Modifications in the Papillary Thyroid Cancer Gene Profile Over the Last 15 Years

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Background: Evidence for an increased prevalence of *BRAF*^{V600E} mutations has been documented in recent decades. The aim of this study was to evaluate the prevalence of both *RET/PTC* rearrangements and *BRAF*^{V600E} mutations in an Italian cohort of papillary thyroid carcinoma (PTC) patients followed at the Endocrine Units of Pisa, Milano, and Perugia from 1996–2010.

Patients and Methods: In total, 401 PTC patients were examined and grouped according to the time of surgery: group 1, 1996–2000; group 2, 2001–2005; and group 3, 2006–2010. Patients were analyzed for clinical, pathological, and molecular features. In parallel, the molecular characteristics of 459 PTC from Sicily were studied.

Results: The genetic profiles of the three groups were significantly different ($P < 0.0001$). In particular, the frequency of *RET/PTC* rearrangements decreased from 1996–2010, occurring in 33 of 100 (33%) of the patients in group 1, 26 of 148 (17%) in group 2, and 15 of 153 (9.8%) in group 3. The incidence of *BRAF*^{V600E} mutations increased over the same period, from 28% in group 1 (28 of 100) to 48.9% in group 2 (73 of 148) and 58.1% in group 3 (89 of 153). A consistent increase in *BRAF*^{V600E} prevalence was observed in the Sicilian group ($P < 0.0001$). Moreover, a statistically significant increase in the mean age at diagnosis and decrease in tumor size over the study period was observed.

Conclusion: The genetic profile of PTC changed over the last 15 yr, with a significant decrease in the prevalence of *RET/PTC* rearrangements and an increase in *BRAF*^{V600E} mutations. In addition, the mean age at diagnosis increased and tumor size decreased over the study period. **(*J Clin Endocrinol Metab* 97: E1758–E1765, 2012)**

Papillary thyroid carcinoma (PTC) is the most common type of thyroid malignant tumor, representing up to 80% of all thyroid cancers (1).

Activating alterations in several genes within the *RET/RAS/RAF/MAPK* signal transduction pathway have been identified in the majority of PTC (2). *RET* oncogene activation in PTC occurs by chromosomal recombination that results in the expression of *RET/PTC* oncoproteins, which consist of the intracellular tyrosine kinase domain of *RET* coupled to the N-terminal fragment of different

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heterologous genes (3). So far, at least 13 different *RET* rearrangements with 11 distinct fusion partners have been reported (2–4). Among the types of *RET* rearrangements, *RET/PTC1* and *RET/PTC3* are the most common and account for more than 90% of the rearrangements identified in sporadic and radiation-induced tumors (3, 5). *RET/PTC* rearrangements are frequent in radiation-induced PTC, occurring in 66–87% of these tumors (5–8). In sporadic tumors, *RET* rearrangements occur in approximately 40% of PTC, with a wide range (3–85%) in different cohorts (3, 6), perhaps due to epidemiological and/or technical reasons.

More recently, a point mutation in the *BRAF* gene ($BRAF^{V600E}$) was identified as the most common genetic change in sporadic PTC (9–12). The prevalence of $BRAF^{V600E}$ mutations ranges from 23–83% in different cohorts, with a cumulative prevalence of 38–44% (12, 13). *BRAF* mutations have been found to be correlated with both the classic and more aggressive variants of PTC (13, 14). Although it is generally accepted that *BRAF* mutation prevalence is higher in older patients, there are still controversial data on this specific relationship (11–13, 15, 16). As matter of fact, *BRAF* mutations are very rare in children and young patients (17). At variance with *RET/PTC* rearrangements, *BRAF* point mutations are uncommon in radiation-induced tumors (18), and only a few tumors containing *BRAF* rearrangements have been observed in children exposed to Chernobyl fallout (19). Finally, *BRAF* mutations are usually correlated with a more aggressive disease at diagnosis (14) and a worse outcome (20).

