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Absence of BRAF mutation in pheochromocytoma and paraganglioma

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Pheochromocytomas and Paragangliomas (PHEO/PARA) are rare endocrine tumors originating from the adrenal medulla. More than 20 genes are involved in the tumorigenesis of these tumors, but a substantial part of the causative genetic events remains unexplained. A recent study has reported the presence of the activating *BRAF* V600E mutation in PCC, suggesting a role for BRAF activation in tumor development. Other studies have not find this mutation. This study investigates the occurrence of the *BRAF* V600E mutation in these tumors.

A cohort of 64 PHEO/PARA were screened for the *BRAF* V600E mutation using direct Sanger sequencing and QRT-PCR.

All cases investigated displayed wild-type without V600E BRAF mutation

Taken together with all previously screened tumors up to date, only 1 V600E *BRAF* mutation has been found among 427 PCCs. These findings imply that the V600E *BRAF* mutation is a rare event in PHEO/PARA.

Key words: pheochromocytoma, paraganglioma, BRAF, mutation

B-Raf is a protein produced by the human gene *BRAF* [1, 2]. B-Raf is a 766-amino acid protein composed of three conserved domains characteristics of the Raf kinase family. In active conformation, B-Raf forms dimers via electrostatic interactions of its kinase domains and hydrogen-bonding [3]. Cell growth is directed by signalling from the B-Raf protein, since it is a member of the Raf kinase family of growth signal transduction protein kinases. Hence, it plays a role in regulating the MAP kinase/ERKs signalling pathway, which in turn affects cell division, differentiation, and secretion. Human cancers have been associated with more than 30 mutations of the *BRAF*. In 90% of these cases, thymine is substituted with adenine at nucleotide 1799 (p. V600E) [4].

The frequency of *BRAF* mutations varies widely in human cancers. Higher frequencies are found in melanomas and nevi, hairy cell leukaemia, and Langerhans cell histiocytosis while rarely in other tumors such as non-Hodgkins lymphoma, colorectal cancer, astrocytoma, papillary thyroid carcinoma, non-small-cell lung carcinoma, lung adenocarcinoma, and neuroblastoma [5-13]. Furthermore, a high frequency of *BRAF* V600E mutations have been detected in ameloblastoma, a locally infiltrative odontogenic benign neoplasm [14].

To date, only one study has identified a *BRAF* mutation with an incidence of 1,2% (1/85) in pheochromocytoma and paraganglioma (PHEO/PGL) [15]. Paulson et al, summarized data from other studies and did not found V600E *BRAF* mutation in 0,3% (1/336) PHEO/PGL tumors [16, 17].

Similary to neuroblastoma which have incidence of *BRAF* mutation about 1%, PHEO is also tumor of the adrenal gland that arises from chromaffin cells located in the adrenal medulla. PGL arise from extra-adrenal chromaffin cells located in sympathetic (abdomen, pelvis) or parasympathetic (head and neck) ganglions [18, 19]. These tumors may produce and

secrete catecholamines and metanephrines [20-22]. Currently, there are about 20 known genes associated with PHEO/PGL pathogenesis [18, 23-25].

Here, we aimed to assess the presence of the *BRAF* mutation on a large population of PHEOs/PGLs and to further contribute to controversial view whether this mutation may or may not occur in these tumors [18, 26]. Since *BRAF* mutation is a very good treatment target, the presence of this mutation in some of these tumors could result in the use of B Raf inhibitors of metastatic forms for which there has not been effective treatment so far.

Materials and methods

Our study included 64 patients with PHEO/PGLs (32 men and 32 women, range from 7 to 77 years). Patient's samples were collected from the 3rd Department of Medicine, 1st Faculty of Medicine, Faculty Hospital and Department of Pediatric Hematology and Oncology, 2nd Faculty of Medicine, Prague, Czech Republic. The informed consent was obtained from all involved patients. Clinical characteristics of study objects are described in Table 1.

