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A different prognostic value of *BRAF*^{V600E} mutation positivity in various age groups of patients with papillary thyroid cancer

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The aim of the retrospective single-center study was to assess the prognostic value of *BRAF*^{V600E} mutation positivity (*BRAF*$^{V600E+}$) on disease persistence/recurrence in patients with papillary thyroid cancer (PTC). A total of 199 patients having had initial surgery with neck dissection in our hospital between 6/2009-6/2012 were included in the cohort. Excluded were patients with unifocal microcarcinoma ≤1cm. *BRAF*^{V600E} mutation was tested from formalin-fixed paraffin-embedded surgicaly removed tumors. All included patients were postoperatively treated with radioiodine. The median duration of follow-up was 43 months, quartiles range 30 – 58 months. Variables included in the final model: *BRAF*$^{V600E+}$, categorised age, sex, and high-risk status, or alternatively lymph node status. Based on differences in persistence/recurrence rates, patients were divided into three age categories (<35, 35-60, ≥60). Multiple regression analysis showed a significant interaction between *BRAF*$^{V600E+}$ and age, modifying the effect of *BRAF*$^{V600E+}$ on persistence/recurrence. *BRAF*$^{V600E+}$ in low-risk patients of any age and in high-risk middle-aged patients did not confer additional hazard compared with *BRAF*^{V600E} mutation negative (*BRAF*$^{V600E-}$) low-risk and *BRAF*$^{V600E-}$ high-risk patients, respectively. However, younger (<35 years) and older (≥60 years) high-risk *BRAF*$^{V600E+}$ patients had 17.28 and 33.49-fold increased hazard of persistence/recurrence, respectively, compared with low-risk *BRAF*$^{V600E-}$ patients. The alternative model including lymph node status yielded similar results for the prognostic significance of *BRAF*$^{V600E+}$ in younger and older patients. In conclusion, the prognostic value of *BRAF*$^{V600E+}$ depends on high-risk status and likely on age-associated factors. Such additional knowledge could change clinical decision-making in treatment modality.

Key words: papillary thyroid cancer, BRAF mutation, neck dissection

Papillary carcinomas, the most common form of thyroid cancers, frequently have genetic alterations leading to the activation of the mitogen-activated protein kinase signaling pathway (1). *BRAF* mutation is the most frequent genetic alteration in thyroid cancer, occuring in around 45% of sporadic papillary thyroid carcinoma (PTC), particularly in the relatively aggressive subtypes, such as the tall-cell PTC, but it does not occur in folicullar, medular thyroid cancers or benign thyroid tumors (2). The most significant hot-spot mutation of the *BRAF* gene is a thymine-to-adenine transversion at nucleotide 1799 in exone 15, resulting in a valine-to-glutamate substitution at residue 600 (V600E) (3).

Many studies have found *BRAF*^{V600E} mutation positivity to be associated with clinicopathological characteristics of PTC that are conventionally known as predictors of tumor progression and recurrence (4-11). This association was also reported for low-risk patients (12, 13).

However, the conclusion for such associations is not unequivocal since several studies have reported that the *BRAF* mutation has no significant association with any of the common high-risk pathological characteristics (14-19).

Personalised therapy is of utmost interest, especially in the treatment of low-risk PTC patients (20). Our primary objective was therefore to explore in detail all known and hypothesized factors that might determine the prognostic value of *BRAF*^{V600E} mutation positivity on disease persistence/recurrence in a single-center retrospective cohort study. More specifically, we were focused on usefulness of *BRAF*^{V600E} mutation testing

in clinical management of low-risk and high-risk patients with PTC after initial surgery with neck dissection which allowed for a more precise determination of nodal status.

Patients and methods

We retrospectively analysed a cohort of 199 PTC patients surgicaly treated in our hospital between 6/2009 and 6/2012. Tumors were staged according to the 7th edition of the TNM-based staging system, recommended by the American Join Committee on Cancer (AJCC) (21). Patients with T1a-multifocal, T1b-T2, N0M0 were classified into a low-risk group and those with T3-T4 or any T, N1 or any M1 or with more aggressive variant of PTC into a high-risk category. Very low-risk patients (unifocal T1 ≤ 1 cm, N0M0) (22) were excluded from the study.

Routine surgical procedure was total thyroidectomy (TTE) with prophylactic or therapeutic central neck lymph node dissection (level VI). In patients with a metastatic lymphadenopathy in lateral neck compartment (level II-V) was central neck dissection combined with therapeutic lateral neck dissection.

