Epithelial to Mesenchymal Transition Is Activated in Metastatic Pheochromocytomas and Paragangliomas Caused by *SDHB* Gene Mutations

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Context: Pheochromocytoma and paraganglioma are rare neural-crest-derived tumors. They are metastatic in 15% of cases, and the identification of a germline mutation in the *SDHB* gene is a predictive risk factor for malignancy and poor prognosis. To date, the link between *SDHB* mutations and malignancy is still missing.

Objective: Epithelial to mesenchymal transition (EMT) is a developmental event, reactivated in cancer cells to promote cell mobility and invasiveness. The aim of this study was to address the participation of EMT in the metastatic evolution of pheochromocytoma/paraganglioma.

Design and Patients: Transcriptomic profiling of EMT was performed on 188 tumor samples, using a set of 94 genes implicated in this pathway. Activation of EMT was further confirmed at protein level by immunohistochemistry in a second set of 93 tumors.

Results: Hierarchical unsupervised classification showed that most *SDHB*-metastatic samples clustered together, indicating that EMT is differently regulated in these tumors. Major actors of EMT, metalloproteases and components of cellular junctions, were either up-regulated (*LOXL2*, *TWIST*, *TCF3*, *MMP2*, and *MMP1*) or down-regulated (*KRT19* and *CDH2*) in *SDHB*-metastatic tumors compared with nonmetastatic ones. Interestingly, within metastatic tumors, most of these genes (*LOXL2*, *TWIST*, *TCF3*, *MMP2*, and *KRT19*) also allowed us to discriminate *SDHB*-mutated from non-*SDHB*-related tumors. In the second set of tumors, we studied Snail1/2 expression by immunohistochemistry and observed its specific nuclear translocation in all *SDHB*-metastatic tumors.

Conclusion: We have identified the first pathway that distinguishes *SDHB*-metastatic from all other types of pheochromocytomas/paragangliomas and suggest that activation of the EMT process might play a critical role in the particularly invasive phenotype of this group of tumors. **(*J Clin Endocrinol Metab* 97: E954–E962, 2012)**

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Abbreviations: EMT, Epithelial to mesenchymal transition; Fc, fold change; LOXL2, lysyl oxidase-like 2; SDH, succinate dehydrogenase; SNAI, snail homolog; TCF, transcription factor; TWIST, Twist-related protein.

Pheochromocytomas and paragangliomas are rare neuroendocrine tumors that arise from neural-crest-derived cells of the sympathetic and parasympathetic nervous systems. Around 30–40% of these tumors are genetically determined (1), and up to 10 susceptibility genes have now been described: the *RET* protooncogene (2) and the *NF1* (3), *VHL* (4), *SDHA* (5), *SDHB* (6), *SDHC* (7), *SDHD* (8), *SDHAF2* (9), *TMEM127* (10), and *MAX* (11) tumor suppressor genes.

Pheochromocytomas/paragangliomas are malignant in 15% of cases, but to date, there is no validated histological criterion that allows predicting malignancy on the analysis of the primary tumor (12). World Health Organization recommendations of 2004 defined pheochromocytomas and paragangliomas as malignant tumors by the presence of metastasis in a nonchromaffin organ (13). Nevertheless, it was proved that the identification of a germline mutation in the *SDHB* gene is a risk factor for malignancy (14) and poor prognosis (15). The reasons for such a specific association between *SDHB* gene mutations and invasiveness remain unexplained.

Cancer mortality is highly correlated with metastatic dissemination (16). Epithelial to mesenchymal transition (EMT) is a phenomenon that normally occurs during embryonic development but that is reactivated in cancer cells that thereby acquire migratory and invasive properties (17). During EMT, different expression (or activation) of specific transcription factors leads to the loss of epithelial markers and to the acquisition of hallmarks of mesenchymal phenotype (18–20). Cell-cell and cell-matrix junctions are lost, and extracellular matrix is digested by metalloproteases, leading to important consequences for cell mobility and motility (21). Numerous genes and signaling pathways involved in the different steps of EMT induction have been identified. Transcription factors associated with EMT comprise transcription factor (TCF)3, TCF4, and TCF12 as well as Twist-related protein (TWIST)1, TWIST2, and SNAIL family [snail homolog (SNAI)1 and SNAI2] (22). Signaling pathways that trigger EMT include β-catenin, integrins, and growth factors pathways (epidermal, fibroblast, insulin-like, and vascular endothelial growth factors, TGF-β, bone morphogenetic proteins, and IL) (23, 24). Cell-cell junctions are tight, adherens, gap junctions, or desmosomes, but only adherens junctions and desmosomes have been described to be deregulated in cancer cells. The most important components of these cell-cell and cell-matrix junctions are cadherins, armadillo proteins, and cytoskeleton elements (actin microfilaments or cytokeratin intermediate filament) (25).

