TITLE: Genetics of pheochromocytoma/paraganglioma in Spanish patients

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ABSTRACT

Context: The presence of familial history in pheochromocytoma/paraganglioma patients, including syndromic antecedents, leads in the majority of cases to a positive genetic testing for mutations in one of the major susceptibility genes described so far. Furthermore, it has been reported that in the absence of familial antecedents (FA), around 11-24% of patients also carry a mutation in one of these related genes. In these cases, other clinical aspects like bilaterality, multiplicity, location of the tumors, or age at onset can help to recognize the underlying genes involved. **Objective:** To discuss clinical criteria helpful in the genetic diagnosis, placing special emphasis on apparently sporadic cases. **Design:** 237 non-related probands were analyzed for the major susceptibility genes: *VHL*, *RET*, *SDHB*, *SDHC*, and *SDHD*. Genetic characterization included both point mutation analysis, and gross deletions in the *SDH* genes performed by multiplex-PCR. **Results:** As expected, all syndromic probands were genetically diagnosed with a mutation affecting either *RET* or *VHL*. 79.1% (19/24) and 18.4% (31/168) of patients presenting either with non-syndromic FA or with apparently sporadic presentation were found to carry a mutation in one of the susceptibility genes. Finally, we found a Spanish founder effect for two mutations: *SDHB* c.166_170delCCTCA and *SDHD* c.129G>A. **Conclusions:** We recommend prioritizing *SDHB* genetic testing in patients developing isolated tumors, especially those with extra-adrenal location or malignant behavior. In fact, for apparently sporadic patients developing isolated tumors in any location, 40 years seems to be an appropriate age limit above which we only advise *SDHB* genetic testing.

INTRODUCTION

Pheochromocytomas (PCCs) are rare neuroendocrine chromaffin-staining tumors arising from the adrenal medulla that usually cause secondary hypertension by oversecretion of catecholamines (1). PCCs developed from extra-adrenal paraganglia, named paragangliomas (PGLs), mainly appear in the intra-abdominal/retroperitoneal (85%) or thoracic (10%) areas as secretory tumors with a high risk of malignancy (2). PGL can also develop as generally non-functioning and benign masses in the head and neck (H&N) region (3). About 30% of cases carry mutations in one of six susceptibility genes identified so far: *VHL, RET, SDHB, SDHC, SDHD,* and *NF1* (4-6). This percentage may be even higher if we consider that to date the contribution of gross *SDH* deletions to the disease has not been established. In addition to this complex scenario, there is also a not well-known percentage of familial cases (i.e. with FA of PCC/PGL) that do not harbor mutations in any of the susceptibility genes mentioned above.

When any syndromic antecedent (e.g. medullary thyroid carcinoma, hemangioblastoma, neurofibromas) is present at the onset of the disease, either familial or personal, this will give clear clues as to which susceptibility gene to analyze for mutations. By contrast, despite some help from various guidelines recently published (4, 7), those cases without antecedents usually follow a consecutive genetic screening in all known susceptibility genes until a mutation is detected. Thus, it is necessary to characterize more series of patients in depth, not only to better understand the involvement of each gene in this "multigenic" disease, but also to elucidate founder effects in specific populations to facilitate genetic diagnosis. In the present study, we have analyzed a large series of Spanish patients for five PCC/PGL susceptibility genes, testing both point mutations and rearrangements.

MATERIALS AND METHODS

Patients

Consecutively registered patients included in this study were clinically diagnosed with functioning or non-functioning PCC/PGL in public Spanish hospitals and sent to our center for genetic testing in five of the six major susceptibility genes: *VHL*, *RET*, *SDHB*, *SDHC*, and *SDHD*. DNA was obtained by standard procedures (8) from blood samples of 237 apparently non-related probands collected from 1995 to 2008. Clinical data were compiled for all patients by means of either direct interviews or detailed clinical data questionnaires. All patients provided informed consent.

Mutation analysis

Complete genetic characterization of the five genes included both point mutation analysis of all exons and intron-exon boundaries, and gross deletions in the *SDH* genes as previously described (9). In the five familial cases without point mutations, gross *VHL* deletions were also discarded by means of multiplex ligation-dependent probe amplification (MLPA), following the manufacturer's instructions (MRC-Holland, Amsterdam, The Netherlands). We used DNA from 1000 unrelated and unaffected individuals as a control population.