A change in the epidemiology of PTC during the last decades has been reported (21), and in particular, it has been observed that the most recently diagnosed PTC are smaller in size and that PTC patients are older at diagnosis (22). Furthermore, two recent separate studies conducted at single institutions demonstrated that the rate of *BRAF* mutations in PTC has increased in recent decades (23, 24). The aim of our multicenter Italian study was to evaluate the variation in the prevalence of $BRAF^{V600E}$ mutations and *RET/PTC* rearrangements in a large series of PTC over a period of 15 yr, taking also into account other epidemiological changes.

Patients and Methods

Study group

Among the patients treated for PTC at the Endocrine Surgery Units of Pisa, Milano, and Perugia, Italy, from 1996–2010, 401 patients were included in this study. Whenever possible, tumor tissues were collected during surgery, immediately frozen in liquid nitrogen, and stored at −80 C. Cases were neither consecutive nor selected, and the same procedures of collection were followed in the three centers.

Patients were classified into three groups according to the time of surgery: group 1, 1996–2000; group 2, 2001–2005; and group 3, 2006–2010. Group 1 had 100 patients (68 females and 32 males, mean age 39.4 ± 15.8 yr, range 9–79 yr); group 2 consisted of 148 patients (111 females and 39 males, mean age 43.9 ± 17.4 yr, range 8–85 yr); and group 3 contained 153 patients (103 females and 50 males, mean age 47.8 ± 17 yr, range 14–84 yr).

Clinical and epidemiological data were available in most cases. Tumors were classified according to the World Health Organization's classification (25) of thyroid malignancy and staged according to the sixth edition of tumor, node metastases, distant metastases staging (26).

In parallel, a group of 459 PTC patients from Sicily were evaluated for the presence of *RET/PTC* rearrangements and $BRAF^{V600E}$ mutations. No clinical data were available in this cohort.

About 70% of patients gave their consent to make the thyroid tissue available for experimental studies at the time of surgical treatment. The remaining 30% of patients gave at enrollment the informed consent to submit to genetic analyses the archival paraffin-embedded tissues. All patients gave their consent for the inclusion of the clinical data in scientific studies. This study was approved by each Internal Review Board.

Analysis of genetic alterations

The core of the tumor nodule was dissected macroscopically for tumors larger than 1.5 cm and analyzed after histological confirmation of malignancy. Tumors with a diameter less than 1.5 cm were microdissected to ensure isolation of tumor tissue.

DNA was extracted from the tumor tissue using commercial kits (Puregene from Gentra Systems, Minneapolis, MN; QIAamp DNA Mini Kit from QIAGEN, Hilden, Germany), and RNA extraction was performed using Trizol reagent (Invitrogen Corp., Carlsbad, CA); both nucleic acid extractions were performed according to the manufacturer's instructions.

For *RET/PTC* analysis, total RNA was reverse transcribed with Superscript II reverse transcriptase (Invitrogen) using a random hexamer primer mixture. The cDNA was analyzed by PCR using appropriate primer sets (6, 27).

For *BRAF* analysis, the DNA was amplified by PCR using specific intronic primers as previously described (12). After purification, PCR products were directly sequenced on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster city, CA).

Although minor methodological differences existed in the protocols used to detect molecular alterations at the individual centers, the same protocol was maintained over the last 15 yr at each institution.

Statistical analysis

Correlations between tumors, clinical features, and genetic alterations were determined by *t* tests, χ^2 tests, and Fisher's exact tests. The difference between two values was considered significant if $P < 0.05$. All tests were performed using the Statistical Package for Social Sciences for Windows (SPSS, Inc., Chicago, IL).