Several arguments are in favor of an implication of EMT in pheochromocytoma and paraganglioma malignancy. In two studies that respectively analyzed 49 (including five metastatic *SDHB* samples) (26) and 50 tumor samples (from 42 patients comprising 10 with a metastatic disease, but without genetic characterization) (27), the overexpression of TWIST1 and SNAI1 was correlated with malignancy of pheochromocytoma and paraganglioma. Besides, TWIST1 and SNAI1 are induced by hypoxia (28, 29), a pathway that is known to be activated in *SDHx*- and *VHL*-related tumors (for review, see Ref. 30) and could therefore participate in *SDHB*-mediated invasiveness. SNAI1 is also activated indirectly by hypoxia, thanks to lysyl oxidase-like 2 (LOXL2), a hypoxia-induced protein widely described as implicated in EMT (18).

In this context, and to progress in the understanding of the metastatic abilities of pheochromocytoma/paraganglioma and in particular of *SDHB*-related tumors, we explored global EMT in these tumors by taking into account both patient genotyping and tumor malignancy.

Patients and Methods

Patients and tumor samples

Gene expression data were obtained from a series of 188 tumor samples (Table 1) (31). It comprised 151 adrenal pheochromocytomas, 27 paragangliomas, and six metastases.

Malignancy was defined by the presence of metastases either at presentation or during a recurrence. Distant metastases were confirmed by histology or as lesions detected by computed tomography or magnetic resonance scans and exhibiting [^{123}I]meta-iodobenzylguanidine or [^{111}In]octreotide uptake. The diagnosis of lymph node metastasis was considered only if validated by pathological analysis or in patients with associated extra-paraganglionic metastases. Follow-up was performed as described (32). Germline sequencing was performed for *RET*, *SDHB*, *SDHC*, *SDHD*, *VHL*, *TMEM127*, and and *MAX* for all patients. *SDHA* sequencing was performed for all patients that displayed a 5p15 loss of heterozygosity (5) or an *SDHA*-negative immunostaining (33). *SDHAF2* sequencing was performed only for the patients that displayed an *SDH*-like tran-

TABLE 1. Genetic characteristics of the tumor samples included in the microarray study

	Total	Nonmetastatic	Metastatic
Sporadic	119	108	11
RETs	6	6	0
VHLs	11	9	2
Inherited	69	54	15
RET	9	9	0
NF1	9	9	0
VHL	27	24	3
SDHA	1	1	0
SDHB	17	6	11
SDHC	2	1	1
SDHD	3	3	0
TMEM127	1	1	0
Total	188	162	26

s, Somatic mutation.

scriptome signature (31). Finally, *NF1* sequencing was not performed, the diagnosis being exclusively based on clinical parameters. Tumor samples lacking germline mutation in the main susceptibility genes are referred to as sporadic tumors.

This collection displays a distribution of benign, referred to as nonmetastatic (86%), and malignant, referred to as metastatic (14%), tumors, and inherited (37%) and sporadic (63%) cases, which is representative of the proportions previously reported in large cohorts of patients. Tumor samples were collected by the COMETE network from 177 patients operated on between 1993 and 2008 in two referral centers in Paris (Georges Pompidou European Hospital and Cochin Hospital). The procedures used for pheochromocytoma/paraganglioma diagnosis and genetic testing were in accordance with institutional guidelines (32).

For immunohistochemistry analyses, we studied 93 paraformaldehyde-fixed, paraffin-embedded tumors collected from 81 patients (Supplemental Table 1, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). Fifty-nine of these samples were also included in the microarray study. In this second set of tumors, 24% were metastatic and 54% presented a mutation in one of the susceptibility genes. Three patients were diagnosed for type 1 neurofibromatosis (NF1). The genetic testing identified a *RET* mutation in four patients, an *SDHB* mutation in 11 patients (five metastatic, six nonmetastatic), an *SDHD* mutation in four patients, an *SDHC* mutation in two patients, a *VHL* mutation in seven patients, and an *SDHA* and a *TMEM127* mutation in one patient, respectively. This collection also contained seven tumors harboring a somatic *VHL* mutation and one with a somatic *RET* mutation (31).

