SCIENCE CHINA
Life Sciences

THEMATIC ISSUE: Frontiers in rare diseases
• RESEARCH PAPER •

July 2017 Vol.60 No.7: 729–738
doi: 10.1007/s11427-017-9083-8

Correlation between *BRAF*^{V600E} mutation and clinicopathological features in pediatric papillary thyroid carcinoma

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Received March 29, 2017; accepted May 21, 2017; published online June 15, 2017

In adults, the presence of the *BRAF*^{V600E} mutation in papillary thyroid cancer (PTC) has been demonstrated to be strongly associated with aggressive cancer-cell characteristics and poor patient prognosis. In contrast, the frequency of this mutation in pediatric PTC has undergone limited study, and the few available estimates range from 0 to 63%. Furthermore, the role of the *BRAF*^{V600E} mutation in pediatric PTC is controversial; thus, the present study aimed to investigate the prevalence and role of the *BRAF*^{V600E} mutation in 48 pediatric patients with PTC, aged 3–13 years. Of these patients, 41 were diagnosed with classic PTC, five were found to have a follicular variant of PTC, and two to exhibit a diffuse sclerosing PTC variant. The *BRAF*^{V600E} mutation was identified to be present in 35.4% of the 48 analyzed patients, and in 41.5% of the patients diagnosed with classical PTC. Furthermore, the presence of the *BRAF*^{V600E} mutation was found to be associated with a patient age at diagnosis of less than ten years (P=0.011), the performance of a thyroidectomy (P=0.03), exhibited tumor multifocality (P=0.02) and/or extra-thyroidal invasion (P=0.003), and both a low MACIS (Metastases, Age, Completeness of resection, Invasion, Size)(P=0.036) and AMES (Age, Metastasis, Extent of tumor, Size)(P=0.001) score. Together, these data suggest that the presence of the *BRAF*^{V600E} mutation may be negatively correlated with partial aggressive clinicopathological features of pediatric PTC.

pediatric papillary thyroid cancer, *BRAF*^{V600E} mutation, clinicopathological characteristics

Citation: Geng, J., Wang, H., Liu, Y., Tai, J., Jin, Y., Zhang, J., He, L., Fu, L., Qin, H., Song, Y., Su, J., Zhang, A., Wen, X., Guo, Y., and Ni, X. (2017). Correlation between *BRAF*^{V600E} mutation and clinicopathological features in pediatric papillary thyroid carcinoma. Sci China Life Sci 60, 729–738. doi: 10.1007/s11427-017-9083-8

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INTRODUCTION

Thyroid carcinoma (TC) is the most common malignant endocrine neoplasm, and its incidence is rapidly increasing in both males and females (American Cancer Society, Cancer Facts and Figures 2012, http://www.cancer.org/research/cancerfacts figures/cancer facts figures/cancer-facts-figures-2012, accessed February 4, 2014). In fact, approximately 62,450 new TC cases are diagnosed in the US each year (Siegel et al., 2015), and similarly, approximately 90,000 new TC cases were diagnosed in China in 2015 (Chen et al., 2016). Recently, a rise in the TC incidence rate has also been reported in pediatric patients, mainly among adolescents (Vergamini et al., 2014). Data recorded by the Surveillance, Epidemiology, and End Results (SEER) Registry program reveals that the incidence of thyroid neoplasm in children aged <5, 5–9, and 10–14 years was 0.01, 0.09, and 0.44 cases per 100,000 persons between 1973 and 2004 (Ward et al., 2014; Hogan et al., 2009).

TCs, including both differentiated (DTCs) and anaplastic thyroid cancers (ATCs), predominantly originate from follicular or parafollicular thyroid cells. The latter cell type produces the hormone calcitonin and is also capable of giving rise to medullary thyroid cancer (MTC). Papillary (PTC) and follicular thyroid cancer (FTC) are differentiated thyroid cancers that comprise more than 90% of all thyroid malignancies in adults (Xing et al., 2013). Notably, significant differences have been observed in the frequency of the TC pathologic types that affect adults versus those that affect children. For example, PTC is more common in children than in adults, comprising approximately 95% and 80% of all TC cases in children and adults, respectively. In contrast, FTCs, ATCs, and Hurthle-cell TCs only rarely occur in children (LaFranchi, 2015; Parisi et al., 2016). Furthermore, the clinicopathological features and biological behaviors of pediatric PTCs are significantly different from those exhibited by adult PTCs (LaFranchi, 2015), in that pediatric PTCs commonly present with more advanced stages than adult PTCs and require aggressive and often repeated treatment; nevertheless, the prognosis for pediatric PTCs is predominantly good (Parisi et al., 2016; Dzodic et al., 2014).

