR technique, Rosenberg et al. (2004) were able to detect one heterozygous \textit{BRAF} mutation-positive cell mixed in 21,692 normal cells. When applying it to blood samples, the authors were able to identify circulating \textit{BRAF} mutation in one of five PTC patients tested. This encouraging method needs to be validated in a larger study. It is hoped that a sensitive and specific method to detect \textit{BRAF} mutation in the blood, which could simplify the diagnostic evaluation of a large number of patients undergoing thyroid nodule evaluation, will be established in the near future; a positive \textit{BRAF} mutation test on the blood may spare the patient from FNAB and other diagnostic procedures and prompt direct surgical treatment.

**Prognostic value of \textit{BRAF} mutation in thyroid cancer**

Because \textit{BRAF} mutation plays an important role in PTC tumorigenesis, it is conceivable that this mutation is a determinant of clinical and pathological behaviors of PTC and could be a novel prognostic factor for this cancer. The relationship between this mutation and the clinicopathological outcomes of PTC have been investigated in several studies (Namba et al. 2003, Nikiforova et al. 2003, Xu et al. 2003, Fugazzola et al. 2004, Kim et al. 2004, Puxeddu et al. 2004; M. Xing et al. unpublished results). In a series of 104 PTCs, comprised mainly of American patients, Nikiforova et al. (2003) reported a significant association of \textit{BRAF} mutation with extrathyroidal invasion [(16/38 (42%) with \textit{BRAF} mutation versus 13/66 (20%) without mutation, \(P=0.03\)] and advanced stages [(for stage III, 10/38 (26%) with \textit{BRAF} mutation versus 2/66 (3%) without mutation, \(P=0.006\); for stage IV, 7/38 (18%) with \textit{BRAF} mutation versus 3/66 (4%) without mutation, \(P=0.03\)] of the primary tumor at the time of initial surgery. In a Japanese series of 126 PTCs, Namba et al. (2003) found a significant association of \textit{BRAF} mutation with advanced stages of the tumor and distant metastasis [(7/38 (18%) with \textit{BRAF} mutation versus 5/88 (6%) without mutation, \(P=0.033\)]. In a recent Korean study, \textit{BRAF} mutation was found to be significantly associated with neck lymph node metastasis [(39/58 (67%) with \textit{BRAF} mutation versus 4/12 (33%) without mutation, \(P=0.048\)] (Kim et al. 2004). In two Italian studies (Fugazzola et al. 2004, Puxeddu et al. 2004) and an American study by Xu et al. (2003), no significant association of \textit{BRAF} mutation with any of the common high-risk pathological characteristics was revealed. The number of the cases examined in these latter three studies was much smaller, however, ranging from 56 to 60. In some of the studies (Nikiforova et al. 2003, Xu et al. 2003, Fugazzola et al. 2004), a trend in association between \textit{BRAF} mutation and lymph node metastasis was observed but did not achieve statistical significance. In the study by Fugazzola et al. (2004), a higher but non-significant recurrence rate of thyroid cancer was found to be associated with \textit{BRAF} mutation. Our recent study on a large series of PTCs demonstrated a significant association of \textit{BRAF} mutation with extrathyroidal invasion, lymph node metastasis, advanced tumor stages, and cancer recurrence, which still existed on multivariate analysis even with adjustment for all the common confounding clinicopathological factors (M Xing et al. unpublished results). In this study, we found a thyroid cancer recurrence rate of 25% in \textit{BRAF} mutation-positive patients versus 9% in \textit{BRAF} mutation-negative patients (\(P=0.004\)). Interestingly, we also observed a significantly higher incidence of the loss of radioiodine avidity in the recurrent thyroid cancer when \textit{BRAF} mutation was positive, suggesting that \textit{BRAF} mutation may not only predict a higher incidence of thyroid cancer recurrence but also predict a poorer response of recurrent thyroid cancers to radioiodine treatment. Only one study (Xu et al. 2003) showed an association of \textit{BRAF} mutation with (male) gender. None of the studies showed association of \textit{BRAF} mutation with larger tumor size, suggesting that \textit{BRAF} mutation increases the aggressiveness of PTC by promoting its invasiveness, metastasis, and recurrence, but not growth in size of the primary tumor.