Statistical analysis

The statistical analysis was performed with R software 2.7.2 (http://www.r-project.org/). We used Fisher's exact test for small samples and the analysis of variance test to compare more than two variables.

Haplotype analysis

In order to detect founder effects in the Spanish population, we performed haplotype analysis. Haplotype distribution was determined for the six c.166_170delCCTCA *SDHB* mutation carriers, and for two additional *SDHD* mutations (c.334_337delACTG and c.129G>A) that were included in the analysis because a Spanish origin was suspected (supplemental table 1).

RESULTS

Patients with syndromic features: multiple endocrine neoplasia type 2 (MEN2) or von Hippel-Lindau (VHL) antecedents

Amongst 237 probands, 45 had personal or FA of MEN2 or VHL syndromes, and all showed a positive carrier status (supplemental table 2). Clinical characteristics of these patients are detailed in the supplemental material.

Non-syndromic patients

Clinical characteristics of the 192 probands without a syndromic presentation (i.e. without personal or FA of MEN2 or VHL) are described in Table 1. Twelve and a half percent of probands (24/192) presented with FA of PCC/PGL, and a mutation was found in 79.1% (19/24) of them (supplemental Figure 1). The involvement of each susceptibility gene was: 8 *SDHB* cases, 7 *SDHD* cases, 3 *VHL* cases and 1 *RET* case. The remaining 20.8% (5/24) of the familial cases showed no mutation in any of the five genes (clinical characteristics of these patients are detailed in the supplemental material). Amongst the non-syndromic cases without FA, a mutation was found in 17.2% (29/168) of probands: 17 in the *SDHB* gene, 7 in *VHL*, 4 in *SDHD* and 1 in *SDHC*. With respect to clinical manifestations, a mutation was detected in: 68% (11/16) of cases with multiple tumors in different organs, 31.6% (6/19) of cases with bilateral PCC, and 60% (3/5) of cases with bilateral carotid PGL. If we only consider those cases with a single tumor, 11.2% (15/133) of patients carried a germline mutation, mainly involving the *SDHB* gene (Figure 1A).

Haplotype analysis

Haplotype analysis revealed the same rare haplotype (frequency, 1.05%) in all the *SDHB* c.166_170delCCTCA mutation carriers (n=8) (supplemental Table 1). Regarding *SDHD* mutations, c.129G>A was found in all carriers (n=12) in the same haplotype, and all carriers (n=9) of the *SDHD* c.334_337delACTG alteration also shared a microsatellite combination. PHASE analysis performed in 100 healthy controls showed that both haplotypes were extremely rare in our population (mean frequency 0-0.73% for the first haplotype and 0.47-4.95% for the second).

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DISCUSSION

Since the year 2000, a great deal of effort has been invested in determining the involvement of the most important susceptibility genes (*VHL*, *RET*, *SDHB*, *SDHC*, and *SDHD*) in the development of PCCs/PGLs. Two major studies based on point mutation analysis performed in large cohorts of patients (4, 10), obtained different percentages (11.6% and 24%, respectively) of apparently sporadic cases (i.e. without FA of PCC/PGL or any related syndrome) carrying a germline mutation. In the present study, based on complete genetic testing of alterations in the five genes (including gross *SDH* deletions), we have found 17% of apparently sporadic patients having a mutation. This proportion is 11% even when we consider only patients with a single tumor. It is important to notice that we have also included in our series cases with non-functioning tumors, while the other two studies only included patients presenting with secreting masses (4, 10).

Non-syndromic patients

Twenty-five percent of non-syndromic probands carried a germline mutation (supplemental Table 3). More than half of the positive carriers harbored a pathogenic *SDHB* variant, which constitutes 10.5% of the total 237 probands and 10.1% of apparently sporadic cases. This percentage was similar to that found by Amar *et al.* (4) in French patients (6.9%). This not statistically significant difference (P=0.28), could be explained because we routinely carry out a gross deletion test of the *SDH* genes, which was not performed in the French series. Regarding this, we have found seven independent families carrying an *SDHB* deletion, which represent 28% of positive *SDHB* cases. The presence of founder mutations in the Spanish population could also explain the high rate of *SDHB* mutations of our series. This latter hypothesis seems more plausible, since we found no significant differences between

apparently sporadic cases carrying a *VHL* or an *SDHD* mutation and those previously described (4).