Ethics statement

Informed signed consent for germline and somatic DNA analysis was obtained from each patient recruited by the COMETE network, and the study was formally approved by an institutional review board [Comité de Protection des Personnes (CPP) Ile de France III, January, 2007].

Microarray analysis of gene expression

Tumor samples (20–30 mg) were powdered under liquid nitrogen. RNA were extracted using RNeasy mini kit (QIAGEN, Courtaboeuf, France). RNA quality was assessed by electrophoresis on a Bioanalyzer 2100 (Agilent Technologies, Massy, France), and quantity was evaluated using a Nano Drop ND-1000 spectrophotometer (Labtech, Palaiseau, France). Stringent criteria for RNA quality were applied to rule out degradation, especially a 28S:18S rRNA ratio above 1.5. Microarray analyses were performed using 3 μg total RNA of each sample as starting material and 10 μg cRNA per hybridization (GeneChip Fluidics Station 400; Affymetrix, High Wycombe, UK). The total RNA was amplified and labeled following the manufacturer's one-cycle target labeling protocol (http://www.affymetrix.com). The labeled cDNA were then hybridized to HG-U133 Plus 2.0 Affymetrix GeneChip arrays (Affymetrix), and chips were scanned with a GCOS version 1.4. Those microarrays were described as being highly reproducible and as displaying a higher level of concordance with quantitative RT-PCR data than others (34).

Statistical analysis

Except when indicated, all transcriptome analysis was carried out using either an assortment of R system software (http://www.R-project.org, version 2.9.1) packages including those of Bioconductor (35) (version 1.8) or original R code. R packages and versions are indicated when appropriate. Unsupervised classification was performed using a list of 94 genes implicated in EMT (Supplemental Table 2). Such a list was established based on The Human Epithelial to Mesenchymal Transition (EMT) RT^2 Profiler PCR Array (SABiosciences, Courtaboeuf, France), and on the literature data.

Supervised analyses of 1) nonmetastatic *vs.* non-*SDHB*-metastatic tumors, 2) *SDHB*-metastatic *vs.* non-*SDHB*-metastatic, and 3) nonmetastatic *vs. SDHB*-metastatic tumors were performed using the StatView software (SAS Institute Inc., Cary, NC). Differences were evaluated by ANOVA Bonferroni test. A P value <0.05 was considered statistically significant.

We used the R package affyQCReport to generate a quality control report for all chips (CEL files) from the Carte d'Identité des Tumeurs (CIT) discovery series. Raw feature data from Affymetrix HG-U133A Plus 2.0 GeneChip microarrays are normalized using robust multiarray average method (R Package affy) (36).

Immunohistochemistry

Paraffin-embedded tissues were used to prepare 6-μm sections, which were mounted on Superfrost plus glass slides.

We used a SNAI1/2 antibody (Abcam, Cambridge, MA; ab85936, 1/100), and heat-mediated antigen retrieval was performed using 10 mM citrate buffer (pH 6) for 15 min. Biotinylated secondary antibody (Vector Laboratories, EUROBIO/ABCYS, Les Ulis, France) was used, because it reacts with avidin-biotin-peroxidase complex (Vectastain ABC Elite; Vector Laboratories). For peroxidase activity detection, we used Histogreen (EUROBIO/ABCYS). Negative control was performed by omitting the primary antibody.

Acquisitions were performed using Leica DM400B microscope, with ×40 objective, thanks to Leica Application Suite software version 2.8.1 and a Leica DFC420C camera.

All slides were coded and evaluated blindly by two independent observers. Slides were either negative or classified according to the localization of immunolabeling: 1) cytoplasmic, when cells presented only a cytoplasmic immunolabeling and never a nuclear staining; 2) cytoplasmic and nuclear, when the section displayed either cytoplasm-restricted or immunostaining in both cell compartments; and 3) nuclear, when the section displayed zones of cells with a positive immunostaining in the nucleus and an absent or very weak signal in the cytoplasm.