All included patients received radioiodine (131I) after initial surgery to ablate the postsurgical remnant. Diagnostic scan with 131I was repeated in all patients six months after the initial ablative 131I dose. In patients with detectable stimulated (endogenously or exogenously by rhTSH) serum thyroglobulin (TG) ≥2 ng/ml or with significant uptake within the thyroid bed (> 1%/24 hours), one more treatment with 131I was given to complete the ablation.

Criteria for identifying persistent/recurrent disease were: presence of pathologic 131I uptake outside thyroid bed on SPECT/CT, and/or presence of pathologic 18-fluorodeoxyglucose (18FDG) uptake on PET/CT, and/or presence of pathologic lesions on neck ultrasound/MRI/CT in patients with elevated level of basal or stimulated (endogenously or exogenously by rhTSH) serum TG ≥2 ng/ml and/or with increasing level of circulating anti-thyroglobulin antibodies (ATG) after initial ablation 131I therapy.

Patients were considered as disease-free when neck ultrasound and diagnostic 131I scan were negative, TSH-stimulated serum TG levels were less than 2 ng/ml, and circulating ATG were undetectable or declining during follow-up. The median duration of follow-up was 43 months, quartiles range from 30 to 58 months.

The study was approved by the institutional review board, with written informed patient consent obtained as per legal requirements.

***BRAF*^{V600E} mutation analysis.** Tumour-rich areas were marked by the pathologist. Genomic DNA form 10 μm thickness was extracted using the RecoverAll ™ Total Nucleic Acid Isolation kit (Ambion). The PCR reaction contained 0.25μM each primer, 12.5 μl Maxima PCR Master Mix (Thermo Scientific) and 100 ng DNA in a final reaction volume of 25 μl. PCR products were purified by Exonuclease I and FastAP (Thermo Scientific) and subsequently analysed in both directions using SNaPshot® Multiplex Kit (Applied Biosystems). We evaluated 4 SNP-specific oligonucleotide primers designed with the ABI PRISM® SNaPshot® Multiplex Kit (Applied Biosystems) and used a systematic assessment protocol. The results were analysed with GeneMapper 3.0 software (Applied Biosystems).

Statistical analysis. All clinically important variables were tested for association with disease persistence/recurrence as well as with *BRAF*$^{V600E+}$ using bivariable tests. Kaplan–Meier analysis was performed to estimate the disease-free survival probability for *BRAF*^{V600E} mutation positive (*BRAF*$^{V600E+}$) *versus* negative (*BRAF*$^{V600E-}$) patient groups as well as for other important patients' characteristics.

Multivariable survival analyses were conducted with Cox proportional hazards method. The explanatory variable in interest (*BRAF*^{V600E} status) and other characteristics which were assumed to influence the disease-free survival were considered for the final model. The male, high-risk and *BRAF*$^{V600E+}$ categories were coded 1 (worse prognosis), and age was transformed into dummy variables. Effect sizes for significant or clinically important explanatory variables and interaction effects were estimated using hazard ratio (HR). The estimates are presented with corresponding 95% confidence intervals (95% CI).

Statistical analyses were performed using Statistica 11 (StatSoft, Inc.-now a part of Dell, USA) and StatsDirect 2.8.0 software (StatsDirect Ltd., UK). Two-sided P-values are reported.

Results

Basic characteristics of the study cohort. The study included a total of 199 PTC patients with the median age at diagnosis of 51 years (range 17-77 years), who underwent TTE with prophylactic – in 125 (62.8%) or therapeutic central neck dissection – in 13 (6.5%) patients. Sixty one patients (30.7%) underwent also therapeutic lateral neck dissection – ipsilateral in 50 out of 61 (82.0%), and bilateral in 11 (18.0%) patients. Patients' characteristics are described in Table 1.

In 40 (85%) out of 47 patients was PTC persistence/recurrence identified on the basis of elevated level of stimulated TG in combination with imaging methods, more specifically: TG ≥ 2 ng/ml + 131I-SPECT/CT in 30 patients, Tg ≥ 2 ng/ml + 18FDG-PET/CT in six patients, Tg ≥ 2 ng/ml + CT/MRI and/or neck ultrasound in four patients. The remaining seven (15%) patients were detected by 131I-SPECT/CT even if they had non-elevated level of serum TG, however, four patients out of them had elevated and/or rising levels of serum ATG antibodies.