Currently, dissimilarities between TCs in pediatric and adult populations are not well characterized (Xing et al., 2013; Francis et al., 2015); however, various tumor molecular markers have been suggested as potential tools to identify aggressive PTC cases, and to thereby improve the clinical risk stratification for adult PTC. For the majority of patients, causative PTC mutations include the B-type RAF kinase (*BRAF*) V600E mutation, RET/PTC rearrangement, and/or RAS mutation (Xing et al., 2013; Yarchoan et al., 2015; Petrulea et al., 2015). Of these, the $BRAF^{V600E}$ mutation is the most frequently observed PTC-associated genetic alteration and induces tumorigenesis via a valine-to-glutamine transversion at position 600 in the *BRAF* amino acid sequence, which constitutively activates the mitogen-activated protein kinase (MAPK) signaling pathway (Liu et al., 2016; Li et al., 2012; Davies et al., 2002; Roring et al., 2012). In adult PTC, the $BRAF^{V600E}$ mutation has been demonstrated to be strongly associated with aggressive tumor-cell characteristics and poor patient prognosis (Xing et al., 2015; Czarniecka et al., 2015; Elisei et al., 2012). Furthermore, a recent meta-analyses of 32 previously conducted studies showed the presence of the $BRAF^{V600E}$ mutation in adult PTC to be associated with lymphatic metastasis, extra-thyroidal extension, multifocality, advanced tumor stage, increased tumor size, male gender, and tall-cell variant PTC (Li et al., 2012).

In contrast, the implications of the $BRAF^{V600E}$ mutation in pediatric PTC have undergone very limited study. Furthermore, the few previously conducted studies addressing this topic reported an incidence for this mutation that ranged from 0 to 63%, and suggested it to play divergent roles in tumorigenesis (Penko et al., 2005; Henke et al., 2014; Sassolas et al., 2012). Thus, the present study aimed to improve the current understanding of the role of $BRAF^{V600E}$ in pediatric PTC, by investigating its prevalence and impact on PTC progression in 48 children aged 3–13 years.

RESULTS

We enrolled a cohort of 48 (29 females and 19 males) pediatric patients aged 3.6–13.8 years. We collected data describing patient gender, age, treatment (e.g., thyroidectomy, CND, modified radical neck dissection (MRND)), tumor characteristics (such as histological subtype, size, multifocality, capsular invasion, and extra-thyroidal invasion), staging (including AJCC, American Thyroid Association (ATA) pediatric PTC, and AMES staging), PTC recurrence, and $BRAF^{V600E}$-mutation status (Table 1). We observed the $BRAF^{V600E}$ mutation to be present in 17 (35.4%) of the 48 patients, all of which exhibited classical PTC. Conversely, all patient tissue samples exhibiting variant PTC pathology (e.g., follicular or diffuse sclerosing variants) were found to harbor only wild-type *BRAF* alleles.

From an analysis of the collected data, we found that the presence of the $BRAF^{V600E}$ mutation was significantly associated with both a low AJCC (P=0.014) and low AMES (P=0.001) tumor stage (Table 1). Conversely, a patient age at diagnosis of less than ten years (P=0.011), the performance of a total thyroidectomy (P=0.030) or MRND (P=0.044), and disease multifocality (P=0.020) or extra-thyroidal invasion (P=0.003) were each negatively associated with the presence of the $BRAF^{V600E}$ mutation. No association was observed between the presence of either a $BRAF^{V600E}$ mutation or wild-type *BRAF* allele and the other analyzed data, for example, patient gender (P=0.867), performance of a CND (P=0.701), tumor histological subtype (P=0.116), size (P=

Table 1 Relationship between *BRAF*^{V600E} mutation and clinicopathological characteristics of pediatric papillary thyroid carcinoma[a)]