Table 1. Clinical and germline mutations characterization

Num.	Age (y)	sex	PHEO/ PARA	site	Volum (ml)	Metastases	Familiar	Germline mutation of SDHB, SDHD, RET, VHL genes
1	36	F	PHEO	Left	39	No	No	Neg.
2	16	M	PHEO	Right	n.d.	No	No	Neg.
3	12	F	PHEO	Bilateral	n.d.	No	No	VHL: c.602T>C, p.Leu201Pro
4	65	F	PHEO	Right	45	no	No	Neg.
5	45	F	PARA	Retroperitoneum	31	no	No	Neg.
6	13	F	PARA	Retroperitoneum	n.d.	no	No	SDHB: c. 589 – 600 dup. AGC ACC AGC TGC, p. Cys 196 dup. Ser 197 Thr 198 Ser 199 Cys 201
7	68	M	PHEO	Right	70	no	No	Neg.
8	35	F	PHEO	Left	120	no	No	Neg.
9	62	F	PHEO	Right	70	no	No	Neg.
10	7	M	PARA	Mediastinum	24	no	No	VHL: c.376G>A, p.Asp126Asn
11	64	F	PHEO	Left	42	no	No	Neg.
12	14	M	PHEO	Right	n.d.	no	No	Neg.
13	22	F	PHEO	Left	46	no	No	Neg.
14	35	M	PHEO	Left	80	no	No	Neg.
15	31	F	PHEO	Bilateral	10;30	no	No	RET: c.1901G>C, p.Cys634Ser; c.1921G>T, p.Ala641Ser
16	52	M	PHEO	Bilateral	6; hyperplasia	no	No	Neg.
17	59	F	PHEO	Left	63	no	No	Neg.
18	68	M	PHEO	Left	28	no	No	Neg.
19	60	M	PHEO	Right	13	no	No	Neg.
20	15	M	PHEO	Right	n.d.	no	No	Neg.
21	73	F	PHEO	Right	35	no	No	Neg.
22	31	F	PARA	Middle ear	n.d.	no	No	Neg.
23	27	F	PARA	Retroperitoneum	60	no	No	Neg.
24	41	M	PHEO	Left	100	no	No	n.d.
25	76	M	PHEO	Right	60	no	No	Neg.
26	23	M	PARA	Mediastinum	60	bone; GC; neck	Yes	Neg.
27	47	M	PHEO	Left	58	no	No	Neg.
28	13	M	PARA	Rertoperitoneum	86+80	no	No	Neg.
29	26	M	PARA	Retroperitoneum	35	no	No	SDHD: c.361C>T, p.Gln121X
30	65	F	PHEO	Right	50	no	No	Neg.
31	40	M	PHEO	Bilateral	30;14	no	No	Neg.
32	40	M	PHEO	Right	70	no	No	Neg.
33	68	F	PHEO	Right	80	no	No	Neg.
34	67	F	PHEO	Right	60	no	No	Neg.
35	77	F	PHEO	Right	65	no	No	Neg.
36	26	F	PHEO	Left	50	no	No	Neg.
37	20	M	PHEO	Bilateral	70;40	no	No	VHL: c.340+2T>C
38	34	M	PHEO	Right	35	no	No	Neg.
39	27	M	PARA	Neck	n.d.	no	No	SDHD: c.2T>A, p.Met1Lys

Table 1. Clinical and germline mutations characterization (continued)

Num.	Age (y)	sex	PHEO/ PARA	site	Volum (ml)	Metastases	Familiar	Germline mutation of SDHB, SDHD, RET, VHL genes
40	65	F	PHEO	Left	17	no	No	Neg.
41	15	F	PHEO	Left	n.d.	no	No	Neg.
42	24	M	PHEO	Bilateral	30;6,9	no	No	VHL: c.374A>C, p.His125Pro
43	47	M	PHEO	Right	55	no	No	Neg.
44	60	F	PHEO	Left	665	no	No	Neg.
45	42	M	PHEO	Right	12	liver; bone;lymph nodes; lung	No	Neg.
46	75	F	PARA	Pelvis	50	no	No	Neg.
47	64	F	PHEO	Left	38	no	No	Neg.
48	60	F	PHEO	Bilateral	30;hyperplasia	no	No	Neg.
49	61	M	PHEO	Left	95	no	No	Neg.
50	33	F	PHEO	Right	55	no	No	Neg.
51	57	F	PHEO	Left	60	no	No	VHL: c.351G>A, p.Trp117Ter
52	21	M	PHEO	Bilateral	60;25	no	No	Neg.
53	50	M	PHEO	Right	8	no	No	Neg.
54	28	F	PHEO	Left	55	no	No	Neg.
55	61	F	PHEO	Right	60	no	No	Neg.
56	51	M	PHEO	Right	50	no	No	Neg.
57	9	F		Retroperitoneum	360	no	No	Neg.
58	68	M	PHEO	Left	40	no	No	Neg.
59	14	F		Retroperitoneum	n.d.	no	No	n.d.
60	77	M	PHEO	Left	90	no	No	Neg.
61	66	M	PHEO	Right	55	no	No	Neg.
62	74	F		Zuckerkandel	27	no	No	Neg.
63	59	M	PHEO	Left	80	no	No	n.d.
64	59	F	PHEO	Left	110	no	No	Neg.

