BRAF mutation in the elderly submitted to thyroidectomy

Mutação BRAF em pacientes idosos submetidos à tireoidectomia

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A B S T R A C T

Objective: To evaluate the frequency of the BRAF V600E mutation in patients over 65 years of age undergoing thyroidectomy, correlating its presence or absence with the different histologic lesions, their variants and with prognostic factors of papillary carcinoma. **Methods**: We evaluated 85 patients over 65 years of age who underwent thyroidectomy, analyzing the BRAF V600E mutation by RT-PCR performed after DNA extraction from the paraffin blocks. **Results**: The study detected the presence or absence of BRAF V600E mutation in 47 patients (55.3%). Among the 17 papillary carcinomas studied, seven had the mutation (41.2%). There was a statistical association between the presence of this mutation and the classic variant of papillary carcinoma, and a trend of association with thyroid extravasation. **Conclusion**: BRAF mutation in the elderly is also exclusive of papillary carcinoma and is often significant. Furthermore, it is related to the classic variant and possibly to thyroid extravasation.

Key words: Thyroid gland. Thyroid neoplasms. Thyroidectomy. Proto-oncogene B-raf. Mutation.

INTRODUCTION

Since their discovery, BRAF mutations have been reported in several types of tumors, with variable frequencies, the most prevalent in melanoma and nevus[1]. An important study showed BRAF mutations in thyroid cancer, with prevalence lower only than the one of melanoma[2]. Mutations in BRAF gene (B-type RAF kinase gene) represent the most common genetic disorders of thyroid cancer and this was the most striking finding in this research field in the last years[3]. The discovery of these changes created the opportunity for developing new treatment strategies for thyroid cancer[4,5]. They are present between 23 and 83% of papillary carcinomas and are highly specific for this histological type [2,6-8]. Kimura *et al.*, in a pioneer study published in 2003, demonstrated that the BRAF mutation occurs in 32.8% of papillary carcinoma, does not occur in benign or follicular lesions and, when present, does not overlap with other RAS mutations or rearrangement of the RET/PTC. These facts point to the MAP kinase pathway as the main responsible for the genesis of papillary carcinoma[2].

This tumor has some histological variants that have also been studied for the presence of BRAF mutations. The classic variant and tall cell variant (related to higher aggression) seem to display a higher incidence of BRAF mutation. The follicular variant, on its turn, rarely presents with such a mutation. The microcarcinomas (tumors smaller than 1.0 cm) may also display it, which confirms the hypothesis that it occurs early in tumorigenesis of papillary carcinoma[9].

As for prognosis, this mutation may appear early in small papillary carcinomas, but it is believed that those with BRAF mutations are associated with poor prognosis and more aggressive variants (eg, tall cell), thyroid extravasation, more advanced clinical stage, distant metastasis and they can still be related to anaplastic carcinoma. These data indicate that tumors with this genotype have a worse prognosis[6,7,9-11].

A correlation was demonstrated between age and the presence of BRAF mutation in papillary carcinomas[9,12] and low frequency in children[10,13]. Currently, no other clear association has been established between genotype and clinico-pathologic changes of papillary

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carcinomas[8]. Some studies demonstrate a correlation between BRAF mutation and advanced stage, lymph node metastasis, distant metastasis and tumor recurrence[5,6,9,11,14].

Xing *et al*. showed that the BRAF mutation is an independent predictor of tumor recurrence, even in patients with stage I and II[14]. Moreover, BRAF mutations have also been related to low uptake of iodine-131 in the postoperative and treatment failure in disease relapse [15]. However, Trovisco et al.[12], among others[8,16,17], showed no association of BRAF mutation with poorer prognosis. Fugazzola *et al*. [8], studying 260 papillary carcinomas, showed a statistically significant association between BRAF mutation and advanced age at diagnosis, without correlation with poor prognosis or poor outcome at 72 months follow-up. In 2009 Ito et al. studied 631 patients with papillary carcinoma and mean follow-up of 83 months, and also failed to demonstrate a worse prognosis associated with BRAF mutation[17].