Results

EMT is specifically regulated in *SDHB*-metastatic pheochromocytomas/paragangliomas

Unsupervised classification of our cohort of 188 pheochromocytomas and paragangliomas was performed using a list of 280 probe sets corresponding to 94 actors of the EMT process (Supplemental Table 2), comprising early EMT inducers (transcription factors), members of signaling pathways that trigger EMT (hypoxia, TGFβ/bone morphogenetic proteins, growth factors, and β-catenin pathways), and components of cell-cell and cell-matrix junctions. Such analysis identified two main clusters in the higher partition (Fig. 1). The

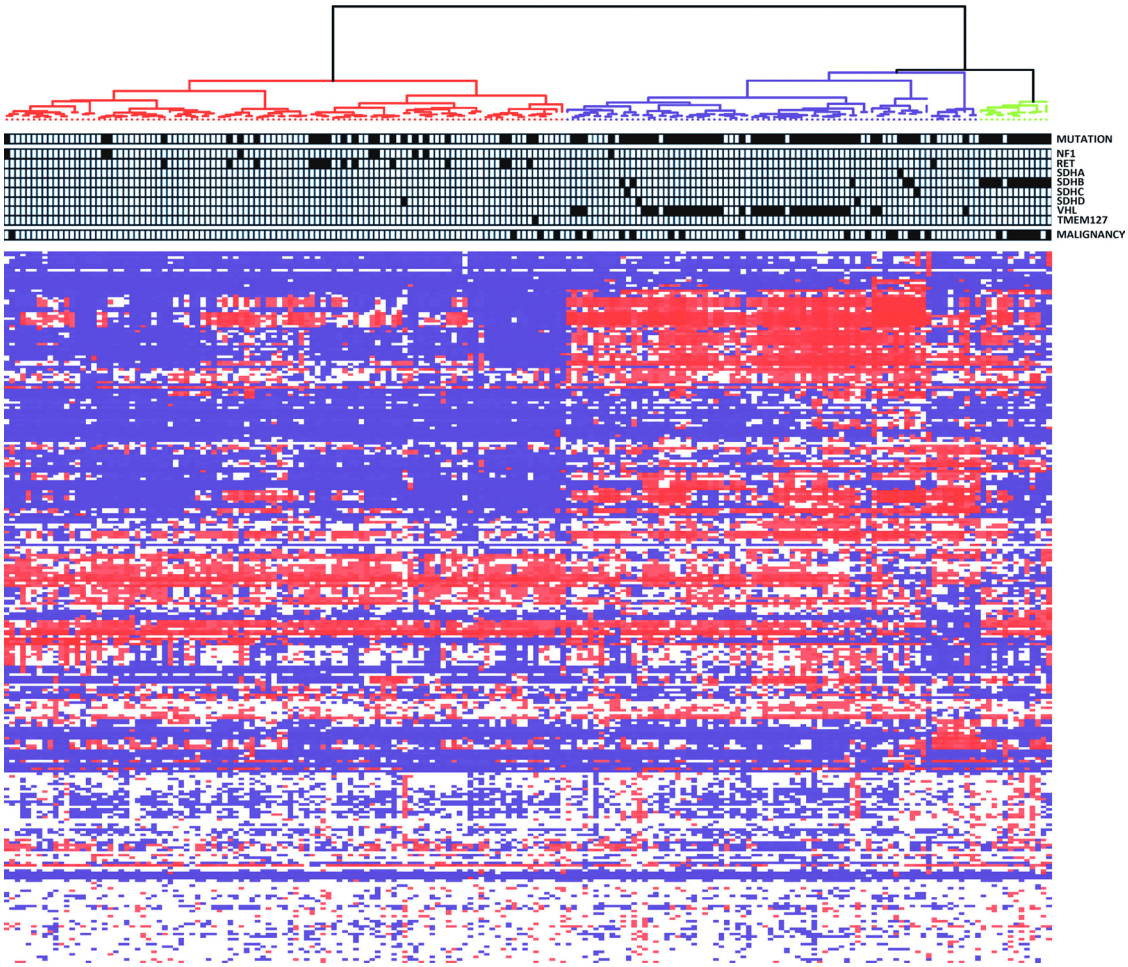

FIG. 1. Microarray analysis of EMT in pheochromocytoma/paraganglioma. Unsupervised hierarchical clustering analysis of 188 samples according to the expression of 94 genes implicated in the EMT pathway. Expression profiles are shown as a heatmap indicating high (*red*) and low (*blue*) expression according to a log2-transformed scale. The higher partition allows distinguishing all *SDHB*-metastatic pheochromocytoma/paraganglioma from all others patients. *Black squares* indicate the presence of an event or of a mutation in the corresponding tumor samples.