To the date of completion of this study (01.05.2015), out of 47 patients with persistent/recurrent disease, 28 (60%) patients were in remission, 10 (21%) patients were in stable disease, and three (6%) patients were in progression. Six (13%) patients have died, five (11%) of them died of thyroid

Table 1. Clinicopathologic features for PTC patients with (+) and without (-) presence of *BRAF*^{V600E} mutation

Patients' characteristics		Total	*BRAF*$^{V600E+}$	*BRAF*$^{V600E-}$	Odds Ratio (95%CI)	P value
Total		199 (100%)	103 (100%)	96 (100%)	n.a.	0.670
Male sex		48 (24.1%)	26 (54.2%)	22 (45.8%)	1.14 (0.56 to 2.30)	0.742
Age (years)	**< 35**	43 (21.6%)	17 (16.5%)	26 (27.1%)	n.a.	0.169
	<35-60)	103 (51.8%)	57 (55.3%)	46 (47.9%)		
	≥ 60	53 (26.6%)	29 (28.2%)	24 (25.0%)		
Histology	**Conventional variant**	140 (70.4%)	82 (79.6%)	58 (60.4%)	n.a.	0.011
	Follicular variant	39 (19.6%)	13 (12.6%)	26 (27.1%)		
	More agressive variant	20 (10.0%)	8 (7.8%)	12 (12.5%)		
Multifocality		68 (34.2%)	33 (32.0%)	35 (36.5%)	0.82 (0.45 to 1.48)	0.551
Extrathyroidal invasion		51 (25.6%)	23 (22.3%)	28 (29.2%)	0.70 (0.37 to 1.33)	0.329
High-risk patients		145 (72.9%)	71 (68.9%)	74 (77.1%)	0.66 (0.35 to 1.25)	0.206
Lymph node metastases		125 (62.8%)	58 (56.3%)	67 (69.8%)	0.56 (0.31 to 1)	0.057
Distant metastases		16 (8.0%)	11 (10.7%)	5 (5.2%)	2.18 (0.66 to 8.28)	0.196
Persistent/recurrent		47 (23.6%)	20 (19.4%)	27 (28.1%)	0.62 (0.32 to 1.19)	0.182

carcinoma, showed recurrence to distant organs and were 131I refractory.

The *BRAF*^{V600E} mutation prevalence within our groups of PTC patients was 103 out of 199 patients; 51.8% (the 95% CI between 44.6% and 58.9), which lies at the slightly higher values, nevertheless, the 95% CI covers the value of mean prevalence of *BRAF*^{V600E} mutation found in the metaanalysis by Xing et al. (5).

BRAF^{V600E} mutation positivity was detected in 45 (60.8%) out of 74 patients without lymph node (LN) metastases (N0), further, in 35 (54.7%) out of 64 patients with LN metastases in the central compartment (N1a) only (N1a prophylactic in 30 (58.8%) out of 51 patients and N1a therapeutic 5 (38.5%) out of 13 patients), and in 23 (37.7%) of 61 patients with LN metastases in the lateral compartment (N1b).

Bivariable analysis. The overall persistence/recurrence in our cohort was 23.6% (47/199; the exact 95% CI, 17.9%-30.1%). Unadjusted persistence/recurrence in *BRAF*$^{V600E+}$ vs *BRAF*$^{V600E-}$ patients was 19.4% (20/103; exact 95% CI, 12.3%-28.4%) vs 28.1% (27/96; the exact 95% CI, 19.4%-38.2%) (P = 0.138).

A graphical inspection of the distribution of persistence/recurrence events (not shown, but can be seen in Figure 1) revealed a non-linear age dependency of data with a lower persistence/recurrence rate for middle-aged patients in comparison with younger and older patients. This finding suggested that the persistence/recurrence rates might not be gradually increasing over the whole range of age from 17 to 77 years in our study cohort, which had to be taken into account in further data analyses. Therefore, the patients were finally categorised into the three groups: <35 (n=43), 35-60 (n=103), and ≥60 years old (n=53) with boundaries representing roughly the 1st and 3rd quartiles reported in the metaanalysis by Xing et al.(5). The persistence/recurrence rates in PTC patients stratified by their high-risk or LN status, and grouped by age and *BRAF*^{V600E} mutation at the time of initial surgery are shown in Table 2 (for the outcome stratified by high-risk status) and Table 3 (for the outcome stratified by LN status). We found a higher proportion of clinically evident LN metastases at the time of initial surgery in the group of younger patients (33 out of 43, i.e. 76.7%) in comparison to the middle-aged (64 out of 103, 62.1%) and the older patients (28 out of 53, 52.8%). On the other hand, a higher proportion of more agressive variants was found in the older patients (9 out of 53, i.e. 17.0%), in comparison to the middle-aged (10 out of 103, 9.7%) and younger patients (1 out of 43, 2.3%).