Thus, this mutation represents an important breakthrough for the investigation of thyroid cancer and is more common in papillary carcinoma. As there are no studies with multivariate analysis, large samples and sufficient follow-up time, it is unclear whether BRAF mutation is associated with poor prognosis, or only occurs in older people where other factors are responsible for it[13,18,19]. Nor is there a specific BRAF mutation study in the elderly in the world literature.

The objective of this study was to evaluate the frequency of mutation of the BRAF V600E in patients over 65 years of age who underwent thyroidectomy, correlating their presence or absence in the different histological lesions with variants and prognostic factors of papillary carcinoma.

METHODS

We conducted a retrospective study between 1994 and 2009 by selecting all patients over 65 years of age subject to any kind of operation on the thyroid gland by the Division of Head and Neck Surgery, Department of Surgery, Faculty of Medical Sciences of Santa Casa de Sao Paulo. This study was approved by the Ethics Committee in Research of the institution under number 116/07.

We identified 104 patients over 65 years of age who had undergone some sort of operation on the thyroid gland. Of these, four were excluded because they had primary tumors in other regions, such as parathyroid, pharynx and larynx, with invasion of the thyroid gland. Of the 100 remaining, 15 had incomplete data records or lacked sufficient tissue in paraffin blocks for DNA extraction, and were then excluded as well. Therefore, the charts of 85 patients were reviewed by gathering information from clinical aspects, surgical procedure, type of histopathological lesion and prognostic factors. Data were transferred to a protocol established for this purpose.

Among the 85 patients, 78 were female. The age ranged between 65 and 89 years old, with a median of 70 years. Total thyroidectomy was performed in 74% of patients. The RT-PCR performed after DNA extraction from paraffin blocks was able to detect the absence or presence of BRAF V600E mutation in 47 patients (55.3%). The BRAF mutation was considered non-assessable in 38 patients (44.7%).

In 47 cases where the mutation was assessable, diseases found are described in figure 1. In 22 cases of papillary carcinoma 17 were assessable, ie 77% of the sample.

Histologic sections were made from paraffin blocks of thyroidectomy specimens to confirm the diagnosis and see if the block had the lesion to be studied. Care was taken to avoid DNA contamination using gloves, disposable razors and manipulation of tissue sections with forceps suitable for this purpose.

We performed DNA extraction after deparaffinization using xylol and ethanol. The successful extraction was confirmed by spectrophotometry.

We then proceeded to the PCR-ARMS (Amplification Refractory Mutation System) in real-time for identification of the mutation of the BRAF gene in DNA extracted using a positive (sample sequenced, demonstrating the presence of the mutation in the gene) and negative (human blood from healthy subject) controls and the reaction without the presence of DNA to ensure the absence of nonspecific amplification reaction on each plate.

The use of the real-time PCR equipment (Applied Biosystems, ABI 7500) allows detection with amplification in a closed tube, eliminating the post-PCR step. This reduces the risks of contamination from the handling of amplified products. Additionally, because the detection is based on

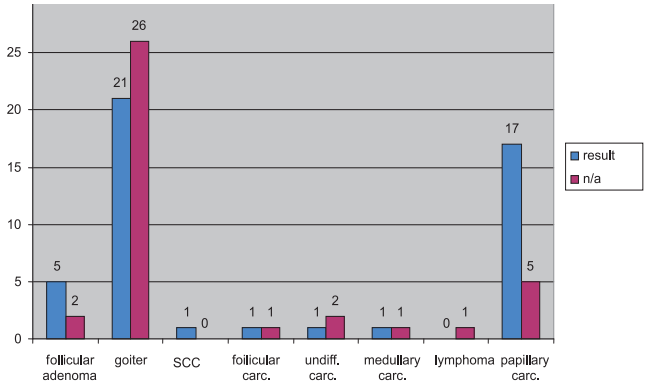

Figure 1 - *Distribution of cases according to histological type and outcome of the RT-PCR.*
** results column represents cases in which the reaction was assessable. ** N / a: not assessable ; SCC: squamous cell carcinoma; follicular carc.: follicular carcinoma; undiff. carc.: undifferentiated or anaplastic carcinoma; medullary carc.: medullary carcinoma; papillary carc.: papillary carcinoma.*

the ability of an intercalating agent (SYBR Green) to emit fluorescence in the presence of amplified double-strand, there is more analytical sensitive when dealing with PCR products smaller than 150 bp.