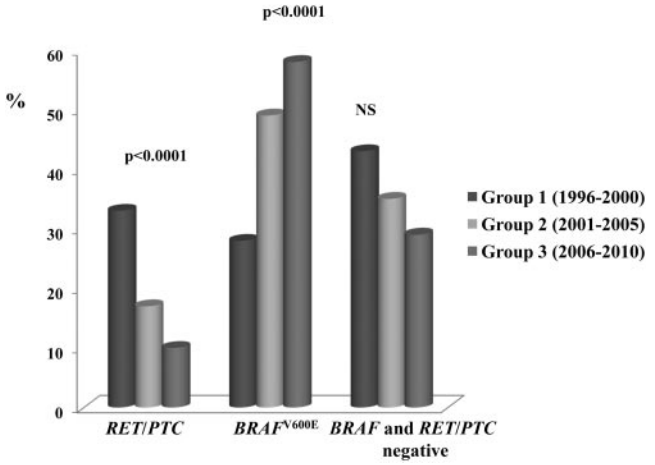

FIG. 1. The prevalence of *BRAF*^{V600E} mutations and *RET/PTC* rearrangements according to the year of surgery [group 1 (1996–2000), group 2 (2001–2005), and group 3 (2006–2010)] in the Pisa, Milano, and Perugia centers was significantly different ($P < 0.0001$). NS, Not significant.

Results

The clinical, epidemiological, pathological, and molecular analysis of cases from Pisa, Milano, and Perugia

As shown in Fig. 1, the genetic profile of the three PTC groups was significantly different ($P < 0.0001$). In particular, the frequency of *RET/PTC* rearrangements decreased from 1996–2010, with *RET/PTC* rearrangements present in 33% of group 1 (n = 33), 17% of group 2 (n = 26), and 9.8% of group 3 (n = 15). The prevalence of *RET/PTC* rearrangements was significantly higher (23%) in younger (*i.e.* < 45 yr) than in older (*i.e.* > 45 yr) patients (13%) ($P = 0.015$). No correlations were found with sex and histological variants.

The prevalence of *BRAF*^{V600E} mutations increased over the same time period, with a frequency of 28% in group 1 (n = 28), 48.9% in group 2 (n = 73), and 58.1% in group 3 (n = 89) ($P < 0.0001$, Fig. 1). As expected, we found an increased prevalence of *BRAF*^{V600E} mutations in the classic variant of PTC in all three groups (Fig. 2A). No differences existed in the incidence of *BRAF*^{V600E} mutations when the patients were analyzed according to age at diagnosis (Fig. 2B), tumor size (Fig. 2C), or the presence of lymph node (Fig. 2D) or distant metastases (data not shown).

The prevalence of PTC without *RET* or *BRAF*^{V600E} mutations decreased over the study period, but this decrease was not statistically significant (Fig. 1).

Despite the different molecular profiles, the three groups of PTC patients were homogeneous regarding sev-