first one comprised 103 samples including 79 sporadic samples, eight of nine *NF1* tumors, eight of nine *RET* samples, all (six of six) samples with a somatic *RET* mutation, the *TMEM127*-related tumor, and one of the three *SDHD*-mutated tumors. It contained only four metastatic samples, all sporadic. The second one, which contained 22 of 26 metastatic samples, was divided into two subclusters. One was composed of 72 samples, including 39 inherited tumors (all *VHL*, *SDHA*, and *SDHC* samples, five of 17 *SDHB*, two of three *SDHD,* one of nine *RET*, and one of nine *NF1* tumors) and 33 sporadic ones. It included 58 nonmetastatic and 14 metastatic tumors (three of 11 *SDHB*, one of one *SDHC*, three of three *VHL*, two of two *VHLs*, and five of nine sporadic). The second subcluster contained 13 samples including eight *SDHB*-metastatic, four *SDHB*-nonmetastatic, and one sporadic nonmetastatic tumor. Hence, all but three *SDHB*-metastatic tumors of the series (eight of 11, 73%) were isolated in this last cluster (Fig. 1).

LOXL2, *TWIST1*, and *TCF3* are overexpressed in *SDHB*-metastatic pheochromocytoma

We precisely assessed the microarray RNA expression profile of some important actors of the EMT pathway, such as LOXL2, and the basic helix-loop-helix proteins TWIST1 and TCF3. We compared the gene expression data of each one in nonmetastatic (n = 162), non-*SDHB*-metastatic (n = 13), and *SDHB*-metastatic (n = 11) tumors (Fig. 2A). *LOXL2* and *TWIST1* RNA levels of expression were markedly increased in *SDHB*-related metastatic tumors compared both with nonmetastatic [fold change (Fc) = 5.02, $P < 0.0001$; Fc = 14.74, $P < 0.0001$] and also with non-*SDHB*-metastatic ones (Fc = 2.9, $P < 0.0001$ and Fc = 6.1, $P < 0.0001$, respectively). Although to a lesser extent, *TCF3* was also significantly up-regulated in *SDHB*-metastatic samples compared with nonmetastatic (Fc = 1.57, $P < 0.0001$) and with non-*SDHB*-metastatic tumors (Fc = 1.2, $P < 0.05$). A slight up-regulation of *TCF3* was also observed in non-*SDHB*-

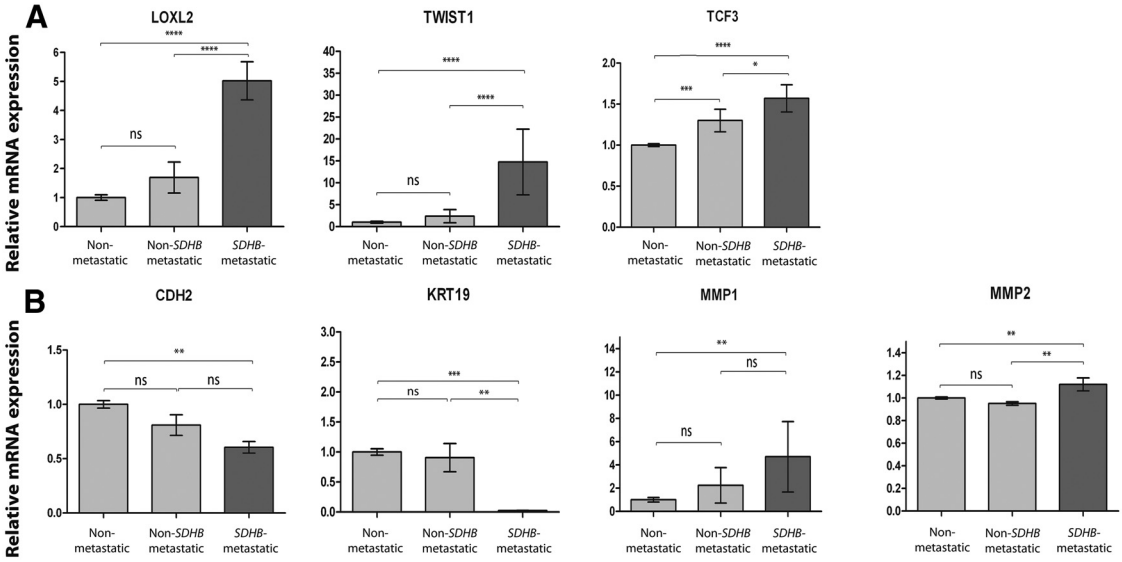

FIG. 2. Early markers of EMT and effects on cell-cell and cell-matrix junction components in pheochromocytoma/paraganglioma. Microarray evaluation of *LOXL2*, *TWIST1*, and *TCF3* (A) and of *CDH2*, *KRT19*, *MMP1*, and *MMP2* (B) expression. Comparisons were between nonmetastatic (n = 162) and non-*SDHB*-metastatic (n = 13), nonmetastatic and *SDHB*-metastatic (n = 11), and non-*SDHB*-metastatic and *SDHB*-metastatic tumors. Expression levels are presented relative to the nonmetastatic group; data are mean ± SEM. ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, not significant.