In preparing the RT-PCR reactions, we used "stock solution" for each condition studied (normal or mutated) (Figure 2). The temperatures used for the PCR were: 95° C for 10 minutes (activation of Taq polymerase) followed by 40 cycles at 66° C for one minute (annealing of the primers) and 72° C for one minute. To ensure specificity of the reaction and detection of a single specific product in real-time PCR (RT-PCR) we added a cycle of dissociation (slow denaturation) to determine the dissociation temperature of each PCR product (Figure 3).

The primers used were[20]:

BRAFwt_s: AGGTGATTTTGGTCTAGCTACAGT;
BRAFmt_s: AGGTGATTTTGGTCTAGCTACAGA, and
BRAF_ASc: TAGTAACTCAGCAGCATCTCAGGGC

All reactions were initially evaluated as to the amplification curve (Figure 3), and in cases where it occurred, the dissociation curve was also observed to ensure that the dissociation temperature (Tm) was similar among the cases with a single amplification peak.

Where the result of RT-PCR was not assessable, it was decided to repeat the whole method for the same sample, looking for positive results. In cases of papillary and anaplastic carcinomas, we repeated DNA extraction and RT-PCR three times.

Finally, the presence or absence of the BRAF mutation in cases with assessable result was correlated with the different histologic variants present (classical variant, follicular variant and tall cells variant) and, with different prognostic factors in papillary carcinoma (age, lymph node metastasis, distant metastases, presence of multifocal tumor, presence of extravasation of thyroid capsule with invasion of adjacent structures, presence of blood or lymphatic vascular invasion and the occurrence of local or regional recurrence).

The statistical analysis included measures of central tendency and dispersion for quantitative variables and absolute and relative frequencies for categorical variables. To investigate the association between these variables, we used the Fisher exact test. To evaluate the difference between the means of quantitative variables (age and size of tumor), we used the Mann-Whitney test according to the status of the mutation. Results were considered significant when $p < 0.05$ (two-tailed tests).

RESULTS

In the 47 cases with assessable results, among the several histological benign and malignant lesions studied,

BRAF V600E		
Reagents	**Wild-type**	**Mutated**
	uL/rx	**uL/rx**
2x Buffer (Tools)	12,5	12,5
Primer sense (WtxMt)	0,3 (Wt)	0,3 (Mt)
Primer antisense common	0,3	0,3
H_2O	10,9	10,9
Volume	**24**	**24**
DNA	1	1
Total volume	**25**	**25**

Figure 2 - *Components of the "stock solution" used in the PCR reaction.*

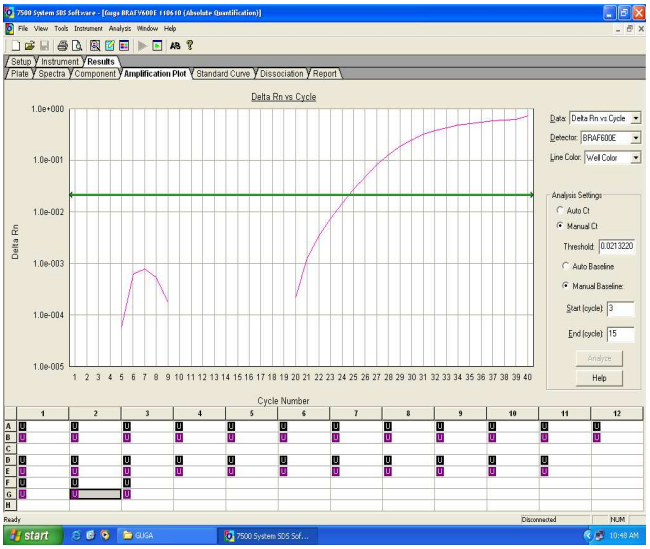

Figure 3 - *DNA amplification curve for RT-PCR samples of papillary carcinoma.*

there was not a single case of BRAF mutation in lesions other than papillary carcinoma (Figure 4).