In order to study the influence of each single characteristic on disease-free survival we performed a Kaplan-Meier analysis. Visual inspection of differences in unadjusted curves among age categories confirmed the assumption that the hazard of persistence/recurrence might not increase with age

Table 2. Persistence/recurrence in PTC patients stratified by their high-risk status, grouped by age and *BRAF*^{V600E} mutation at the time of initial surgery

Age category (years)			Persistence/Recurrence		No persistence/Recurrence	
			High risk n=46	**Low risk n=1**	**High risk n=99**	**Low risk n=53**
<35	**n=43**	*BRAF*$^{V600E+}$	6/13 (46.2%)	0/0 (0.0%)	8/21 (38.1%)	3/9 (33.3%)
<35-60)	**n=103**	*BRAF*$^{V600E+}$	5/20 (25.0%)	0/1 (0.0%)	32/54 (59.3%)	20/28 (71.4%)
≥60	**n=53**	*BRAF*$^{V600E+}$	9/13 (69.2%)	0/0 (0.0%)	11/24 (45.8%)	9/16 (56.3%)

Table 3. Persistence/recurrence in PTC patients stratified by their LN status, grouped by age and *BRAF*^{V600E} mutation at the time of initial surgery

Age category (years)			Persistence/Recurrence		No persistence/Recurrence	
			LN metastases	No LN metastases	LN metastases	No LN metastases
			n=43	n=4	n=82	n=70
<35	n=43	*BRAF*$^{V600E+}$	6/13 (46.2%)	0/0 (0.0%)	7/20 (35.0%)	4/10 (40.0%)
<35-60)	n=103	*BRAF*$^{V600E+}$	3/18 (16.7%)	2/3 (66.7%)	27/46 (58.7%)	25/36 (69.4%)
≥60	n=53	*BRAF*$^{V600E+}$	8/12 (69.2%)	1/1 (100.0%)	7/16 (43.8%)	13/24 (54.2%)

(Figure 1, upper left). Although differences across the whole time course were nonsignificant (P = 0.137), a comparison of the curves at fixed time points for early times (between 8-16 months) yielded significant differences in disease-free survival rates.

Consistently with the results from published studies, PTC-persistence/recurrence was significantly associated with sex (P << 0.001), tumor size (P << 0.001; Figure 1, lower left) and LN status (presence of LN metastases) (P << 0.001; Figure 1, lower right). Also survivor curves by extrathyroidal invasion (P << 0.001), distant metastasis status (P << 0.001), and high-risk status (P << 0.001; Figure 1, upper right) were found to be significantly different by their respective categories. However, extrathyroidal invasion was not independently associated with PTC-persistence/recurrence events when added to the Cox model of disease-free survival that included high-risk status.