In the absence of any syndromic background, the genetic management of PGL/PCC patients can be guided by other clinical aspects like the presence of FA, bilaterality/multiplicity and location of tumors, age, and malignancy.

Familial antecedents (FA)

Though the presence of FA is the clinical feature with the strongest association to germline mutations in PCC/PGL patients (4, 10), its absence does not preclude the existence of an inherited disease. It seems likely that in the case of *SDHD* patients, maternal imprinting accounts for the absence of FA in one third of our probands. Regarding *SDHD* inheritance, we found no affected carriers inheriting the mutation from the mother in our series (n=19), so the recently reported maternal transmission of *SDHD*-linked PGLs (11), if confirmed (12), seems to be a very rare event. In addition, since we can discard a potential high rate of *de novo* presentations, only a lower penetrance of *SDHB* mutations would explain why two thirds of all *SDHB* mutation carriers from our series appeared as apparently sporadic cases. Probably, the existence of genetic modifiers could be responsible for this incomplete penetrance. Further discussion regarding the five probands with FA and no mutation can be found in supplemental data.

Multiplicity or bilaterality

In the absence of FA, multiplicity (two or more tumors in different organs) was the clinical feature with the strongest association with the presence of a germline alteration ($P<0.001$) (Table 1). Bilaterality affecting the adrenal gland was clearly associated to *VHL* mutations,

making the susceptibility gene choice to be studied easier. As expected, the majority of patients developing only one tumor were truly sporadic, but 9% of them carried an *SDHB* mutation (Figure 1A). *SDHB* mutations are often associated to the development of non-multiple tumors (13), so in patients developing isolated tumors, especially those with abdominal/thoracic location or malignant behavior, genetic analysis of *SDHB* should be prioritized.

Tumor location and age at onset

Concerning the tumor location, almost half of the patients who developed at least one abdominal/thoracic PGL carried a mutation, with *SDHB* being the main gene involved. If we only consider patients with a single abdominal/thoracic tumor, the percentage of cases harboring a mutation is maintained, but the involvement of *SDHB* is even higher (Figure 1A). On the other hand, 36% of patients with H&N tumors showed a mutation, but in this case, the presence of an isolated tumor decreases almost by half the probability of finding a mutation. In addition, in those patients with adrenal tumors, the probability of carrying a genetic alteration is even lower (8.4% of apparently sporadic cases), and it dramatically decreases to 2.3% in patients developing a single tumor. It is worthy to note that one of the two positive cases had an *SDHB* mutation. So we strongly recommend *SDHB* genetic testing in patients with apparently sporadic tumors, even those with adrenal masses (Figure 1B). Regarding age, *SDHB*-positive non-familial probands showed the highest age at onset (31 years) amongst all positive cases, as occurred in patients with FA. It is interesting to note that when we consider patients with single tumors, only 3.9% of probands diagnosed above 40 years showed a germline mutation, two of them in *SDHB* (Figure 1A). Thus, 40 years seems to be an appropriate age limit above which we recommend only *SDHB* genetic testing for apparently sporadic patients developing isolated tumors, with the exception of H&N cases in which we

also recommend *SDHC* (Figure 1B), since the mean age of onset reported for *SDHC* cases is 45 years (14).

Malignancy

Half of the patients from our series who developed metastases carried a germline *SDHB* mutation, which agrees with previous reports (15). Though five patients showed *SDHB* deletions (supplemental Table 3), this could be due to the existence of founder effects in the Spanish population, and not to a genotype-phenotype association. We also found 27.2% (3/11) of *SDHD* patients showing metastases at onset, but the small size of our series could explain this high percentage. Two out of these three cases had a primary tumor in an abdominal extra-adrenal location, suggesting that the location is more important than the gene involved.