The frequency of BRAF mutations found, considering only the papillary carcinomas, was 41.2% (seven cases BRAFm within the 17 cases with assessable results). Among these 17 patients, the median age was 71 years (mean 72.5, standard deviation 6.18) and there was only one man. Regarding the histological variants of papillary carcinoma, 12 (70.6%) were the classic variant, four were follicular (23.5%) and one case was tall cells (5.9%).

The median of the tumor size was 3.0 cm, with a mean 3.1 cm and standard deviation of 2.32. As for the other prognostic factors, we found multicentricity in eight cases, lymph node metastases in six, distant metastases in one, capsule extravasation in seven, vascular invasion in ten and local or regional recurrence in two cases.

The correlation between BRAF mutation and gender was not significant by the Fisher's exact test ($p = 0.412$), neither the correlation between BRAF mutation and the presence of vascular invasion ($p = 1.0$), recurrence ($p =$

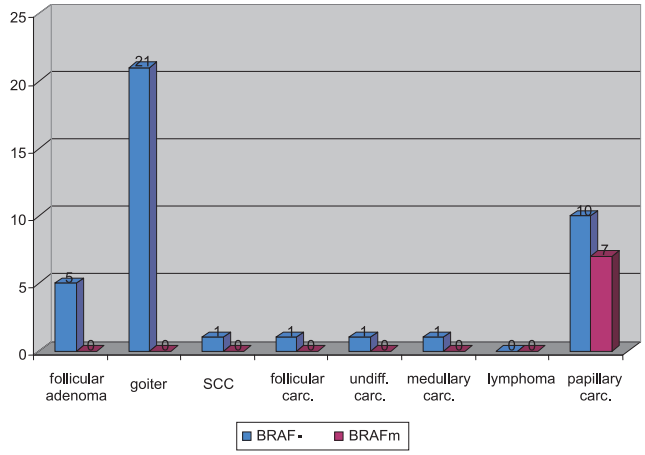

Figure 4 - *Distribution of cases with assessable results, according to the histological type and outcome of the RT PCR for the presence or absence of the BRAF mutation.*

** BRAF-: absence of BRAF mutation; BRAFm: presence of BRAF mutation. SCC: squamous cell carcinoma; follicular carc.: follicular carcinoma; undiff. carc.: undifferentiated or anaplastic carcinoma; medullary carc.: medullary carcinoma; papillary carc.: papillary carcinoma.*

0.154), multicentricity (p = 1.0) and extravasation of the thyroid capsule (p = 0.058). In the latter prognostic factor, statistical significance was observed near the limit of 5%, with a difference in the percentage of leakage, according to the status of BRAF mutation (71.4% in BRAFm cases and 28.6% in BRAF- cases) (Table 1).

When analyzing the group of papillary carcinomas, we observed a significant association between the classical variant and BRAF mutation status (p = 0.044) when compared with other grouped histological variants (Table 1).

The variants of tumor size and age were analyzed by Mann-Whitney test and no statistically significant association was found, with levels of statistical significance of 0.8066 and 0.1052, respectively.

However, the mean and median age of the group of patients with the BRAF mutation was 75.3 and 74 years, respectively. In the group of patients with papillary carcinoma and absence of the mutation studied, the average was 70.6 and median of 70 years.

In short, studying only the elderly, we found the presence of BRAF V600E mutation only in cases of papillary carcinomas. Among 17 patients with this lesion, seven had the mutation (41.2%). Among 47 patients with assessable results – 17 papillary carcinomas, four other types of malignant tumors and 26 benign lesions – no other type of histological lesions had the mutation. When analyzing prognostic factors in papillary carcinoma, we found a statistical trend of association between extravasation and the presence of BRAF mutation, and observed a statistically significant association between the classic variant of papillary carcinoma and the presence of BRAF mutation.