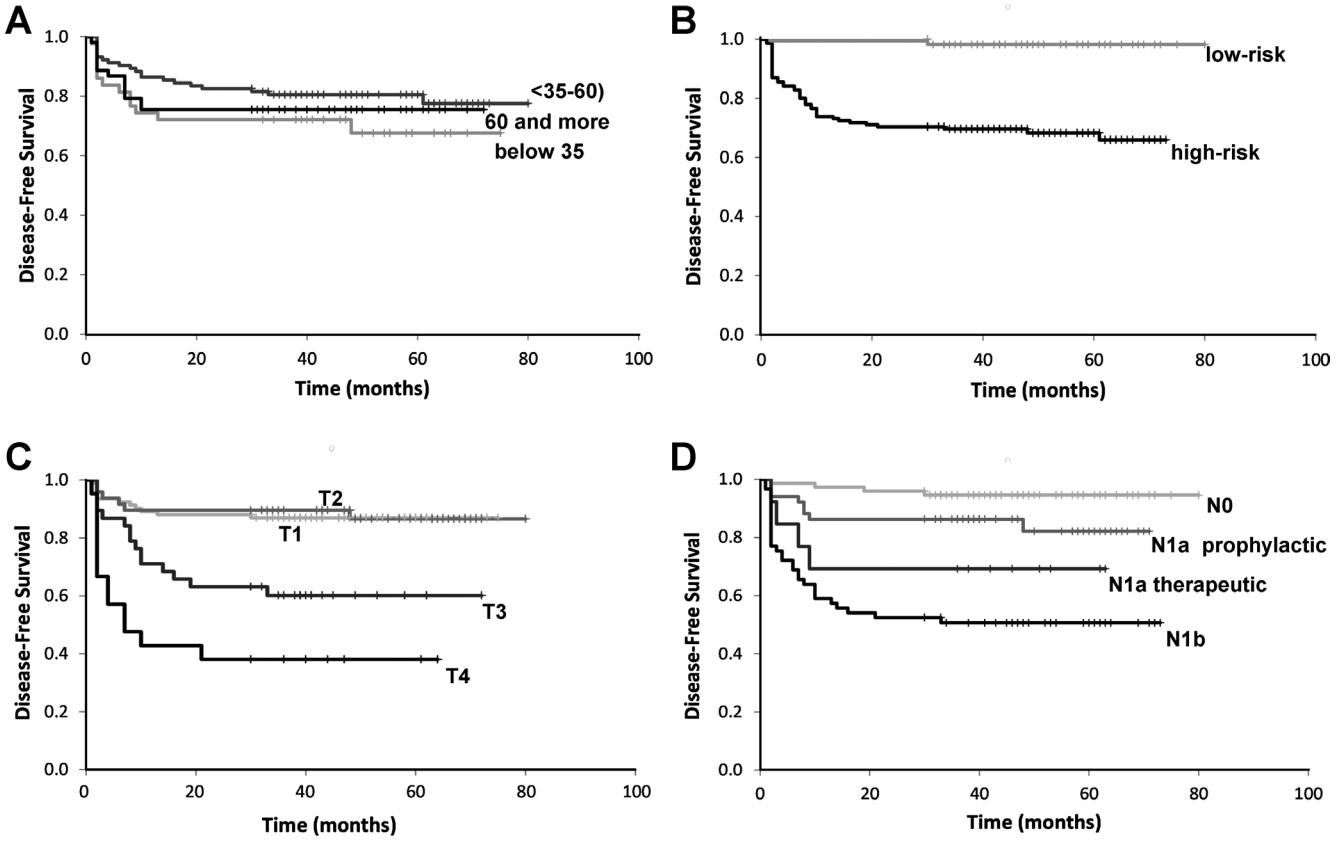

Figure 1. Comparison of Kaplan-Meier disesase recurrence-free survival curves by age categories (upper left), by high-risk status (upper right), by tumor staging (lower left) and by nodal status (lower right).
Testing for curve differences among age categories a) overall: log-rank P = 0.357, b) age category below 35 vs <35-60): generalised Wilcoxon (Gehan-Breslow) P = 0.137.
Testing for curve differences among high-risk status categories: log-rank P << 0.001.
Testing for curve differences among tumor stage categories: overall log-rank P << 0.001.
Testing for curve differences among nodal status categories: overall log-rank P << 0.001.

A plausible explanation for this lies in a significant association of extrathyroidal invasion with LN status (P = 0.002) and with tumor size (P < 0.001). In addition, distant metastases status was also not included in the multivariable model since it was rather an intervening variable preceding the outcome than an explanatory variable.

In our sample of 199 patients, there were not clear differences among survivor curves of $BRAF^{V600E}$ status (P = 0.180), multifocality (P = 0.602) and histological type (P = 0.666) in the whole cohort of PTC patients.

Multivariable analysis. In order to identify the prognostic potential of variables identified by bivariable analysis, we performed Cox regression analyses. The fitted final model (Table 4) was composed of four explanatory variables ($BRAF^{V600E}$ mutation and high-risk status, adjusted to age and sex) and an interaction term (*BRAF**Age) reflecting that effect of the primary „exposure" ($BRAF^{V600E}$ mutation) on the outcome (i.e., persistence/recurrence) might differ depending on the level of a third variable (age category). In fact, we have confirmed the presence of interaction between $BRAF^{V600E}$ mutation status and age categories in their effects on disease-free-survival outcome, which implies that the association of $BRAF^{V600E+}$ with persistence/recurrence was dependent on patient's age. No other interaction effects or significant higher order terms were found.

The patient disease-free survival was clearly dependent on sex and high-risk status. As anticipated, the risk of persistence/recurrence was increased 14.5-fold in high-risk patients compared with the reference group of low-risk patients. The interaction pattern, such that younger and older PTC patients had a different relationship between $BRAF^{V600E}$ mutation status and outcome than middle-aged patients, is also apparent in Figure 2. Based on values of regression coefficients from the Cox model (Table 4) the combined effects of the predictors on the outcome can be calculated. For patients aged <35 and ≥60, in the same category of sex and high-risk status, the relative risk of a $BRAF^{V600E+}$ patient to a $BRAF^{V600E-}$ patient was equal to 1.190 ($HR=e^{(-1.421+1.595)}=1.190$) and 2.305 ($HR=e^{(-1.421+2.256)}=2.305$), respectively. The combined effect of $BRAF^{V600E+}$ and high-risk category on persistence/recurrence, yielded the hazard ratio of 17.28 ($HR=e^{(-1.421+1.595+2.676)}=17.2809$) and 33.49 ($HR=e^{(-1.421+2.256+2.676)}=33.4918$) for patients <35 and ≥60, respectively.