Founder mutations

To date, a founder effect has been described for two missense *SDHD* and one *SDHB* gross deletion in Dutch and Spanish populations, respectively (16, 17). The c.166_170delCCTCA mutation, first described in a Spanish patient (13), was found in our series in six unrelated probands sharing the same infrequent haplotype (supplemental table 1). A previous reported case carrying this mutation (18) was a Sephardic Jew whose ancestors left Spain in 1453 (personal communication), being likely the second example of a Spanish founder mutation in *SDHB*. Regarding the *SDHD* c.129G>A haplotype, four out of the five French patients carrying the mutation were native to Spain, and there is another Spanish case reported in the literature carrying this mutation (19), so a Spanish origin for this variant seems clear. Finally, despite the fact that the four families carrying the c.334_337delACTG mutation also share an

uncommon haplotype, this variant was previously found in five French patients (20), so that the origin of the variant is uncertain.

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FIGURE LEGEND

Figure 1. A, Schematic representation, considering age at onset and tumor location, of probands that developed a single tumor and had no personal or FA of any related syndrome. The broken line marks 40 years of age, and the genetic status is denoted with a empty rhombus (no mutation found), filled rhombus (*SDHB* mutation), filled circle (*SDHD* mutation), or filled square (*SDHC* mutation). B, Scheme proposed for genetic analysis of non-syndromic PCC/PGL patients based on our data. *, Although we have no positive cases in our series, we cannot rule out the involvement of these genes in the disease based on the literature.

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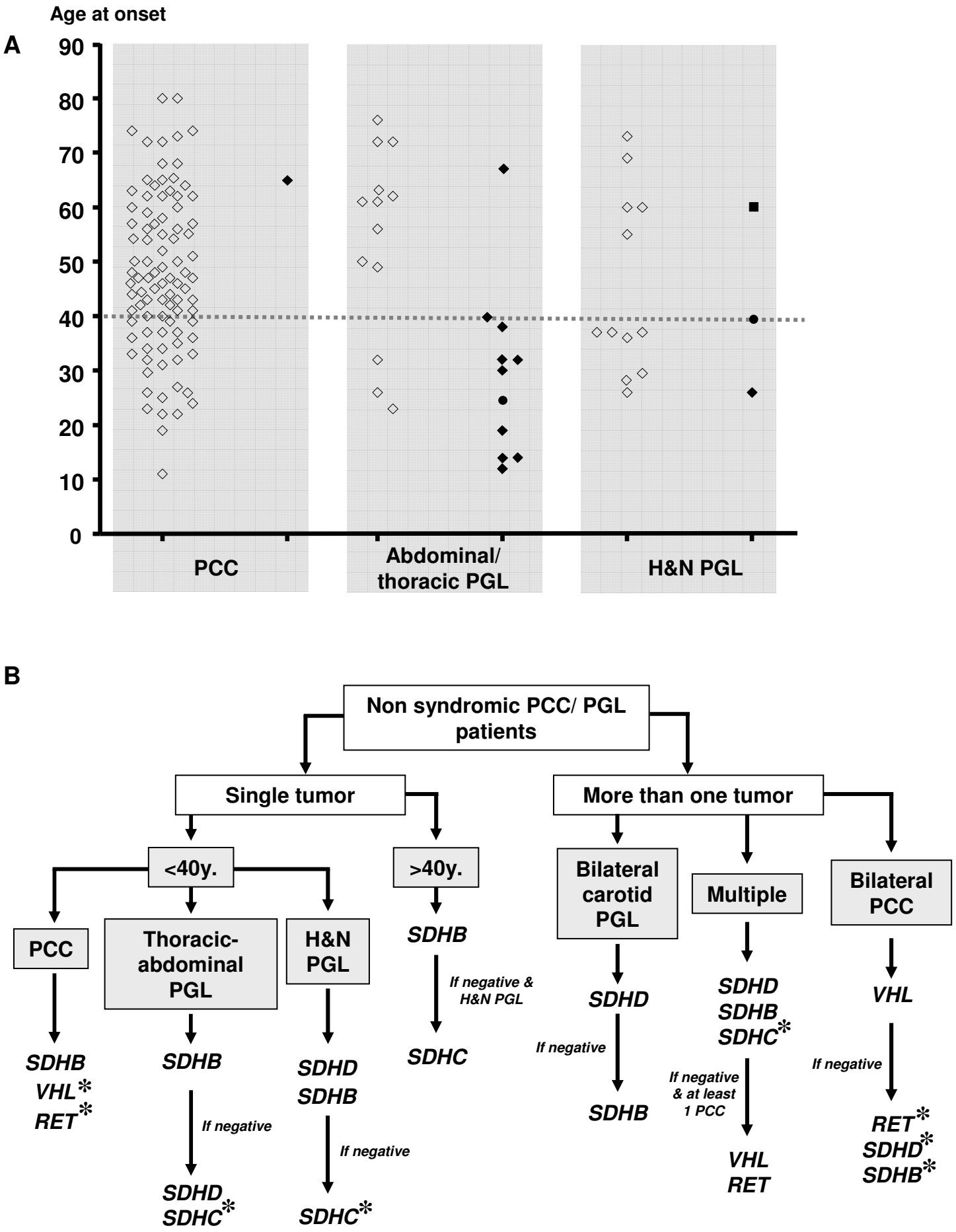
Table 1. Clinical data of non-syndromic index patients