metastatic tumors compared with nonmetastatic ones (Fc = 1.3, $P < 0.001$).

SDHB-metastatic tumors lose cell-cell and cell-matrix junction markers

We further studied the expression of cell-cell and cell-matrix junction components. In adrenal medulla, which derives from neural crest cells, the only expressed cadherin is neural cadherin (N-cadherin) (37).

We found a major down-regulation of *KRT19* that encodes an intermediate filament required for desmosomes and hemi-desmosomes formation, in *SDHB*-metastatic *vs.* nonmetastatic and non-*SDHB*-metastatic samples (Fc = −42.4, $P < 0.001$; and Fc = −38.4, $P < 0.01$, respectively). *CDH2,* which encodes N-cadherin, was moderately but significantly down-regulated in *SDHB*-metastatic *vs.* nonmetastatic samples (Fc = −1.65, $P < 0.05$) (Fig. 2B).

We also observed differences in the expression of *MMP1* and *MMP2*, two metalloproteases implicated in cell-matrix adhesion. In *SDHB*-metastatic tumors *vs.* nonmetastatic tumors, *MMP1* was clearly up-regulated (Fc = 4.7, $P < 0.01$), whereas *MMP2* presented a minor up-regulation (Fc = 1.12, $P < 0.01$). *MMP2* was also slightly increased in *SDHB*-metastatic tumors *vs.* non-*SDHB*-metastatic samples (Fc = 1.18, $P < 0.01$).

Pseudohypoxia partially participates in the regulation of EMT in pheochromocytomas and paragangliomas

LOXL2, *TWIST1*, and *TCF3* are transcriptionally induced by hypoxia. We thus postulated that their overexpression in *SDHB*-metastatic tumors may reflect the pseudohypoxic drive known to be associated with succinate dehydrogenase (SDH) inactivation (38). We thus evaluated the levels of expression of the previously analyzed EMT-associated genes in several subgroups of inherited tumors and compared them with those observed in the group of nonmetastatic sporadic tumors, used as a control (Supplemental Fig. 1).

Although to a lesser extent than that observed in *SDHB*-metastatic samples, we did observe a significant overexpression of *LOXL2* and partly of *TCF3* in pseudo hypoxic tumors harboring *VHL* and/or *SDHD* genes mutations. Accordingly, *CDH2* tended to be down regulated in *SDHD* and *VHL* samples compared with nonmetastatic sporadic. In contrast, *TWIST1, KRT19* and *MMP1* seemed to be specifically regulated in *SDHB*-mutated metastatic tumors.

SDHB-related tumors display SNAI1/2 nuclear translocation

SNAI1/2 is a transcription factor that represses the expression of ectodermal genes within the mesoderm and plays a critical role during mesoderm formation in the embryo. It is implicated in metastasis and is sufficient to induce EMT (18, 22, 39).

We analyzed its mRNA expression in our large cohort of 188 samples but found no major difference in its expression in any of the groups studied by microarray (Supplemental Fig. 1). We then studied SNAI1/2 protein expression by immunohistochemistry on 93 paraffin-embedded tumor samples (Supplemental Table 1). We

TABLE 2. SNAI1/2 localization according to tumor type

Tumor type	No expression	Cytoplasmic expression	Nuclear and cytoplamsic expression	Nuclear translocation
NF1 nonmetastatic (n = 3)		2	1	
RET nonmetastatic (n = 5)		5		
SDHA nonmetastatic (n = 2)		2		
SDHB metastatic (n = 9)				9
SDHB nonmetastatic (n = 6)	1	3	2	
SDHC nonmetastatic (n = 2)			2	
SDHD nonmetastatic (n = 5)	1	2	2	
TMEM127 nonmetastatic (n = 2)		2		
VHL metastatic (n = 2)		2		
VHL nonmetastatic (n = 14)	1	9	1	3
Sporadic metastatic (n = 11)		7	2	2
Sporadic nonmetastatic (n = 32)	3	17	9	3