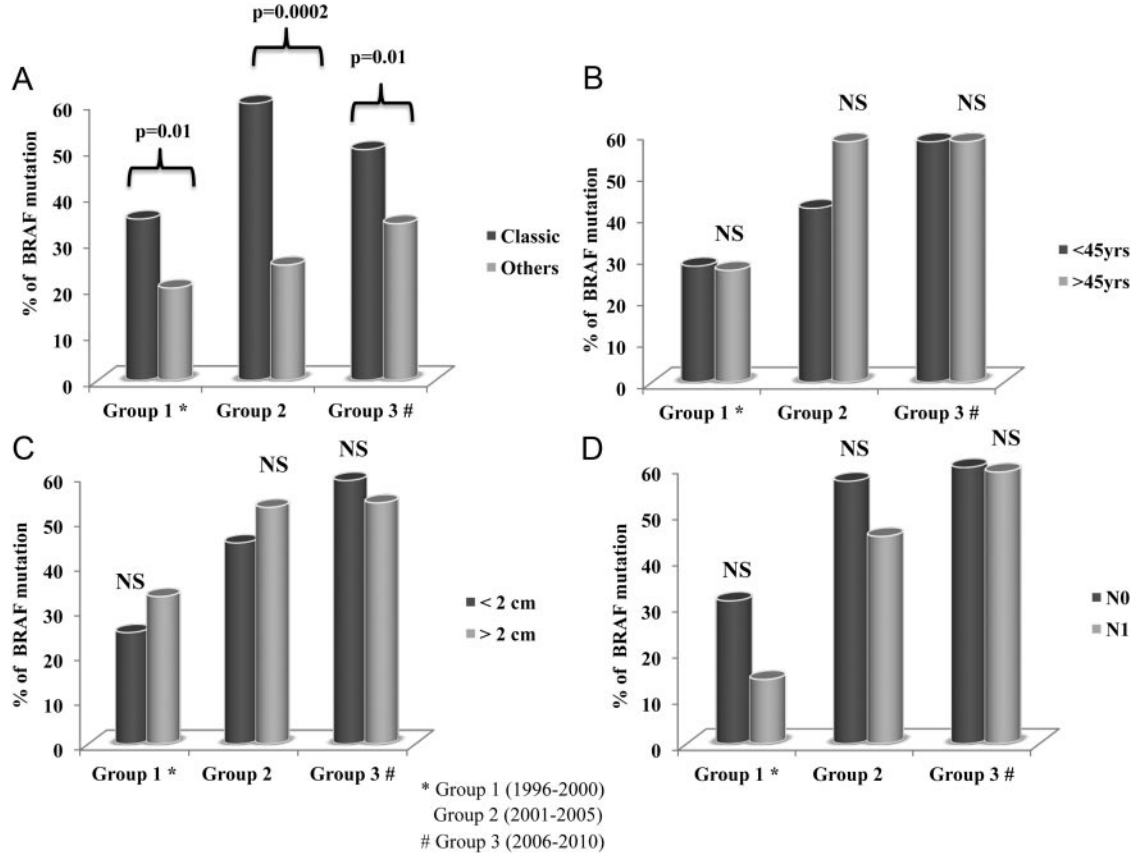

FIG. 2. Correlations between the presence of a *BRAF*^{V600E} mutation and histological variants (A), age at diagnosis (B), tumor size in centimeters (C), and lymph node metastases (D) in the three groups [group 1 (1996–2000), group 2 (2001–2005), and group 3 (2006–2010)]. A statistically significant correlation was observed only between *BRAF*^{V600E} mutations and the classic variant of PTC. NS, Not significant.

TABLE 1. The distribution of clinical, pathological, and epidemiological features of PTC in the three groups stratified by the year of surgery

	1996–2000 [n (%)]	2001–2005 [n (%)]	2006–2010 [n (%)]	*P*
Mean age	40.9 ± 15.6	44 ± 17.4	47.6 ± 16.7	**0.014**
Sex (n)				
Male (119)	32 (32)	37 (25)	50 (33)	NS
Female (282)	68 (68)	111 (75)	103 (67)	
Tumor size (379)				
<2 cm	39 (39)	85 (57)	89 (58)	**0.01**
≥2 cm	51 (51)	58 (43)	57 (42)	
Tumor (361)				
T1 (149)	20 (27)	57 (41)	72 (49)	
T2 (73)	21 (28)	28 (20)	24 (16)	**0.0048**
T3 (115)	23 (30)	46 (34)	46 (31)	
T4 (24)	11 (15)	7 (5)	6 (4)	
Node metastases (333)				
N0 (166)	35 (49)	67 (52)	64 (48)	NS
N1 (167)	36 (51)	62 (48)	69 (52)	
Distant metastases (203)				
M0 (194)	50 (96)	84 (93)	60 (98)	NS
M1 (9)	2 (4)	6 (7)	1 (2)	
Histological variants				
Classic	56 (65)	98 (69)	106 (74)	
Follicular	10 (12)	26 (18)	22 (15)	NS
Others	15 (17)	17 (13)	16 (11)	
External radiation				
Yes	0	1 (1.4)	2 (1.7)	NS
No	100	70 (98.6)	111 (98.3)	