Most of the studies on the relationship between \textit{BRAF} mutation and the clinicopathological outcomes of PTC were conducted without subtype stratification of PTC. As discussed above, \textit{BRAF} mutation occurs mostly in conventional and tall-cell PTC and uncommonly in follicular-variant PTC. Compared with conventional PTC, follicular-variant PTC is infrequently associated with high-risk pathological characteristics such as lymph node metastasis and extrathyroidal invasion. Therefore, the inconsistent results from different reports on the association of \textit{BRAF} mutation with high-risk pathological characteristics could be partially due to different combinations of various subtypes of PTC that were included in the study. For example, a significant association of \textit{BRAF} mutation with high-risk pathological factors could be shown on a series of PTC that is comprised of certain proportions...
of follicular-variant PTC and conventional PTC, while this association may be lost on analysis within a specific subtype of PTC, particularly when the sample number is small. This illustrates the importance of the use of multivariate analysis with adjustment for various confounding factors, including histological subtypes of PTC, as we did recently to establish an independent prognostic role of BRAF mutation (M Xing et al. unpublished results). As the BRAF mutation is so prevalent in conventional or tall-cell PTC, a large series of such cases may be needed to reveal an association of BRAF mutation with poorer clinicopathological outcomes within these subtypes of PTC. A recent Korean study by Kim et al. (2004) focused specifically on conventional PTC and showed a significant association of BRAF mutation with lymph node metastasis. Overall, the data available to date support the idea that BRAF mutation is an independent prognostic factor that predicts a poorer prognosis of PTC. As mentioned above, the demonstration of BRAF mutant-induced development of PTC and its transition into ATC in transgenic mice (Knauff et al. 2004) is consistent with the clinical findings on the role of BRAF mutation in predicting a poor outcome of PTC.

Whether to treat a PTC patient with radioiodine, and how vigilantly and aggressively to guard against recurrence, are often questions without straightforward clinical answers. Use of BRAF mutation status may help clarify such clinical situations and assist clinical decision making. It is expected that BRAF mutation may also be useful in risk and prognostic evaluation of micro PTC. Although this type of thyroid cancer is generally thought to be indolent and associated with a relatively good prognosis, local and distant metastasis and recurrence do occur, and no specific independent prognostic clinicopathological factors were identified on multivariate analysis for this type of PTC (Chow et al. 2003). As BRAF mutation often occurs in micro PTC as well (Nikiﬁrova et al. 2003, Sedliarou et al. 2004, Trovisco et al. 2004), it would be interesting to investigate BRAF mutation as an independent prognostic factor to help manage these patients more appropriately.

As BRAF mutation can be readily analyzed on FNAB specimens (Baloch et al. 2004, Cohen et al. 2004, Salvatore et al. 2004, Xing et al. 2004c), preoperative BRAF mutation analysis, in conjunction with routine FNAB cytology study, could help surgeons better tailor their surgical procedures by helping them choose, for instance, between vigilant exploration and resection of suspicious regional lymph node and no neck dissection, and between total thyroidectomy and lobectomy. The current standard prognostic evaluation of thyroid cancer is based largely on clinicopathological criteria, which is often incomplete, particularly preoperatively, when the pathological characteristics of the tumor are not known. BRAF mutation represents the first molecular marker that can be used, even preoperatively, for more efficient prognostic evaluation and clinical management of PTC. Therefore, it may be reasonable to examine BRAF mutation on preoperative FNAB specimens for every patient not only for diagnostic purposes, but also for risk evaluation. In this sense, BRAF mutation may be examined on FNAB specimens even if a diagnosis of PTC is already known based on cytological studies. This approach may assist clinicians in optimizing both the short-term (surgical) and long-term (medical) management of their thyroid cancer patients.