did not find any quantitative difference in the level of protein expression in nonmetastatic *vs.* metastatic tumors, but interestingly, we observed modifications in its cellular sublocalization between tumor types. Tumors were thus classified in four different subgroups according to SNAI1/2 localization (Table 2). There was no expression of the protein in six nonmetastatic tumors (Fig. 3A). Fifty-one tumor samples displayed a cytoplasmic expression of SNAI1/2 (Fig. 3B), and 19 samples showed both cytoplasmic and nuclear expression (Fig. 3C). The latter groups contained various types of tumors (indifferently nonmetastatic, metastatic, genetically determined, and sporadic). Finally, SNAI1/2 was restricted to the nucleus in 17 samples (Fig. 3D). This group included all *SDHB*-related metastatic tumors.

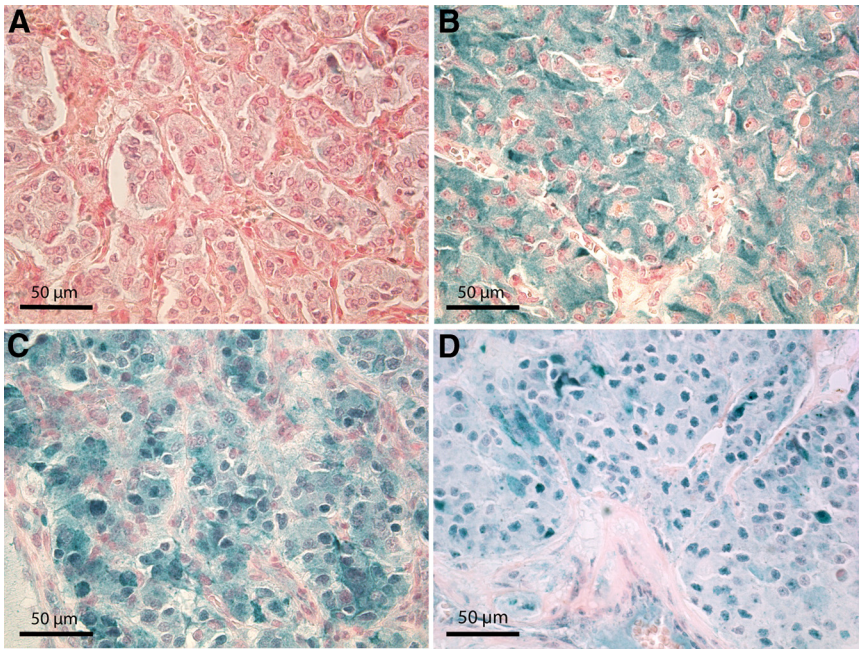

FIG. 3. Differential localization of SNAI1/2 in pheochromocytoma/paraganglioma. SNAI1/2 immunohistochemistry was performed on 93 samples. Tumors were classified regarding SNAI1/2 protein localization, which was either absent (A), restricted to the cytoplasm (B), cytoplasmic and nuclear (C), or strictly nuclear (D). *Calibration bar*, 50 μm.

Discussion

In this study, we evaluated the expression of early markers of epithelial to mesenchymal transition such as LOXL2, TWIST1, TCF3, or SNAI1/2, as well as cell-cell and cell-matrix junction components, in a large cohort of pheochromocytomas and paragangliomas. Our results reveal that EMT is specifically induced in *SDHB*-related metastatic tumors and suggest that this process may be responsible, or at least involved, in the acquisition of the particular metastatic properties of this subset of tumors. These data provide the first relevant clue to elucidate the link between *SDHB*-related pheochromocytoma/paraganglioma and metastatic diffusion.

To evaluate the hypothesis of a role of EMT in metastatic pheochromocytoma and paraganglioma, we used the microarray expression profile of 94 EMT-associated genes to perform an unsupervised classification of 188 pheochromocytomas and paragangliomas that comprised 26 metastatic samples. This study led to a partition in two clusters. Interestingly, all but four metastatic samples were classified in the second cluster. These four samples all corresponded to patients affected by sporadic, slowly evolving adrenal pheochromocytomas (three are still alive 22, 23, and 25 yr after the first surgery, respectively, and one died 36 yr after the first surgery). In the second cluster, a subgroup of 13 tumors was isolated; it comprised all *SDHB*-mutated metastatic tumors, except three samples. To our knowledge, this is the first transcriptome-based classification