NS, Not significant. *Bold* values represent statistically significant values.

eral clinical and pathological characteristics, such as sex, node and distant metastases, histological variant, and neck irradiation history. Conversely, we observed a statistically significant increase in the mean age at diagnosis and decrease in tumor size, this latter observation being consistent with the increase of the tumors owning to the T1 category (Table 1). To rule out the possibility that the results were biased by the nonconsecutive enrollment of patients, the analysis of more than 4000 PTC cases followed at the Department of Endocrinology in Pisa from 1996–2010 was performed. In particular, patients were separated into three groups according to the year of diagnosis, and the trend in clinical and pathological features was not different from that reported in the present study for the multicentric series, indicating its representativeness (data not shown).

Molecular analysis of the Sicilian cases

A similar statistically significant ($P < 0.0001$) increase in the prevalence of $BRAF^{V600E}$ mutations was observed in the group of Sicilian patients (Table 2). Interestingly, *RET/PTC* rearrangements were virtually absent in these samples; only two cases of 459 were positive for these rearrangements, one with *PTC1* in the 1998–2000 group and one with *RET/PTC3* in the 2006–2010 group.

When the molecular data from Sicily and the other centers were combined, we confirmed on a larger scale (total cases n = 860) that the prevalence of $BRAF^{V600E}$ mutations has been increasing in the last decades, from 33.6% in group 1 to 47.8% in group 2 and up to 61.5% in group 3 ($P < 0.0001$) (Fig. 3A). Similarly, the statistically significant decrease in the prevalence of *RET/PTC* rearrangements was confirmed in the entire cohort of 860 cases ($P < 0.0001$) (Fig. 3B).

Discussion

PTC is characterized by genetic lesions, such as chromosomal rearrangements of the *RET* gene or activating point mutations in *BRAF*, that lead to activation of the MAPK signaling pathway (2). According to the literature, approximately 70% of PTC harbor one of these primarily mutually exclusive lesions (13). Although significant cor-

TABLE 2. The prevalence of $BRAF^{V600E}$ mutations in the Sicilian cohort stratified by the year of surgery

	1996–2000 (n = 101)	2001–2005 (n = 183)	2006–2010 (n = 175)	*P*
$BRAF^{V600E}$ mutation (%)	39 (38.6)	86 (46.9)	113 (64.5)	**<0.0001**