Characteristics	No. (%)	*BRAF* positive, No. (%)	*BRAF* negative, No. (%)	*P* value
Gender				0.867
Male	19 (39.6)	7 (36.8)	12 (63.2)	
Female	29 (60.4)	10 (34.5)	19 (65.5)	
Age (years)				0.011
<10	26 (54.2)	5 (19.2)	21 (80.8)	
10−13	22 (45.8)	12 (54.5)	10 (45.5)	
Thyroidectomy				0.030
Lobectomy	21 (43.7)	11 (52.4)	10 (47.6)	
Total	27 (56.3)	6 (22.2)	21 (77.8)	
CND				0.701
Yes	39 (81.2)	13 (33.3)	26 (66.7)	
No	9 (18.8)	4 (44.4)	5 (55.6)	
MRND				0.044
Yes	29 (60.4)	7 (24.1)	22 (75.9)	
No	19 (39.6)	10 (52.6)	9 (47.4)	
Histological subtype				0.116
Classical type	41 (85.4)	17 (41.5)	24 (58.5)	
Follicular variant	5 (10.4)	0 (0.0)	5 (100.0)	
Diffuse sclerosing variant	2 (4.2)	0 (0.0)	2 (100.0)	
Tumor size (cm)				0.092
<2	2 (4.2)	2 (100.0)	0 (0.0)	
2−4	28 (58.3)	11 (39.3)	17 (60.7)	
>4	18 (37.5)	4 (22.2)	14 (77.8)	
Multifocality				0.020
Yes	25 (52.1)	5 (20.0)	20 (80.0)	
No	23 (47.9)	12 (52.2)	11 (47.8)	
Capsular invasion				0.080
Yes	33 (68.8)	9 (27.3)	24 (72.7)	
No	15 (31.2)	8 (53.3)	7 (46.7)	
Extrathyroidal invasion				0.003
Yes	25 (52.1)	4 (16.0)	21 (84.0)	
No	23 (47.9)	13 (56.5)	10 (43.5)	
AJCC Tumor Stage				0.014
T1	3 (6.3)	3 (100.0)	0 (0.0)	
T2	16 (33.3)	7 (43.8)	9 (56.2)	
T3	6 (12.5)	3 (50.0)	3 (50.0)	
T4	23 (47.9)	4 (17.4)	19 (82.6)	
AJCC nodal stage (loco-regional metastases)				0.228
N0	12 (25.0)	6 (50.0)	6 (50.0)	
N1a	11 (22.9)	5 (45.5)	6 (54.5)	
N1b	25 (52.1)	6 (24.0)	19 (76.0)	
AJCC distant metastases				1.000
M0	41 (85.4)	15 (36.6)	26 (63.4)	
M1	7 (14.6)	2 (28.6)	5 (71.4)	
ATA pediatric PTC stage (2015)				0.053
Low risk	11 (22.9)	7 (63.6)	4 (36.4)	
Intermediate risk	10 (20.8)	4 (40.0)	6 (60.0)	
High risk	27 (56.3)	6 (22.2)	21 (77.8)	
Recurrence				0.643
Yes	5 (10.4)	1 (20.0)	4 (80.0)	
No	43 (89.6)	16 (37.2)	27 (62.8)	
AMES				0.001
Low risk	21 (43.7)	13 (61.9)	8 (38.1)	
High risk	27 (56.3)	4 (14.8)	23 (85.2)	

a) AJCC, American Joint Committee on Cancer 7th Edition (2010). ATA pediatric PTC stage, Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer developed by The American Thyroid Association Guidelines Task Force on Pediatric Thyroid Cancer 1st Edition (2015).

0.092), and/or capsular invasion (P=0.080), AJCC nodal (P=0.228), distant metastasis (P=1.000), and/or ATA pediatric PTC stage (P=0.053), and/or disease recurrence (P=0.643).

Among patients diagnosed with classical pediatric PTC (Table 2), the presence of the *BRAF*^{V600E} mutation was found to be significantly associated with a small tumor size (P=0.045), a low AJCC tumor (P=0.001) and nodal stage (P=0.028), a low ATA pediatric PTC stage (P=0.007), and a low AMES score (P=0.000). Conversely, the presence of the *BRAF*^{V600E} mutation was negatively associated with a patient age at diagnosis of less than 10 years (P=0.004), the performance of a total thyroidectomy (P=0.011) or MRND (P=0.005), and tumor multifocality (P=0.009), capsular invasion (P=0.029) and/or extra-thyroidal invasion (P=0.000). No association was observed between the presence of the *BRAF*^{V600E} mutation and/or a wild-type *BRAF* allele and patient gender (P=0.975), the performance of a CND (P=0.141), AJCC distant metastases (P=0.679), and/or disease recurrence (P=0.382).

For pediatric PTC diagnosed in children aged less than 10 years, the presence of the *BRAF*^{V600E} mutation was significantly associated with a low AJCC tumor (P=0.012) and nodal (P=0.007) stage, a low ATA pediatric PTC stage (P=0.009), and a low AMES score (P=0.034); however, in contrast, it was negatively associated with the performance of a total thyroidectomy (P=0.004) or MRND (P=0.010), and tumor multifocality (P=0.007) and/or extra-thyroidal invasion (P=0.034). No association was observed between the presence of the *BRAF*^{V600E} mutation and/or a wild-type *BRAF* allele and patient gender (P=1.000), the performance of a CND (P=0.488), tumor size (P=0.121) or capsular invasion (P=0.101), AJCC distant metastasis staging (P=1.000), and/or disease recurrence (P=1.000) (Table 3).

For pediatric PTC diagnosed in children aged 10–13 years, the presence of the *BRAF*^{V600E} mutation was negatively associated with tumor multifocality (P=0.007). No association was observed between the presence of the *BRAF*^{V600E} mutation and/or a wild-type *BRAF* allele patient gender (P=1.000), the performance of a total thyroidectomy (P=0.691), CND (P=1.000), or MRND (P=1.000), tumor size (P=0.668), capsular invasion (P=0.675), and/or extra-thyroidal invasion (P=0.378), AJCC tumor (P=1.000) or nodal stage (P=0.853), AJCC distant metastasis (P=1.000), ATA Pediatric PTC stage (P=1.000), disease recurrence (P=0.455), and/or a low AMES score (P=0.192) (Table 4).