Including the variable LN status (Figure 1, lower right) as a clinico-pathological characteristic instead of high-risk status in the Cox regression model did not change either the overall statistical significance of the model, or the significance of the covariates and the $BRAF^{V600E}$*age interaction. The combined hazard ratios for $BRAF^{V600E+}$ and LN status for all age categories are presented in Table 5.

Table 4. Multivariable analysis of selected clinicopathologic features and disease-free survival in PTC patients.

Variable	B	Standard Error	P value	HR	95% CI
Sex M	0.904	0.298	0.002	2.470	1.376 to 4.435
Age < 35	-0.099	0.454	0.826	0.906	0.371 to 2.204
Age <35-60)	[Ref]				
Age <60-78>	-0.676	0.559	0.227	0.509	0.169 to 1.523
Age < 35*$BRAF^{+}$	1.595	0.757	0.035	4.928	1.116 to 21.75
Age <60-78>* $BRAF^{+}$	2.256	0.795	0.005	9.545	2.009 to 45.37
$BRAF^{+}$	-1.421	0.514	0.006	0.242	0.088 to 0.661
Low risk	[Ref]				
High risk	2.676	1.017	0.009	14.527	1.976 to 106.7

The likelihood χ^2 test statistic was 45.862 and corresponding P-value<<0.0001

Abbreviations: B, regression coefficients; HR, hazard ratio; 95%CI, 95% confidence interval; M, male.

Table 5. The combined effect of $BRAF^{V600E+}$ and LN status on hazard ratio in PTC. Hazard ratios for $BRAF^{V600E+}$ include interaction term Age*$BRAF^{V600E+}$

Variable	Reference	Age < 35	Age <35-60)	Age <60-78>
	$BRAF^{V600E-}$	$BRAF^{V600E+}$	$BRAF^{V600E+}$	$BRAF^{V600E+}$
N0	[1]	1.522	0.328	2.629
N1a prophylactic	2.425	3.690	0.796	6.376
N1a therapeutic	5.707	8.683	1.874	15.005
N1b	9.300	14.150	3.054	24.452

The first column refers to hazard ratios for patients with LN metastases compared with patients without metastases when they both are $BRAF^{V600E-}$ and in the same category of age and sex. Abbreviations: LN, lymph node; PTC, papillary thyroid cancer.