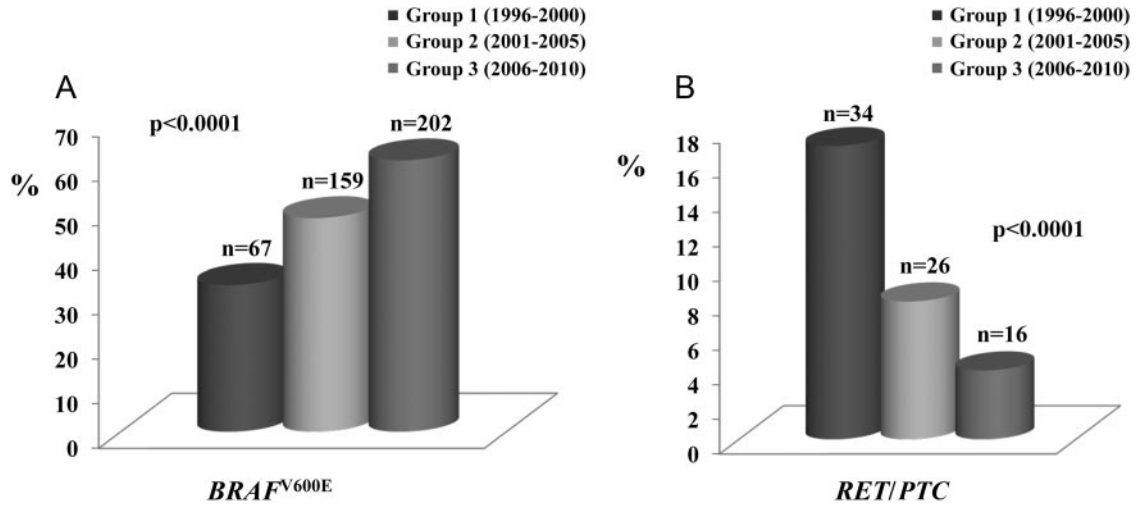

FIG. 3. A statistically significant difference ($P < 0.0001$) was observed in both the prevalence of *BRAF*^{V600E} mutations (A) and *RET/PTC* rearrangements (B) according to the year of surgery [group 1 (1996–2000), group 2 (2001–2005), and group 3 (2006–2010)] in the entire cohort (860 cases).

relations between *BRAF* mutations and several pathological, clinical, and epidemiological features have been described (11–13, 15, 20), the role of *RET/PTC* rearrangements in determining clinical and pathological clinical features is not fully understood (28). Among *RET/PTC* rearrangements, only *RET/PTC*3 has been found to correlate with a larger tumor size, a more aggressive variant, and a more advanced stage at diagnosis (5, 27, 28).

This multicenter Italian study of a large PTC cohort demonstrated that the prevalence of *RET/PTC* rearrangements has significantly decreased by comparing cases diagnosed in three different time periods (1996–2000, 2001–2005, and 2006–2010).

External and internal ionizing radiation exposure is the most established risk factor for PTC (29). A correlation between radiation exposure and a higher prevalence of *RET/PTC* rearrangements, particularly of *RET/PTC*3, has been documented in cases of radiation-induced PTC (5, 6, 8). In addition, a relationship between radiation exposure and the development of *RET/PTC* rearrangements *in vitro* has been reported (30).

Four years after the Chernobyl nuclear accident, an increase in thyroid cancer incidence was observed in the highly exposed areas (31, 32). However, with increased latency, the molecular and morphological pathology of post-Chernobyl tumors has changed (32). Over time, the proportion of radiation-induced tumors with a *RET/PTC* rearrangement has declined, but within *RET*-positive tumors, the prevalence of *RET/PTC1* rearrangements has increased, whereas the incidence of *RET/PTC*3 rearrangements has decreased (33). Although our PTC were sporadic cases with no history of radiation exposure, the temporal variation in the prevalence of particular *RET/PTC* rearrangements was similar to that observed in post-Chernobyl cases. This indicates that PTC cases diagnosed between 1996 and 2000 could be related to post-Chernobyl radioactive fallout. In addition, the decrease of *RET/PTC* rearrangements was not observed in a recent study of a PTC cohort diagnosed during the same time period in California (United States), which is far enough from Chernobyl to be unaffected by post-Chernobyl radioactive fallout. Furthermore, in the California cohort, the prevalence of *RET/PTC* rearrangements was lower even in previous decades, when the radiation exposure could happen. Although difficult to prove, the decreased incidence of *RET/PTC* rearrangements observed in Italy might be related to reducing radiation exposure in recent decades. However, epidemiological studies have demonstrated that in Italy, particularly in the north, the incidence of thyroid cancer was indistinguishable in children born in 1985–1986 and in a control group not exposed to radioactive pollution (34).

In this large multicenter Italian study, we discovered a statistically significant increase in the prevalence of *BRAF*^{V600E} mutations in PTC cases diagnosed from 1996–2010. This corresponds with two smaller, single-center PTC studies in the United States and Ireland (23, 24). It is worth noting that, in our study, the conclusions were similar when the cohort was analyzed individually by center and when all of the cases were combined.

Interestingly, *BRAF* point mutations are uncommon in post-Chernobyl tumors (18), and only a few tumors containing *BRAF* rearrangements have been observed in children exposed to Chernobyl fallout (19). These data agree with the fact that ionizing radiation induces double-strand DNA breaks, which can lead to gene rearrangements (35). Just as ionizing radiation is responsible for gene rearrange-

ments, other environmental factors should be identified that induce DNA point mutations (35).