	RET (n=1)	*VHL* (n=10)	P^a	*SDHB* (n=25)	P^b	*SDHD* (n=11)	*SDHC* (n=1)	P^c	No mutation (n=144)	Total (n=192)
Sex										
Male	0	6		14		4	1		56	81
Female	1	4		11		7	0		88	111
Age at onset (years)										
Mean	50	26.4	.458	30.9	.281	24.9	60	**<.001**	46.4	42.0
Range	-	10-52		12-67		11-42	-		11-80	10-80
Cases with familial antecedents	1	3	.698	8	.141	7	0	**<.001**	5	24
Only one tumor	0	1	.546	5	**.026**	0	-	.326	3	9
PCC (bilateral)	1 (0)	3 (2)	.061 (.055)	2 (0)	>.99 (>.99)	2 (0)	-	.630 (.521)	3 (1)	11 (3)
H&N PGL (carotid bilateral)	0 (0)	0 (0)	.491 (>.99)	3 (0)	**.026** (**.001**)	7 (6)	-	>.99 (>.99)	2 (1)	12 (7)
Abdominal/thoracic PGL	1	0	.061	6	**.041**	1	-	.130	0	8
Multiple	1	0	>.99	2	.132	5	-	.615	1	9
Malignant	0	0	.491	3	>.99	2	-	.544	0	5
Cases without familial antecedents	0	7	>.99	17	.141	4	1	**.001**	139	168
Only one tumor	-	0	**.005**	12	.574	2	1	**.001**	118	133
PCC (bilateral)	-	7 (6)	**<.001 (<.001)**	2 (0)	.489 (.191)	1 (0)	0	**<.001** (.104)	109 (13)	119 (19)
H&N PGL (carotid bilateral)	-	1 (0)	>.99 (>.99)	3 (1)	.053 (.080)	3 (2)	1 (0)	**.029** (**.037**)	14 (2)	22 (5)
Abdominal/thoracic PGL	-	1	**.004**	14	.228	2	0	**<.001**	20	37
Multiple	-	3	>.99	6	.558	2	0	**<.001**	5	16
Malignant	-	1	.625	6	>.99	1	0	**<.001**	4	12

Multiple: 2 or more tumors in different organs; P^a, *P* value for the comparison between *VHL* and *SDHB* data; P^b, *P* value for the comparison between *SDHB* and *SDHD* data; P^c, *P* value for the comparison between positive and negative mutation probands

Figure 1


A
Age at onset
90
80
70
60
50
40
30
20
10
0
PCC
Abdominal/ thoracic PGL
H&N PGL
B
Non syndromic PCC/ PGL patients
Single tumor
More than one tumor
<40y.
>40y.
Bilateral carotid PGL
Multiple
Bilateral PCC
PCC
Thoracic-abdominal PGL
H&N PGL
SDHB
If negative & H&N PGL
SDHC
SDHD
If negative
SDHB
SDHD SDHB SDHC*
If negative & at least 1 PCC
VHL RET
VHL
If negative
RET* SDHD* SDHB*
SDHB VHL* RET*
SDHB
If negative
SDHD SDHC*
SDHD SDHB
If negative
SDHC*