DISCUSSION

The BRAF mutation has been extensively studied worldwide, playing a crucial role in tumorigenesis of colorectal cancer, melanoma and papillary thyroid carcinoma[4]. Although many authors have demonstrated techniques for extraction of DNA from paraffin embedded tissue, other attempts had been made for this purpose at the institution of the authors, without success. This difficulty was attributed to the presence of formaldehyde and probable DNA damage. Finally, using a simple protocol for deparaffinization and DNA extraction, a routine for the development of this study could be established. Thus a wide field of research emerged, minimizing dependence on fresh tissue and tumor banks.

There were difficulties in extracting DNA from some samples, without any correlation with their age or their lot. Some cases were PCR unassessable, it is impossible to tell whether the mutation in question was present or not, reducing the number of cases resulting in increased expenditure of material, since all the methodological process was repeated.

Initially this study was designed to verify the presence of BRAF mutation in elderly subjects who underwent thyroidectomy for benign and malignant lesions. These initial results demonstrated the absence of

Table 1 - Distribution of prognostic factors evaluated according to the presence or absence of BRAF mutation.

Prognostic factors		BRAFm	%	BRAF-	%	P
		n=7		n=10		
Gender	Male	1	14.3	0	0.0	0.412
	Female	6	85.7	10	100.0	
Vascular invasion		4	57.1	6	60.0	1.0
Recurrence		2	28.6	0	0.0	0.154
Multicentricity		3	42.9	5	50.0	1.0
Extravasation		5	71.4	2	20.0	0.058
Classic variant		7	100.0	5	50.0	0.044

this mutation in benign and malignant lesions other than papillary carcinoma, even in elderly individuals, which is consistent with the literature[2,6-8]. In 2003, Xu *et al*. also described the presence of BRAF mutation in papillary carcinomas and its absence in goiters and follicular lesions[21].

We chose to investigate only the cases of interest, ie, papillary carcinomas and anaplastic carcinomas that could have their origin in papillary carcinomas. This explains the higher number of cases for which results were obtained between the papillary carcinomas when compared to goiters.

The BRAF mutation is absent in benign lesions – colloid goiter and Hashimoto's thyroiditis –, as well as in malignant lesions other than papillary carcinoma – follicular carcinoma – in elderly. Those anaplastic carcinomas arising from papillary carcinomas may contain the mutation[22], although this study examined only one case of anaplastic carcinoma that had not.

Oler and Cerutti studied 120 cases of papillary carcinoma, of which 48% showed BRAF mutation[23].Fugazzola et al. cite a worldwide prevalence of BRAF mutation in papillary carcinomas around 40% (858 of 2174 cases studied) in the introduction of their study [8]. The same authors, in a multicenter Italian research, studied 260 papillary carcinomas and found BRAF mutation in 38% of them [8]. In 2009 Araújo *et al*. showed an incidence of 28.1% in papillary carcinomas[24]. This study demonstrates a frequency just above the reported (41.2%), which is easily justified in view of the population.

BRAF mutation is also linked to locally invasive papillary carcinoma, as well as the papillary carcinomas that display capsule extravasation[19,25], or even non-encapsulated lesions. In this study we found a trend towards a significant association between BRAF mutation and extravasation ($p = 0.058$), which can give the BRAF mutation prognostic significance.

Lupi *et al*.[11] studied 500 patients with papillary thyroid carcinomas (230 microcarcinomas, 82 classic variants, 114 follicular variants, 40 tall cell variants and 34 other variants). BRAF mutation frequency was 43.8%, being higher in tall cell variant (80%) and in the classic variant (68.3%) and lowest in the follicular variant (18.8%). Among the group of microcarcinomas, the frequency was 39.4%. This study also demonstrated a high frequency of BRAF mutation in papillary carcinomas of the classic variant (58.3%), with statistical association between them. Also in this series, there was no statistically significant relationship between age, gender and the presence of the mutation in the univariate analysis, but there was statistical association of mutation with extrathyroid invasion, multicentricity, lymph node metastasis, stage III versus stage I and II, and absence of tumor capsule. In the multivariate analysis, only the presence of tumor capsule was associated with the absence of the BRAF mutation, which, in this aspect, suggests a better prognosis. Despite the small sample of this study, we were able to demonstrate a tendency of association between thyroid extravasation and the presence of BRAF mutation, which may mean a worse prognosis.