The iodine prophylaxis carried out in iodine-deficient regions has been associated with an increased prevalence of PTC and a reduction of follicular histotype (36–38). A significant association between $BRAF^{V600E}$ mutations and high iodine intake has been also demonstrated, suggesting that high iodine levels could be a risk factor for *BRAF* mutations and therefore the development of PTC (39). For these reasons, the increased occurrence of *BRAF* mutations in PTC observed recently by us and others (23, 24) could be related to the intake of prophylactic iodine that has become increasingly recommended. However, this may be particularly true for Italy and several other European regions (38), but not for the United States, which has one of the oldest iodine prophylaxis programs and is thus considered one of a few iodine-sufficient regions (http://www.iccidd.org/pages/global-scorecard.php).

Another hypothesis to explain the increased prevalence of *BRAF* mutations is that younger populations are exposed to new pollutants that could induce DNA point mutations (40). In support of this hypothesis, a high incidence of PTC associated with an increased prevalence of *BRAF* mutations was identified in the volcanic area of Mount Etna in Sicily (16, 41). No association with iodine levels was demonstrated in that study, although the drinking water often contains several mineral elements that exceed the maximum admissible concentrations (16, 41). Regarding this, it is worth noting that excessive levels of radon have been found in the volcanic area of Mount Etna. Both radon and thoron are the most important components of the background radiation that has been demonstrated to be increasing worldwide and that could be also able to induce DNA point mutations (42). Sicilian samples are also characterized by the absence of *RET/PTC* rearrangements. The peculiarity of this series could lie both in the common genetic origin of the Sicilian population that is relatively different from that of the rest of Italian people and in the geographical location of Sicily that is so far from central Europe to be not reached by post-Chernobyl fallout, like it happened in California where the *RET/PTC* rearrangements were not absent but very low prevalent.

In this multicenter Italian study, we observed that both age at diagnosis and tumor size changed over the study period. In particular, patients diagnosed most recently were significantly older and had significantly smaller tumors. One could argue that a bias has been produced at the time of tissue collection. However, the increasing age and the reducing size were features observed separately in the three centers. Furthermore, these findings agree with other recent observations (22, 43), particularly those regarding the increased prevalence of micro-PTC (21, 43). *BRAF* mutations have been controversially reported to be more prevalent in older patients (20), whereas *RET/PTC* rearrangements have been demonstrated to be more frequent in children and younger patients (5, 6), as it happened also in the present series. Taking into account these two observations, one possible explanation for our results is that the age at diagnosis has becoming progressively older in recent years, thus favoring the increase of *BRAF* mutations and the decrease of *RET/PTC* rearrangements. This hypothesis is supported by the evidence that both the different age of the groups of patients and the year of diagnosis resulted to be independent variables at multivariate analysis for the presence of the *RET/PTC* rearrangements. However, this phenomenon could be interpreted also in the opposite way, and the increase of age at diagnosis could be the consequence of an increase of *BRAF* mutation determined by new pathogenic factors mainly affecting older patients. Conversely, the increased incidence of smaller tumors cannot be explained by variations in genetic characteristics because both *RET/PTC* rearrangements and *BRAF* mutations occur with the same prevalence in micro-PTC and in larger tumors (44). The increase of PTC with a smaller size could be not related to genetic events but perhaps to anticipation of diagnosis due to the widely use of neck ultrasound.

In conclusion, this large multicenter Italian PTC study clearly demonstrated a significant change in the molecular profile of PTC, which is becoming progressively more and less frequently associated with *BRAF* mutations and *RET/PTC* rearrangements, respectively. One plausible explanation is that recently, the mean age at diagnosis has become progressively older, as demonstrated by us and others (22). At the same time, new pollutants that induce point mutations could be contributing to the increase in *BRAF* mutations, whereas a progressive elongation of the latency period from the Chernobyl event may be responsible for decreased *RET/PTC* rearrangements.

Acknowledgments

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