Table1. characterized clinical data and germline mutation status of SDHB, SDHD, VHL and RET genes.
PHEO/PARA-Pheochromocytoma/Paraganglioma; neg.- negative; n.d.- not done, M-male; F- female; GC-glomus caroticum

Genomic DNA was extracted from fresh or frozen peripheral blood using QIAamp DNA Mini Kit (Qiagene, USA). Somatic DNA was extracted from frozen tumour's samples after histological confirmation of PHEO/PGL. DNA was extracted by Puregene Core kit A (Qiagene, USA). Quality of DNA was checked by NanoDrop™ 2000/2000c Spectrophotometers (ThermoScientific)

Sanger sequencing. PCR primers for *BRAF* gene have been designed based on GenBank sequences using the Primer 3 software including intron-exon boundaries, reverse primer 5′- CTGTTCAAACTGATGGGACCC– 3′, forward primer 5′– TGCTTGCTCTGATAGGAAAATG– 3′. The BRAF PCR conditions are as follows 25 µl reaction mixture contained 1x PCR buffer (Fermentas), between 50-300 ng of genomic DNA as template, 1.5 mM MgCl2 (Fermentas), 25 pmol of each primer, 200 µM of each deoxynucleotide triphosphate (Fermentas), and 1.0 unit of TaqDNA polymerase (MBI Fermentas). Amplification conditions were included an initial denaturation at 94C° for 3 min., followed by 35 cycles of 45 sec at 94 C°, 45 sec at 60 C°, 1 min. at 72C° and final extension step running 5 min. at 72 C°. DNA fragments were sequenced in both directions using an automatic fluorescent ABI PrismTM 3130 Genetic Analyzer (PE Applied Biosystems) according to the manufacturer′s instructions.

QRT-PCR for detection of V600E *BRAF* mutation. PCR primers and probes (accession No. NG_007873), PCR conditions and results classification were designed by Lang et al. [27]. These primers and probes are targeted against each mutation, and a mutation-unspecific region was used as a reference amplicon. All unlabelled primers were synthesized by EastPort Praha, Czech Republic; and probes (TaqMan) were purchased from Applied Biosystems, Foster City, CA. Real-Time PCR Reference PCR was performed in a 25 µl reaction volume with HotstarTaq DNA polymerase, Qiagene, 900 nmol/L of each BRAF mutation-un-specific primer, 100 nmol/L of the BRAF probe, 112.5 nmol/L of each internal control primer, 25 nmol/L of internal control probe, and 5 µl of DNA of varying concentration. Allele-specific PCRs were performed according to the same protocol but using a concentration of 450 nmol/L of allele-specific primer. All real-time PCRs were performed on a system (TaqMan 7300 PCR System Applied Biosystems, Foster City, CA) under the following thermocycling conditions: 95°C for 10 minutes,

followed by 50 cycles of 90°C for 15 seconds and 60°C for 1 minute. Cycle threshold (Ct) values were recorded for reference PCR and for each allele-specific PCR, and corresponding Δ Ct values (ie, allele-specific Ct minus reference Ct) were calculated.

Results

Somatic activating *BRAF* mutations in exon 15 were investigated in 64 tumor samples. Initially, we used Sanger sequencing. Results for V600E *BRAF* mutation were validated by QRT-PCR. All of these samples were negative for *BRAF* mutation in exon 15.

Discussion

In the present study, which included 66 PHEOs/PGL, we did not confirm the presence of any *BRAF* mutations. This contrasts Luchetti et al., who detected V600E *BRAF* mutation in 1,2% (1/85) of these tumors. Until now, 427 PHEOs/PGLs were investigated for the presence of a *BRAF* mutation, which was only found] in 1 of these tumors, suggesting that the *BRAF* V600E mutation is a extremely rare genetic event in PHEO/PGL and would not serve as a target for new treatment options in metastatic PHEO/PGL.

Previous studies have demonstrated that tumor oncogene activation such as *RET*, *HIF2A*, and *HRAS* in PHEO/PGL may result in tumorigenesis of these tumors [15, 24, 26, 28, 29]. Furthermore, additional gene expression studies suggested that most PHEO/PGLs can be classified into two distinct groups (cluster1 and cluster 2) by transcription profiling: cluster 1 includes tumors that harbour mutations in genes linked to pseudohypoxia (*VHL, HIF2A, SDHA, SDHB, SDHC*, and *SDHD*) and cluster 2 contains tumors harbouring mutations in genes that are involved in the kinase signalling characterized by the activation of the PIK3/AKT/mTOR and RAS/RAF/ERK pathways *(RET, NF1, TMEM127, MAX,* and *HRAS*), both converging on the HIF-signaling pathway [30]. The proto-oncogene *RET* is a tyrosine kinase receptor primarily expressed in the neural crest cells. *RET* mutations have been associated with increased activation of PI3K/v-Akt signals. *NF1* encodes for the neurofibromin protein, a GTPase- activating protein in the RAS signaling cascade and mTOR signaling pathway. Thus, *RET* and *NF1* mutations lead to activation of the PI3K/AKT/mTOR and RAS/RAF/MAPK signaling pathways [24]. Thus, RAS/RAF/MAPK signaling pathways genes are a promising aim of mutations in PHEO/PGL. That supposition was confirmed by Luchetti et al. and other which found somatic *HRAS* mutation in PHEO/PGL [26, 15] very recently Luchetti et al. found V600E *BRAF* mutation in 1,2% (1/85 cases). We investigated 64 cases without any detection of the V600E *BRAF* mutation.

In conclusion, our results along with previous results, suggest that the BRAF V600E mutation is an extremely rare genetic event in PHEO/PGL.

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