A histopathological analysis of patient PTC samples showed that the observed papillary-morphology cancer nests exhibited infiltrative growth in thyroid follicles, in which the cancer cells were arranged in a papillary shape and found to vigorously proliferate. Furthermore, these cells exhibited atypical, vesicular-shaped nuclei, and a visible nuclear sulcus that was scattered in the gravel body (Figure 1A). Immunochemical (IHC) staining of PTC samples with thyroglobulin showed the papillary cancer nests and marginal residual thyroid follicles to be cytoplasm positive (Figure 1B).

DISCUSSION

In recent decades, the incidence of pediatric TC, and especially that of pediatric PTC, has rapidly increased. In addition, while pediatric PTC incidence and mortality rates are currently lower than those observed in adult PTC, the frequency of disease recurrence is much higher in children. Pediatric PTCs commonly present as large, painless, palpable thyroid and/or neck lumps that are often neglected, leading to the diagnosis of PTC at advanced stages. Despite this common delay in PTC diagnosis, effective treatment regimes can be used to reduce the risk of PTC recurrence and/or induced patient mortality.

Recent studies have shown the *BRAF*^{V600E} mutation to be the most common genetic alteration in adult PTC, affecting an estimated 36%–83% of all adult PTC cases (Francis et al., 2015; Li et al., 2012). To date, available estimates for the frequency of the *BRAF*^{V600E} mutation in pediatric PTC are both limited and conflicting, largely due to the low incidence of the disease. While one study reported the *BRAF*^{V600E} mutation to be very rare in pediatric PTC (Francis et al., 2015), more recent studies have produced very different results (Henke et al., 2014; Daniel et al., 2014). The results of the present study

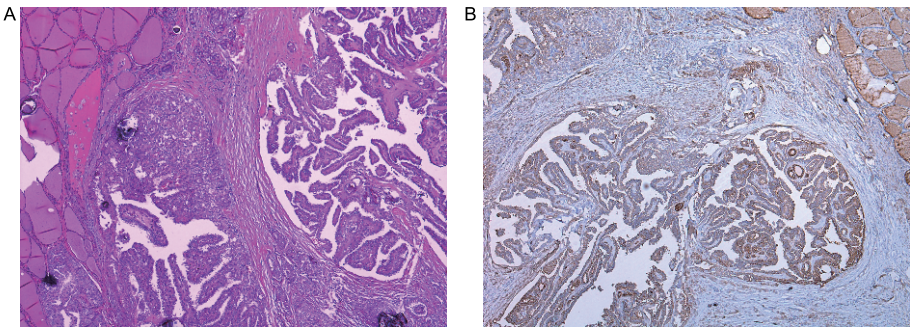

Figure 1 A histopathological analysis of patient PTC samples. A, Papillary thyroid carcinoma (hematoxylin-eosin staining, ×50). B, Papillary thyroid carcinoma (immunochemical staining, ×50).

Table 2 Relationship between *BRAF*^{V600E} and clinicopathological characteristics of pediatric classic papillary thyroid carcinoma[a)]