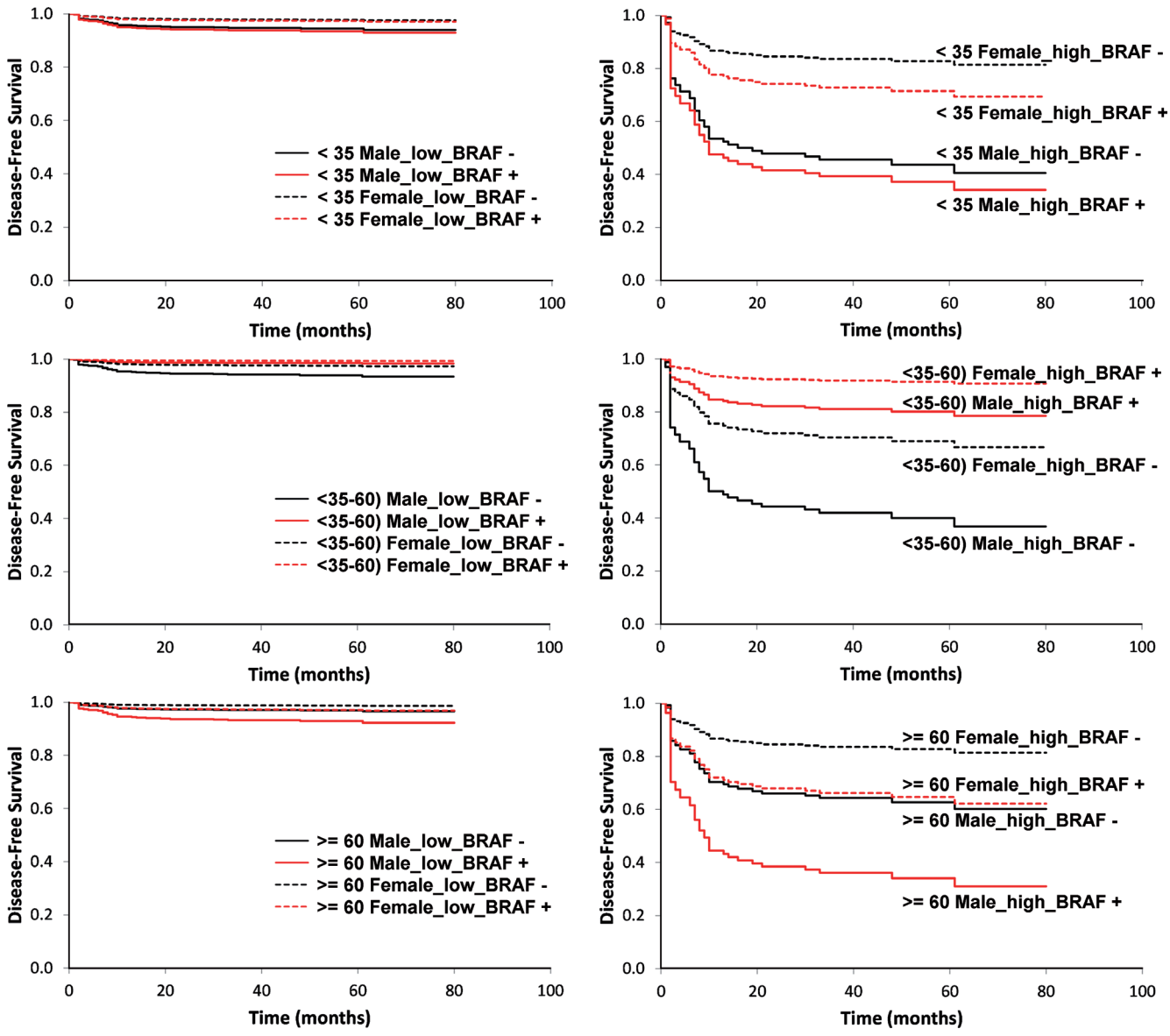

Figure 2. Adjusted Kaplan-Meier curves corresponding to the Cox model (Table 4) on the effect of *BRAF*^{V600E} status on disease-free survival probability in PTC patients. The interaction between age and *BRAF*^{V600E} mutation is apparent. Abbreviations: low, low-risk status; high, high-risk status; BRAF-, *BRAF*^{V600E} mutation absent; BRAF+, *BRAF*^{V600E} mutation present.

Discussion

Though mortality from thyroid cancer is low, the risk of persistence/recurrence is not insignificant, up to 30% (23, 24). Therefore, it is important to accurately identify those who require more aggressive treatment, *versus* those in whom the risk of treatment of their malignancy may outweigh the benefit. Contrary to the studies that have found a significant positive association between *BRAF*^{V600E} mutation and a more aggressive clinical course, we did not succeed in proving the presence of *BRAF*$^{V600E+}$ to be an independent prognostic factor for predicting persistent/recurrent disease in both, bivariable and multivariable analysis, when patients were grouped by age into two categories, ≤45 and >45 years in the initial analyses (for the sake of comparability with other studies). Nevertheless, we found the younger (< 35 years) and older patients (≥ 60 years) at higher risk of PTC persistence/recurrence in comparison to middle-aged patients. It can be assumed that the higher proportion of LN metastases in the younger and higher proportion of more agressive variants in the older patients made a strong independent contribution to reduced disease-free survival in these groups of patients. The low

number of identified persistence/recurrence in the group of middle-aged patients was rather surprising and could not be neglected, since that group was the most numerous. Moreover, bivariable analysis of the association between the $BRAF^{V600E+}$ and rate of persistent/recurrent disease categorised by age revealed even significantly lower risk of persistent/recurrent disease for that age category. With categorisation of patients into three age groups we took into account the distribution of persistence/reccurence events and also different clinicopathological characteristics of younger and older patients, which would not be sufficiently reflected if stratification by age at 45 was made.

Noting the ambiguous role of age in $BRAF^{V600E+}$ participation in the PTC persistence/recurrence (e.g., Brzeziańska et al. (16) found no correlation, Basolo et al. (6) reported a negative and Kebebew et al. (9) positive correlation between age and *BRAF* positivity prevalence), we investigated the possibility of nonproportionality due to an age effect. We estimated the rates and survival functions for the patients stratified according to age, utilizing the boundary limits of 35 and 60 years old separately to see if the shapes would differ. Though the disease-free survival functions of the younger and older $BRAF^{V600E+}$ patients were found to have higher risk in comparison to $BRAF^{V600E-}$, the middle-aged patients' survival functions were reversed with respect to $BRAF^{V600E}$ positivity. Therefore, to model the effect of $BRAF^{V600E+}$ we included an interaction term between $BRAF^{V600E}$ mutation status and age in the analysis in addition to sex and high-risk status.