Although the BRAF mutation is the most common genetic abnormality in papillary thyroid carcinoma, its meaning for the long-term prognosis is not well established. Contradictory studies have been published, probably due to heterogeneity of papillary carcinoma around the globe and different phenotypes that overlap due to different genetic alterations [18,26].

The association between the classic variant of papillary carcinoma and BRAF mutation had already been demonstrated [9,12,27,28]. In this study, we found a significant association between the presence of BRAF mutation and the classic variant of papillary carcinoma. But due to the small number of cases analyzed, there was no statistical association between the other prognostic factors and BRAF mutation status. The association between the classic variant of papillary carcinoma and BRAF mutation with a more aggressive phenotype due to reduced expression of iodine-metabolizer genes suggests the detection of BRAF mutation as a prognostic factor, an assistant in therapy choice for patients with papillary carcinoma[23].

It is believed that the papillary carcinomas with BRAF mutation may have lower uptake of iodine in the postoperative period, which translates into more aggressive phenotype and a higher chance of recurrence[15,29,30], indicating that in these cases, at least the follow-up must be strict.

Elisei *et al*. published the first series of cases with consistent follow-up to suggest that BRAF mutation is associated with poor prognosis and appears to be a factor independent from age[25]. In 102 patients with papillary carcinoma and mean follow up of 15 years, the mutation frequency was 37.3%, but was higher in patients over 60 years of age, which confirms the data found in this study. They also showed associated of the mutation with more advanced stages, vascular invasion, and worse prognosis[25]. Despite having been studied only individuals over 65 years of age, it is known that the higher the age, the worse the prognosis of papillary carcinoma, which led to the study of the association of age with the BRAF mutation that BRAFm patients had with more advanced age.

Thus, one can say that even in elderly patients the BRAF mutation was found only in papillary carcinomas, with a frequency of 41.2%. This study also demonstrated the association between the classic variant of papillary carcinoma and the presence of BRAF mutation, even in a small sample of elderly patients. It was also found a statistical trend of association between extravasation and the presence of thyroid BRAF mutation, and higher median age in this group, without statistical significance.

However, these results do not indicate that the presence of BRAF mutation is associated with worse prognosis in view of the small sample studied and also

because follow-up time and patient survival were not interests in the study.

Perhaps these are goals to further studies, with increasing sample size, involvement of other institutions and extended clinical follow-up, trying to define the prognostic value of BRAF mutation in papillary thyroid carcinoma.

In conclusion, the BRAF mutation in the elderly is also exclusive of papillary carcinoma and is often significant. Furthermore, it is related to the classical variant and possibly to thyroid extravasation.

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R E S U M O

Objetivo: *Avaliar a frequência da mutação V600E do gene BRAF em pacientes com mais de 65 anos de idade submetidos à tireoidectomia, correlacionando sua presença ou ausência com as diferentes lesões histológicas, com as variantes e com fatores prognósticos do carcinoma papilífero.* ***Métodos:*** *Foram avaliados 85 pacientes com mais de 65 anos de idade submetidos à tireoidectomia, analisando a mutação BRAF V600E através de reação de PCR-RT realizada após a extração do DNA dos blocos de parafina.* ***Resultados:*** *Detectou-se ausência ou presença da mutação BRAF V600E em 47 pacientes (55,3%). Entre os 17 carcinomas papilíferos estudados, sete apresentavam a mutação (41,2%). Demonstrou-se associação estatística entre a presença desta mutação e a variante clássica do carcinoma papilífero, além de tendência de associação com o extravasamento tireoideano.* ***Conclusão:*** *A mutação BRAF nos pacientes idosos também é exclusiva do carcinoma papilífero e tem frequência expressiva. Além disso, está relacionada à variante clássica e, possivelmente, ao extravasamento tireoideano.*

Descritores: *Glândula tireoide. Neoplasias da glândula tireoide. Tireoidectomia. Proteínas proto-oncogênicas B-raf. Mutação.*

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