Characteristics	No. (%)	*BRAF* positive, No. (%)	*BRAF* negative, No. (%)	*P* value
Gender				0.975
Male	17 (41.5)	7 (41.2)	10 (58.8)	
Female	24 (58.5)	10 (41.7)	14 (58.3)	
Age (years)				0.004
<10	23 (56.1)	5 (21.7)	18 (78.3)	
10−13	18 (43.9)	12 (66.7)	6 (33.3)	
Thyroidectomy				0.011
Lobectomy	17 (41.5)	11 (64.7)	6 (35.3)	
Total	24 (58.5)	6 (25.0)	18 (75.0)	
CND				0.141
Yes	36 (87.8)	13 (36.1)	23 (63.9)	
No	5 (12.2)	4 (80.0)	1 (20.0)	
MRND				0.005
Yes	27 (65.9)	7 (25.9)	20 (74.1)	
No	14 (34.1)	10 (71.4)	4 (28.6)	
Tumor size (cm)				0.045
<2	2 (4.9)	2 (100.0)	0 (0.0)	
2−4	22 (53.7)	11 (50.0)	11 (50.0)	
>4	17 (41.5)	4 (23.5)	13 (76.5)	
Multifocality				0.009
Yes	22 (53.7)	5 (22.7)	17 (77.3)	
No	19(46.3)	12(63.2)	7(36.8)	
Capsular invasion				0.029
Yes	30 (73.2)	9 (30.0)	21 (70.0)	
No	11 (26.8)	8 (72.7)	3 (27.3)	
Extrathyroidal invasion				0.000
Yes	24 (58.5)	4 (16.7)	20 (83.3)	
No	17 (41.5)	13 (76.5)	4 (23.5)	
AJCC tumor stage				0.001
T1	3 (7.3)	3 (100.0)	0 (0.0)	
T2	11 (26.8)	7 (63.6)	4 (36.4)	
T3	4 (9.8)	3 (75.0)	1 (25.0)	
T4	23 (56.1)	4 (17.4)	19 (82.6)	
AJCC nodal stage (loco-regional metastases)				0.028
N0	8 (19.5)	6 (75.0)	2 (25.0)	
N1a	9 (22.0)	5 (55.6)	4 (44.4)	
N1b	24 (58.5)	6 (25.0)	18 (75.0)	
AJCC distant metastases				0.679
M0	34 (82.9)	15 (44.1)	19 (55.9)	
M1	7 (17.1)	2 (28.6)	5 (71.4)	
ATA pediatric PTC stage (2015)				0.007
Low risk	9 (22.0)	7 (77.8)	2 (22.2)	
Intermediate risk	6 (14.6)	4 (66.7)	2 (33.3)	
High risk	26 (63.4)	6 (23.1)	20 (76.9)	
Recurrence				0.382
Yes	5 (10.4)	1 (20.0)	4 (80.0)	
No	36 (89.6)	16 (44.4)	20 (55.6)	
AMES				0.000
Low risk	16 (39.0)	13 (81.3)	3 (18.7)	
High risk	25 (61.0)	4 (16.0)	21 (84.0)	

a) AJCC, American Joint Committee on Cancer 7th Edition (2010). ATA pediatric PTC stage, Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer developed by The American Thyroid Association Guidelines Task Force on Pediatric Thyroid Cancer 1st Edition (2015).

Table 3 Relationship between *BRAF* and clinicopathological characteristics of papillary thyroid carcinoma in children under the age of 10[a)]

Characteristics	No. (%)	*BRAF* positive, No. (%)	*BRAF* negative, No. (%)	*P* value
Gender				1.000
Male	9 (34.6)	2 (22.2)	7 (77.8)	
Female	17 (65.4)	3 (17.6)	14 (82.4)	
Thyroidectomy				0.004
Lobectomy	10 (38.5)	5 (50.0)	5 (50.0)	
Total	16 (61.5)	0 (0.0)	16 (100.0)	
CND				0.488
Yes	23 (88.5)	4 (17.4)	19 (82.6)	
No	3 (11.5)	1 (33.3)	2 (66.7)	
MRND				0.010
Yes	19 (73.1)	1 (5.3)	18 (94.7)	
No	7 (26.9)	4 (57.1)	3 (42.9)	
Tumor size (cm)				0.121
<2	0 (0.0)	0 (0.0)	0 (0.0)	
2−4	16 (61.5)	5 (31.2)	11 (68.8)	
>4	10 (38.5)	0 (0.0)	10 (100.0)	
Multifocality				0.007
Yes	15 (57.7)	0 (0.0)	15 (100.0)	
No	11 (42.3)	5 (45.5)	6 (54.5)	
Capsular invasion				0.101
Yes	19 (73.1)	2 (10.5)	17 (89.5)	
No	7 (26.9)	3 (42.9)	4 (57.1)	
Extrathyroidal invasion				0.034
Yes	17 (65.4)	1 (5.9)	16 (94.1)	
No	9 (34.6)	4 (44.4)	5 (55.6)	
AJCC tumor stage				0.012
T1	2 (7.7)	2 (100.0)	0 (0.0)	
T2	7 (26.9)	2 (28.6)	5 (71.4)	
T3	1 (3.8)	0 (0.0)	1 (100.0)	
T4	16 (61.5)	1 (6.3)	15 (93.7)	
AJCC nodal stage (loco-regional metastases)				0.007
N0	3 (11.5)	2 (66.7)	1 (33.3)	
N1a	8 (30.8)	3 (37.5)	5 (62.5)	
N1b	15 (57.7)	0 (0.0)	15 (100.0)	
AJCC distant metastases				1.000
M0	20 (76.9)	4 (20.0)	16 (80.0)	
M1	6 (23.1)	1 (16.7)	5 (83.3)	
ATA pediatric PTC stage (2015)				0.009
Low risk	4 (15.4)	3 (75.0)	1 (25.0)	
Intermediate risk	5 (19.2)	1 (20.0)	4 (80.0)	
High risk	17 (65.4)	1 (5.9)	16 (94.1)	
Recurrence				1.000
Yes	4 (15.4)	1 (25.0)	3 (75.0)	
No	22 (84.6)	4 (18.2)	18 (81.8)	
AMES				0.034
Low risk	9 (34.6)	4 (44.4)	5 (55.6)	
High risk	17 (65.4)	1 (5.9)	16 (94.1)	

a) AJCC, American Joint Committee on Cancer 7th Edition (2010). ATA pediatric PTC stage, Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer developed by The American Thyroid Association Guidelines Task Force on Pediatric Thyroid Cancer 1st Edition (2015).