that leads to the isolation of such a subgroup of *SDHB*-metastatic tumors. This cluster also contained four *SDHB* nonmetastatic tumors (Supplemental Table 3, patients 1, 4, 5, and 6). The question of the true benignity of a tumor is always difficult in pheochromocytomas and paragangliomas, particularly in an *SDHB*-mutated context. These tumors corresponded to four young patients at diagnosis (10–33 yr old). One of them presents a lung nodule, which is putatively a metastasis of his pheochromocytoma. However, in the absence of histological confirmation or [^{123}I]meta-iodobenzylguanidine or [^{111}In]octreotide uptake, we cannot classify him in the metastatic group, according to our diagnostic criteria. One has a very long disease-free survival without recurrence (30 yr) compared with the two others (respectively, 0 and 11 yr after diagnosis) (Supplemental Table 3). Hence, we cannot exclude that three of these four patients are actually affected by a malignant, not yet metastatic form of their disease. They should now benefit from specific attention in follow-up. The last tumor was a sporadic nonmetastatic paraganglioma, which systematically clusterized with *SDHx*-related tumors in global unsupervised classification and which we recently defined as a pseudo-*SDHx* tumor (31). Here, we confirm this observation and suggest that this tumor is actually similar to *SDHB*-metastatic ones.

TWIST1, LOXL2, TCF3, and SNAI1/2 have been described to be implicated in the metastatic process of numerous malignant diseases such as breast (20, 39), pancreatic, adrenal (26, 40), or renal (19) cancer. Here, we show that not only *LOXL2* and *TWIST1* but also *TCF3* are significantly up-regulated in *SDHB*-metastatic samples, which suggests that they may also be key partners of *SDHB*-dependant pheochromocytoma/paraganglioma malignancy. These genes are known to be overexpressed in hypoxic conditions (19, 28, 29). Although pseudohypoxia does appear to participate in the induction of *LOXL2* or *TCF3* in *SDHB*-related tumors, this process does not explain the global activation of EMT observed in this subset of tumors. Indeed, the global activation of this pathway (up-regulation of *LOXL2*, *TWIST1*, *TCF3*, *MMP1*, and *MMP2*, combined with the down-regulation of *CDH2* and *KRT19*) seemed to be restricted to the association of an *SDHB* mutation with a metastatic phenotype. For example, *TWIST* overexpression and SNAIL nuclear translocation seem to be very specific of *SDHB*-metastatic samples. Hence, we speculate that *SDHB* may modulate EMT-associated genes in a distinctive way, not found in other *SDHx*- or *VHL*-related tumors.

SNAIL is sufficient to induce EMT in cancer cells (18, 39), and two studies recently described a correlation between its overexpression and malignancy in pheochromocytoma and paraganglioma (26, 27). In this study, we did not observe a significant increase in SNAI1/2 expression in the group of metastatic tumors, neither by microarray nor by immunohistochemistry. In contrast, we observed a dramatic change in SNAI1/2 localization, which was specifically translocated to the nucleus in all *SDHB*-metastatic tumors. The reason for such a discrepancy with the previously reported studies may be due to differences in antibodies or immunohistochemistry techniques but is difficult to address because one did not report the genotype of the patients and the other contained only three *SDHB* metastatic patients. Such nuclear translocation, which reflects the activation of SNAI1/2 as a transcription factor, may be the initial step in the activation of EMT, leading to metastasis in *SDHB*-mutated cells. The mechanism involved in this process remains unknown but also appears to be independent of the pseudohypoxic status. It suggests an unexpected and new function of SDHB that will have to be further addressed. Anyhow, SNAI1/2 nuclear translocation could be a criterion allowing gathering of all *SDHB*-metastatic tumor and should be further studied as an innovative prognostic or therapeutic target for patients affected by *SDHB*-related metastatic pheochromocytoma or paraganglioma.

In conclusion, this study identifies EMT as the first pathway that may be responsible for the specific metastatic properties of *SDHB*-related pheochromocytoma or paraganglioma and suggests that SNAI1/2 nuclear translocation may be used as a histological marker of malignancy for *SDHB*-related tumors. Although these observations will have to be validated prospectively in other large series of tumors, they may lead to important clinical consequences in the management of patients harboring *SDHB* mutations.

Acknowledgments

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Complete data sets are available online as ArrayExpress entry E-MTAB-733 (http://www.ebi.ac.uk/arrayexpress/).

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