**Therapeutic potential of inhibiting the MAP kinase pathway using novel inhibitors in thyroid cancer**

Although thyroid cancer is usually indolent and curable with the current standard treatments of surgery, often followed by adjuvant radioiodine therapy, there remain many patients whose conditions are incurable, disabling, and even fatal. The most difficult cases, for which there is no effective current treatment, are those that are inoperable and have lost radioiodine avidity. This includes ATC, which is often positive for BRAF mutation (Table 1). A novel effective treatment is needed desperately for these patients (Sherman 2003). As activation of the MAP kinase pathway by various genetic alterations, including BRAF mutation, plays a pivotal role in thyroid tumor genesis and progression, efforts targeted at inhibiting this pathway may lead to development of novel effective therapy for thyroid cancer.

A therapeutic approach targeted at the Raf kinases has been tested for human cancers using specific inhibitors with encouraging results in in vitro cell studies and in vivo animal studies (Wilhelm & Chien 2002, Bollag et al. 2003, Dumas et al. 2004). Among these inhibitors, the Bay 43–9006 compound seems to be a promising one as it has excellent safety profile in human subjects and effectiveness in inhibiting Raf kinases (Bollag et al. 2003, Lee & McCubrey 2003). The Bay 43–9006 compound is in several clinical trials at various phases targeted at several types of human cancer (Lee & McCubrey 2003). Although this compound most potently inhibits the C-type Raf kinase, it also has excellent potency in inhibiting
The discovery of the \textit{BRAF} mutation in thyroid cancer represents one of the most important recent advancements in thyroid cancer research and is of significant clinical potential in thyroid cancer medicine. Since the initial report on \textit{BRAF} mutation in thyroid cancer nearly 2 years ago, rapid progress has occurred due to an explosion of research in this area. The T1799A \textit{BRAF} mutation is the most common activating genetic alteration in thyroid cancer. Advancements have also been made in understanding the relationship between wild-type and V600E mutant \textit{BRAF} kinases (Karasarides \textit{et al.} 2004, Wan \textit{et al.} 2004). X-ray crystallography has recently demonstrated the binding of this inhibitor with the kinase domain in both the wild-type and V600E \textit{BRAF} kinases (Garnett & Marais 2004, Wan \textit{et al.} 2004). By binding with the kinase domain of \textit{BRAF}, Bay 43–9006 locks the kinase in an inactive state. Treatment with this compound can block kinase signaling downstream of Raf kinase, inhibit \textit{BRAF}-stimulated DNA synthesis and cell proliferation, induce apoptosis in melanoma cells harboring \textit{BRAF} mutation, and delay the growth of melanoma tumor xenografts in mice (Karasarides \textit{et al.} 2004). A recent preliminary study by Kumar \textit{et al.} (2004) has shown that Bay 43–9006 can inhibit the growth and proliferation, and induce apoptosis, of KAT-5 cells, a PTC-derived cell line harboring the \textit{BRAF} mutation. As \textit{BRAF} is the predominant type of Raf kinase in follicular thyroid cells (Fagin 2004) and as \textit{BRAF} mutation is highly prevalent in PTC (Table 1), strategies targeted at inhibition of \textit{BRAF} may be particularly effective for the treatment of PTC. Several other MAP kinase pathway inhibitors acting at steps other than Raf kinases have also been developed, including MEK inhibitors (Sebolt-Leopold 2004). A good example is the MEK-specific inhibitor CI-1040, which is the first MEK-targeted drug candidate to undergo clinical trials, although monotherapy with this drug in some cancers did not clearly prove to be effective on a multicenter phase II study (Rinehart \textit{et al.} 2004). It remains to be investigated whether these MAP kinase pathway inhibitors may have therapeutic effects in thyroid cancer patients.