showed the *BRAF*^{V600E} mutation to be present in 35.4% (17) of the 48 analyzed patients, and 41.5% (17/41) of the analyzed patients with classical pediatric PTC. These estimates are significantly higher than that of the early literature, but lower than that reported by the more recent studies. The young age of the analyzed patients may be one of the main factors underlying the observed low proportion of *BRAF*^{V600E} mutations in the present study. This is supported by the results of a recent report by the ATA, which considered the mutation to be virtually absent from very young patients (Francis et al., 2015). In fact in the present study, the *BRAF*^{V600E} mutation was present in only five of the 26 (19.2%) pediatric patients with PTC aged <10 years, but in 12 of the 22 (54.5%) pediatric patients with PTC aged 10–13 years (P=0.011).

In adults, extensive research has shown the *BRAF*^{V600E} mutation to be associated with PTC lymphatic metastasis, extra-thyroidal extension, male patient gender, disease multifocality, and advanced tumor stage and size (Li et al., 2012). Furthermore, two very recent studies determined the *BRAF*^{V600E} mutation to be present in 36.8% (n=19) and 63.0% (n=27) of patients with pediatric PTC, respectively (Henke et al., 2014; Daniel et al., 2014). Consistent with these reported estimates, the overall incidence of the *BRAF*^{V600E} mutation among the 48 pediatric patients with PTC in the present study was found to be 35.4%.

A previous study by Henke et al. showed that the presence of the *BRAF*^{V600E} mutation did not correlate with the PTC stage at diagnosis, nor with tumor size, capsular invasion, vascular invasion, soft tissue invasion, and/or margin status (Henke et al., 2014). In contrast, another previous study by Daniel et al. suggested the mutation to be significantly associated with a lower rate of metastases, age at diagnosis, and size of the tumor score and invasion, and with a higher rate of successful resection (P=0.087). Neither the presence of lymphatic/pulmonary metastases, tumor size, patient age, nor extra-thyroidal extension was found to be associated with the presence of the *BRAF*^{V600E} mutation (Daniel et al., 2014). For all 48 pediatric patients with PTC in the present study, the presence of the *BRAF*^{V600E} mutation was significantly associated with both a low AJCC tumor stage (P=0.014) and low AMES score (P=0.001), while conversely, it was negatively associated with a patient age of less than ten years (P=0.011), the performance of a total thyroidectomy (P=0.030) or MRND (P=0.044), and tumor multifocality (P=0.020) and/or extra-thyroidal invasion (P=0.003). Although Henke et al. previously found the presence of the *BRAF*^{V600E} mutation in pediatric PTC to be associated with male patient gender (Henke et al., 2014), this was not supported by the results of the present study, which found no association between the presence of the mutation and either patient gender.

As discussed, previous research data estimated the incidence of thyroid neoplasm in children aged 10–14 years to be 0.44 cases per 100,000 persons between 1973 and 2004 (Ward et al., 2014), but only 0.09 and 0.01 cases per 100,000 persons among children aged 5–9 and <5 years, respectively (Hogan et al., 2009). Given this discrepancy, we selected 10 years of age as the point of division for patient grouping in the present study

Of the 41 analyzed pediatric patients diagnosed with classical PTC in the present study (Table 2), 41.5% were identified to harbor the *BRAF*^{V600E} mutation. The presence of the *BRAF*^{V600E} mutation in these patients was found to be significantly associated with both a small tumor size (P=0.045), and a low AJCC nodal (P=0.028) and ATA pediatric PTC (P=0.007) stage; conversely, it was negatively associated with PTC capsular invasion (P=0.029).

For pediatric patients with PTC aged 10–13 years (Table 4), the presence of the *BRAF*^{V600E} mutation was not found to be significantly associated with any of the analyzed patient variables, but was negatively associated with tumor multifocality (P=0.007). In contrast, the presence of the mutation in pediatric patients with PTC aged less than 10 years (Table 3) was associated with a low AJCC tumor (P=0.012) and nodal (P=0.007) stage, a low ATA pediatric PTC stage (P=0.009), and a low AMES score (P=0.034). Conversely, it was demonstrated to be negatively associated with the performance of a total thyroidectomy (P=0.004) or MRND (P=0.010), and tumor multifocality (P=0.007) and/or extra-thyroidal invasion (P=0.034).

It is likely that the observed differences in the prevalence of the *BRAF*^{V600E} mutation between children aged less than 10, and those aged 10–13 years in the present study reflects inherent differences in the clinical features of PTC in these two age groups.