Xing et al. (5) have reported finding a significant interaction of $BRAF^{V600E}$ with several conventional clinicopathological risk factors in affecting PTC-related mortality, specifically LN metastases, distant metastases, stage IV disease, and patient age at diagnosis stratified by 45 or 60 years. They have found a significant difference between disease-free survival of $BRAF^{V600E+}$ and $BRAF^{V600E-}$ patients followed up to 12 years.

In our study, when age, sex, and high-risk status or LN metastases were included in the Cox's proportional hazards model, we were able to confirm a significant interaction between $BRAF^{V600E}$ mutation status and age (Table 4). The significance of the interaction term confirms that age modified the effect of $BRAF^{V600E+}$ on the outcome persistence/recurrence of PTC in our cohort. Thus, the presence/absence of $BRAF^{V600E}$ mutation did not yield an unambiguous independent effect on disease-persistence/recurrence in our cohort. Our results suggest that for the category of middle-aged patients (with higher proportion of $BRAF^{V600E}$ negative patients among those with persistent/recurrent disease) other, intraneous/extraneous (e.g. genetic/environmental) prognostic factors might contribute to prediction of persistent/recurrent disease (25).

Contrary to the middle-aged patients, in the high-risk patients both, <35 and ≥60 years, the $BRAF^{V600E}$ mutation positivity appeared to be a useful independent prognostic factor for predicting persistent/recurrent disease in our cohort. Even if it was not a significant prognostic marker *per se*, it could be used as a subsidiary diagnostic marker and/or as a prediction variable in multivariable models. These can be used for developing decision support tools and calculators to assist in identifying PTC patients who are most likely to experience persistence/recurrence and therefore require more aggressive treatment: higher dose of 131I and more frequent follow up.

The study by Elisei et al. (13) has found that the $BRAF^{V600E}$ mutation was a poor, but statistically significant prognostic factor for the persistence of the disease independently of other clinical-pathological features in the entire group of low-risk intrathyroid PTC patients. However, we did not find a statistically significant association between $BRAF^{V600E}$ mutation positivity and persistence/recurrence in the low risk patients in either age group. The explanation may lie in the fact that we investigated a homogenous group of patients – all patients underwent TTE with a routine neck dissection. With this approach the LN status of all treated patients was known at the beginning of 131I therapy, similarly, as it was performed in the study by Czarniecka et al. (17). In our opinion, low-risk patients without LN metastases after neck dissection might have had less aggressive therapy regardless of the $BRAF^{V600E}$ mutation status.

Our study has also several limitations: investigated cohort was of a small-to-medium sample size, and the follow up did not exceed 6.5 years. Further, our study was a single-institution study, which may be considered as both, an advantage (homogeneity of the patient population) and a drawback (compromised external validity).

Obviously, the range of factors associated with the outcome can be wider than that explored in our study and the effect of another, so far undetected predictor that is highly correlated with $BRAF^{V600E}$, cannot be excluded. Apart from that possibility, there was a clear interaction between $BRAF^{V600E}$ positivity and age on the underlying scale (of three age subgroups) in the association with the outcome persistence/recurrence of PTC in maximum 6.5 years of follow-up of 199 individuals. Since we have not found any mention of age categorisation into more than two categories in the available literature, we cannot directly compare or contrast our findings with those in previous research. Thus, for the time being, neither literature sources, nor our data analysis can offer a conceptual explanation for the different prognostic value of $BRAF^{V600E}$ mutation positivity in various age groups of patients with PTC. Nevertheless, large-scale clinico-epidemiological studies and subsequent modeling to simulate the observed data may help to find out whether $BRAF^{V600E}$ mutation effect on PTC persistence/recurrence is modified by age and/or other factors.

In conclusion, our study points to a varying prognostic value of $BRAF^{V600E}$ mutation positivity across age categories. Investigation of $BRAF^{V600E}$ mutation at the time of initial therapy is unlikely to be useful *per se* for predicting persistent/recurrent disease in low/high risk middle-aged (35-60) year patients. However, the marker has been confirmed as a significant factor independently associated with persistent/recurrent disease in both, high-risk young (<35 years) and high-risk older (≥60 years) patients, which can change

clinical decision-making in favour of more aggressive treatment. In low-risk patients of any age, who underwent neck dissection, the prognostic significance of $BRAF^{V600E}$ mutation positivity was low. Performing neck dissection enables the clinician to effectively target subsequent 131I therapy in patients at higher risk for persistence/recurrence and, at the same time, to spare low-risk patients unnecessary overtreatment.

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