Several earlier studies demonstrated that the transformation of thyroid cells with \textit{ras} oncogene induced loss of expression of thyroid-specific proteins such as thyroid-stimulating hormone (TSH) receptor (TSHR) (Berlingieri \textit{et al.} 1990) and thyroglobulin (Avvedimento \textit{et al.} 1991). A recent study by Knauf \textit{et al.} (2003) demonstrated that acute expression of \textit{RET/PTC3}, H-Ras, or constitutively activated MEK-1 could all block TSH-induced expression of thyroglobulin and sodium-iodide symporter (NIS) in PCCL3 thyroid cells. This study also demonstrated that treatment of cells with MEK inhibitors could restore the expression of thyroglobulin and NIS. Interestingly, the transgenic mice in which development of PTC and its transition to ATC were induced by V600E \textit{BRAF} mutation had absent or decreased expression of thyroglobulin and developed hypothyroidism (Knauf \textit{et al.} 2004). Normal expression of these thyroid-specific molecules is essential for the unique function of thyroid cells to take up and metabolize iodide and synthesize thyroid hormones (Nilsson 2001). It therefore appears that silencing of thyroid-specific genes by aberrant activation of the MAP kinase pathway may be the basis for the loss of radioiodine avidity seen clinically in some thyroid cancer patients. Aberrant methylation was shown to be a mechanism for silencing some of the thyroid-specific genes involved in iodide metabolism, including those for NIS (Venkataraman \textit{et al.} 1999), TSHR (Xing \textit{et al.} 2003a), and pendrin (Xing \textit{et al.} 2003b) in thyroid cancer. It is thus plausible to propose that inhibiting the MAK kinase pathway could reverse the aberrant methylation of these genes and restore their expression and the lost iodide-concentrating ability of thyroid cancer cells. In this sense, the MAP kinase pathway inhibitors could be particularly useful as a conjunction therapy with radioiodine treatment of those patients whose thyroid cancers have decreased or lost radioiodine avidity. These hypotheses need to be tested.

The MAP kinase pathway-activating \textit{BRAF} mutation, \textit{ras} mutations, \textit{RET/PTC}, and \textit{RASSF1A} methylation may together account for nearly all follicular epithelial cell-derived thyroid cancers (Xing \textit{et al.} 2004a), and these common genetic alterations may all induce thyroid tumor genesis and progression through the MAP kinase pathway, either entirely or partially. Therefore, the MAP kinase pathway inhibitors may be effective in treating a wide range of thyroid cancers, irrespective of \textit{BRAF} mutation status. With the proven safety profiles of the Raf kinase inhibitor Bay 43–9006 and the MEK inhibitor CI-1040 in clinical trials on other cancers, a well-designed phase II clinical trial on these novel MAP kinase pathway inhibitors is now needed for thyroid cancer patients, particularly for those with incurable disease. Before such a clinical trial is conducted, more preclinical studies on the anticancer effects of these compounds in thyroid cancer cell lines and tumor xenograft animal models will provide important implications and necessary support for such clinical trials.

\textbf{Summary and future directions}

The discovery of the \textit{BRAF} mutation in thyroid cancer represents one of the most important recent advancements in thyroid cancer research and is of significant clinical potential in thyroid cancer medicine. Since the initial report on \textit{BRAF} mutation in thyroid cancer nearly 2 years ago, rapid progress has occurred due to an explosion of research in this area. The T1799A \textit{BRAF} mutation is the most common activating genetic alteration in thyroid cancer. Advancements have also been made in understanding the relationship between...
BRAF mutation and other common genetic alterations in thyroid cancer, and of particular note is the discovery of BRAF mutation’s mutual exclusivity with other well-established genetic alterations, a finding which points toward an independent role of BRAF mutation in thyroid tumorigenesis. The results from transgenic mouse studies have unequivocally established the role of BRAF mutation in the initiation and progression of PTC. The reciprocal age association between BRAF mutation and RET/PTC rearrangements is interesting, although it remains without a clear explanation at this time. More recent studies have been focused on the clinical significance of the BRAF mutation, particularly its diagnostic and prognostic values. As the T1799A BRAF mutation does not occur in benign thyroid tumors, it is a specific diagnostic marker for thyroid cancer when used in conjunction with FNAB, albeit with a low sensitivity for cases with indeterminate cytology. The association of BRAF mutation with poor clinicopathological outcomes, demonstrated by several relatively large studies, has established that this mutation is a novel prognostic molecular marker and may add a new dimension to the conventional risk evaluation of thyroid cancer. Preoperative knowledge of the BRAF mutation status of the thyroid tumor, through mutation analysis on FNAB cytological specimens, may be particularly valuable as it can assist clinicians in more efficiently planning and optimizing both the short- and long-term managements of thyroid cancer patients. Studies aimed at the therapeutic potential of novel inhibitors of the MAP kinase pathway for the treatment of thyroid cancer may yield important breakthroughs.