MATERIALS AND METHODS

Patients and clinicopathological data

The present study assessed a retrospective cohort of 48 consecutive pediatric patients with PTC, comprising 29 females and 19 males aged 3.6–13.8 years, who were treated and clinically observed from 1994 to 2014 at the Beijing Children's Hospital. None of the patients had any prior history of radiation exposure, (note that one patient with Hodgkin lymphoma was excluded from the study because they had a prior history of external radiation exposure). Patient clinical data including gender, age, tumor size, histological subtype, multifocality, extra-thyroidal extension, soft-tissue invasion, lymphovascular invasion, loco-regional metastases, distant metastases, treatment, and long-term surveillance were obtained both from institutional medical records and by direct communication with patients and/or their parents. Each patient was independently diagnosed by two pathologists according to the American Joint Committee on Cancer (AJCC) Staging Manual (7th Edition, 2010), and the Management Guidelines

Table 4 Relationship between *BRAF* and clinicopathological characteristics of papillary thyroid carcinoma in children over the age of 10[a)]

Characteristics	No. (%)	*BRAF* positive, No. (%)	*BRAF* negative, No. (%)	*P* value
Gender				1.000
Male	10 (45.5)	5 (50.0)	5 (50.0)	
Female	12 (54.5)	7 (58.3)	5 (41.7)	
Thyroidectomy				0.691
Lobectomy	10 (45.5)	6 (60.0)	4 (40.0)	
Total	12 (54.5)	6 (50.0)	6 (50.0)	
CND				1.000
Yes	16 (72.7)	9 (56.3)	7 (43.7)	
No	6 (27.3)	3 (50.0)	3 (50.0)	
MRND				1.000
Yes	11 (50.0)	6 (54.5)	5 (45.5)	
No	11 (50.0)	6 (54.5)	5 (45.5)	
Tumor size (cm)				0.668
<2	2 (9.1)	2 (100.0)	0 (0.0)	
2−4	12 (54.5)	6 (50.0)	6 (50.0)	
>4	8 (36.4)	4 (50.0)	4 (50.0)	
Multifocality				0.007
Yes	10 (45.5)	5 (50.0)	5 (50.0)	
No	12 (54.5)	7 (58.3)	5 (41.7)	
Capsular invasion				0.675
Yes	14 (63.6)	7 (50.0)	7 (50.0)	
No	8 (36.4)	5 (62.5)	3 (37.5)	
Extrathyroidal invasion				0.378
Yes	8 (36.4)	3 (37.5)	5 (62.5)	
No	14 (63.6)	9 (64.3)	5 (35.7)	
AJCC tumor stage				1.000
T1	1 (4.6)	1 (100.0)	0 (0.0)	
T2	9 (40.9)	5 (55.6)	4 (44.4)	
T3	5 (22.7)	3 (60.0)	2 (40.0)	
T4	7 (31.8)	3 (42.9)	4 (57.1)	
AJCC nodal stage (loco-regional metastases)				0.853
N0	9 (40.9)	4 (44.4)	5 (55.6)	
N1a	3 (13.6)	2 (66.7)	1 (33.3)	
N1b	10 (45.5)	6 (60.0)	4 (40.0)	
AJCC distant metastases				1.000
M0	21 (95.4)	11 (52.3)	10 (47.6)	
M1	1 (4.6)	1 (100.0)	0 (0.0)	
ATA pediatric PTC stage (2015)				1.000
Low risk	7 (31.8)	4 (57.1)	3 (42.9)	
Intermediate risk	5 (22.7)	3 (60.0)	2 (40.0)	
High risk	10 (45.5)	5 (50.0)	5 (50.0)	
Recurrence				0.455
Yes	1 (4.6)	0 (0.0)	1 (100.0)	
No	21 (95.4)	12 (57.1)	9 (42.9)	
AMES				0.192
Low risk	13 (59.1)	9 (69.2)	4 (30.8)	
High risk	9 (40.9)	3 (33.3)	6 (66.7)	

a) AJCC, American Joint Committee on Cancer 7th Edition (2010). ATA pediatric PTC stage, Management Guidelines for Children with Thyroid Nodules and Differentiated Thyroid Cancer developed by The American Thyroid Association Guidelines Task Force on Pediatric Thyroid Cancer 1st Edition (2015).

for Children with Thyroid Nodules and Differentiated Thyroid Cancer (2015).

Laser microdissection

Several (n=3–6) 10-μm thick serial sections were cut from each formalin-fixed, paraffin-embedded (FFPE) PTC sample and prepared for laser microdissection. A 4-μm thick adjacent "reference" section was also cut, and then stained with hematoxylin and eosin (HE). The PTC samples for microdissection were next deparaffinized using xylene and stained with toluidine blue (1% cresyl violet). A pathologist visually delineated microscopic PTC target regions prior to automatic dissection of each area by an Arcturus laser microdissection system (Arcturus XTLCM instrument; Life Technologies, Carlsbad, CA, USA) (Leica AS LMD; Wetzlar, Germany), used according to the manufacturer's instructions. Micro-dissected tissues were individually collected, and used for subsequent analyses.

DNA extraction

Several (n=3–6) 10-μm thick serial sections were cut from each FFPE PTC tissue block for DNA extraction. The sections were dewaxed, HE stained, and covered with coverslips, before areas containing representative tumor/benign nodules were identified, dissected, and placed in 1.5-mL tubes. DNA was extracted from each sample using the QIAamp DNA FFPE tissue kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions.

Genotyping

BRAF exon 15 was amplified using a *BRAF* mutation testing kit (ADx-ARMS; Amoy Diagnostics, Xiamen, China) containing Taq DNA polymerase, oligonucleotide primers, hydrolysis oligonucleotide probes, nucleotides, and buffers. Briefly, 5 μL of extracted DNA was mixed with 0.4 μL of Taq DNA polymerase, and appropriate amounts of the other kit reagents (totaling 35 μL). qRT-PCR was performed using the ABI 7500 PCR system (Applied Biosystems, Foster City, CA, USA) to achieve cycling conditions comprising 15 min initial denaturation at 95°C, followed by 35 cycles of 30 s at 94°C (denaturation) and 20 s at 64°C (annealing), and 10 min at 72°C (final extension).

BRAF amplicons were purified using the Wizard SV Gel and PCR Clean-up System, and sequenced using a Big Dye Terminator v3.1 kit (Applied Biosystems). Following DNA sequence analysis, specimens were incubated for 30 min with 5 μL of 5 mol L^{-1} NaAC and 50 μL of absolute ethanol, and then centrifuged (14,000×g, 30 min). The resulting pellets were washed with 70% ice-cold ethanol, dried, and diluted with 20 μL deionized formamide before being processed by the ABI 3130 DNA sequence analyzer.

Patient treatment and surveillance

Total thyroidectomy was performed for patients with PTC and concomitant benign thyroid disease, multifocal disease, lymphovascular invasion, extra-thyroidal invasion, loco-regional metastases, distant metastases, and/or a tumor size >1 cm, based on preoperative observations. A therapeutic cervical lymphadenectomy was performed for patients with clinical evidence of central and/or lateral neck metastases, based on preoperative staging and/or intraoperative observations. A prophylactic ipsilateral central neck dissection (CND) was performed on patients determined to have N0 or NX PTC. All surgical operations were performed by experienced surgeons, and all patients received postoperative thyroid-stimulating hormone (TSH) suppression therapy with levothyroxine (LT4). Radioactive iodine (RAI) ablation with iodine-131 (131I) was selectively performed for patients with a tumor size >2 cm, extra-thyroidal invasion, loco-regional metastases, and/or distant metastases. Long-term patient surveillance of patients with no evidence of disease comprised cervical ultrasonography, and an analysis of serum thyroglobulin (Tg), thyroglobulin antibody, and thyroid stimulating hormone (TSH) levels. Patients with suspected disease recurrence were assessed via magnetic resonance imaging (MRI) and/or contrast-enhanced computed tomography (CT), and diagnostic whole-body scanning.

Statistical analysis

A chi-squared analysis was used to assess whether the presence of the $BRAF^{V600E}$ mutation correlated with dichotomous patient data (comprising gender, histological subtype, thyroidectomy, multifocality, extra-thyroidal invasion, lymphovascular invasion, loco-regional metastasis, distant metastasis, ATA pediatric tumor stage, MACIS score, age-metastases-extent-size (AMES) score, and disease recurrence). Conversely, a Student's t-test was used to assess whether the presence of the $BRAF^{V600E}$ mutation correlated with continuous variables (such as patient age and tumor size), and a Fisher's exact test was used to assess its association with categorical data. Statistical analyses were performed using SPSS for Windows version 21 software (SPSS, Inc., Chicago, IL, USA). A P value <0.05 was considered to indicate statistical significance.

Compliance and ethics *The author(s) declare that they have no conflict of interest. This clinical study was approved by the Institutional Review Board of the Beijing Children's Hospital, and all procedures involving human participants were conducted in strict accordance with both hospital ethical standards, and the 1975 Helsinki declaration (as revised in 2008, including amendments). Informed parental consent was obtained for all patients prior to participation in the study.*

Acknowledgements *This work was supported in part by Beijing Municipal Administration of Hospitals Clinical Medicine Development of Special*

Funding Support (ZYLX201508), Beijing Municipal Science and Technology Project (D131100005313014), and Beijing Health System Top Level Health Technical Personnel Training Plan (20153079).

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