Further work is needed in the following several areas: (1) the elucidation of the specific molecular and cellular alterations and events that are caused by BRAF mutation and MAP kinase pathway activation in thyroid cancer; (2) the possible restoration of the ability of thyroid cancer cells to metabolize iodide by interfering with BRAF mutation-initiated aberrant signaling; (3) the improvement of the diagnostic utility of BRAF mutation, possibly through combination with other specific molecular markers for thyroid cancer in conjunction with FNAB, and through the establishment of a BRAF mutation-based blood test; (4) the clinical application of the prognostic value of BRAF mutation in guiding the optimal short- and long-term managements of thyroid cancer patients and (5) further preclinical and clinical studies on the therapeutic potential of novel inhibitors of MAP kinase pathway. It is anticipated that rapid advancements in these areas will occur in the next few years.

Acknowledgements
I wish to thank Dr P W Ladenson and Dr D Sidransky for their critical comments and inputs. I also wish to thank Dr W H Westra, Dr R P Tufano, Dr E Rosenbaum, Dr Y Cohen, Dr K J Rhoden, Ms K A Carson, Dr V Vasko, Dr A Larin, Dr G Tallini, Dr S Tolaney, Dr E H Holt, Dr P Hui, Dr C B Umbricht, Dr S Basaria, Ms M Ewertz, Dr A P Tufaro, Dr J A Califano, Dr M D Ringel, Dr M A Zeiger, Dr G Wu, Dr B Trink, and other colleagues for their generous support and collaboration. I admire the investigators cited in this review for their outstanding work. I intended to cite all the published work in this area, but apologize for the possible, unintended, omission of any relevant references.

Funding
This work is supported by a research grant from the Flight Attendant Medical Research Institute and a Johns Hopkins Clinician Scientist Award, which partially support the research work in my laboratory. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References
Baloch Z, Kumar MS, Lai M, Volpe P, LiVolsi VA, Mandel SJ & Brose MS 2004 Rate of BRAF and N-Ras mutations in thyroid nodules undergoing fine needle aspiration. Thyroid 14 745.
Braf mutation in thyroid cancer

Bongarzone I & Pierotti MA 2003 The molecular basis of thyroid epithelial tumorigenesis. Tumori 89 514–516.
Conard RA, Dobyns BM & Sutow WW 1970 Thyroid neoplasia as late effect of exposure to radioactive iodine in fallout. Journal of the American Medical Association 214 316–324.
Fagin JA 2002 Minireview: branded from the start-distinct oncogenic initiating events may determine tumor fate in the thyroid. Molecular Endocrinology 16 903–911.
and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. *Oncogene* **23** 7436–7740.


Krohn K & Paschke R 2004 BRAF mutations are not an alternative explanation for the molecular etiology of ras-mutation negative cold thyroid nodules. Thyroid 14 359–361.


Kumar MS, Moore KE & Brose MS 2004 Functional analysis of BRAF in a papillary thyroid cancer cell line. Thyroid 14 712.


Penko KS, Livezey J, Fenton CL, Patel A, Nicholson D, Flores M, Oakley K, Tuttle RM & Francis GL 2004 BRAF mutations are less common in childhood papillary thyroid cancer (PTC) than adult PTC. Thyroid 14 750.

carcinomas, rare in endocrine tumors of the gastrointestinal tract and not detected in other endocrine tumors. Endocrine-Related Cancer 11 855–860.


