ANTICANCER RESEARCH *25*: 2809-2814 (2005)

Active Succinate Dehydrogenase (SDH) and Lack of *SDHD* Mutations in Sporadic Paragangliomas

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Abstract. *Background: Paragangliomas are benign, slow-growing tumours of the head and neck region. The candidate gene for familial and some sporadic paragangliomas, SDHD (succinate dehydrogenase, subunit D), has been mapped to the PGL1 locus in 11q23.3. Materials and Methods: Normal and tumour DNA of 17 patients with sporadic paragangliomas were analysed by sequencing (SDHD, SDHB and SDHC genes), fluorescence in situ hybridisation (FISH). In addition, loss of heterozygosity (LOH) and succinate dehydrogenase (SDH) enzyme activity assays were performed. Results and Conclusion: Only two patients from our collective showed SDH gene mutations, one in SDHD and one in SDHB, respectively. Moreover, SDH activity detected in 5/8 patients confirmed the fact that SDH inactivation is not a major event in sporadic paragangliomas. LOH and FISH analysis demonstrated a frequent loss of regions within chromosome 11, indicating that additional genes in 11q may play a role in tumour genesis of sporadic paragangliomas.*

Non-chromaffin paragangliomas (PGL, OMIM 16800) represent a rare form of head and neck tumours, mostly originating from neural crest-derived chief cells of paraganglia, with an incidence of 1:100,000 to 1:1,000,000 (1). They occur between the ages of 14 and 65 (2). In the head and neck region, PGLs seem to appear predominantly at the glomus caroticum, which is a parasympathetic paraganglion located in the wall of the carotid bifurcation. The glomus caroticum reacts as a chemoreceptor to changes in the blood pH and the O_2 and CO_2 partial pressures, thereby contributing to the regulation of respiration and circulation. Even though mainly of benign character, PGLs, especially in the glomus tympanicum region, are usually removed because their spread can lead to symptoms such as hearing impairment, tinnitus or facial nerve paralysis (3). A malignant progression is uncommon, but may emerge in lymph nodes, lung and liver (4). PGLs mostly appear to be sporadic, however, depending on populations, 10 to 50% occur as familial cases (5, 6).

Heutink and co-workers determined 11q22.3-q23 (PGL1) as a chromosomal region frequently involved in the development of PGLs with familial history (7). Two further loci (PGL2-11q13.1, PGL3-1q21) have also been described, implicating the tumour's high level of genetic heterogeneity (8, 9). In 1997, Hirawake *et al.* localised the gene *SDHD* in 11q23.3 and characterised it two years later (10, 11). *SDHD* encodes a subunit of succinate dehydrogenase (SDH) belonging to the mitochondrial complex II (succinate quinone oxidoreductase) of the respiratory chain. SDH consists of four subunits. Two subunits, a flavoprotein (SDHA) and an iron-sulphur protein (SDHB), constitute the catalytic part, which is anchored to the mitochondrial inner membrane by subunits SDHC and SDHD. Thus, the SDHC and SDHD subunits link the citric acid cycle to the respiratory chain. PGL mutations in *SDHB*, *SDHC* and *SDHD* have been described recently and mutation frequencies were determined for these three genes (12-16). *SDHB* and *SDHD* mutations account for up to 70% in familial PGLs, but only for approximately 8% in sporadic cases, with *SDHD* alterations as the leading cause (13). *SDHC* seems not to be frequently involved: the only two mutations in *SDHC* were shown in familial PGLs by Niemann and co-workers (17, 18).

Loss of heterozygosity (LOH) in this region has also been shown, mainly in familial cases (4, 19-22). Consequently, *SDHD* is regarded as the first tumour suppressor gene encoding a mitochondrial protein. The functional loss of both alleles by the "two hit mechanism" (23), *i.e.* point mutation and LOH, leads to a complete inactivation of the

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Key Words: Glomus tumours, *SDHD*-, *SDHB*-, *SDHC*-gene, succinate dehydrogenase (SDH) enzyme activity, loss of heterozygosity (LOH).

mitochondrial complex II (24). Thus, it is assumed that *SDHD* is involved in cellular responses of the carotid body on hypoxia and, if lost, results in a hypoxic stimulus, which may lead to proliferation and tumour genesis (3). Although several studies were performed that indicate an involvement of *SDHD* in the development of hereditary PGLs, mutation analyses in sporadic cases are rather uncommon. Additionally, only a few studies have reported LOH data in sporadic head and neck PGLs (25). For this reason, we aimed to evaluate the significance of *SDHD* and the corresponding chromosomal region (11q23.3-q24) in connection with sporadic paragangliomas.

Materials and Methods

Patients and samples. Seventeen paraffin-embedded tumour samples and corresponding normal tissues (lymph nodes) were retrieved from patients of German origin with sporadic PGLs (glomus caroticum, glomus tympanicum and glomus jugulare). Three patients had malignant tumours (Table I). DNA was derived from the samples by dissection, followed by phenol-chloroform purification and ethanol precipitation. For 8 patients, fresh tumour biopsies were available. According to the Ethics Committee of the University of Tübingen, Germany, written informed consent was obtained from all patients.

SDH activity assay. Freshly-prepared 10-μm frozen sections, derived from PGL tumours of 8 patients, were spread to poly-L-lysine pre-treated slides. For every patient, three sections were prepared: one for histochemical determination of SDH activity, a second one that served as a negative control (SDH assay-protocol without substrate) and a third one for HE staining. SDH activity was determined histochemically, according to protocols described elsewhere (26).

Mutational screening. All four exons of *SDHD* were amplified as described elsewhere (27) and sequenced using the ABI 377 sequencer (ABI, CA, USA). The sequences were compared with NCBI accession number NT_035088 using DNAsis software (MWG, Germany).

Mutation c.341A>G (p.Y114C) in exon 4, which was detected in one patient in heterozygosity, was screened in 114 German control individuals by polymerase chain reaction (PCR) and restriction digest. A 170-bp-PCR product was amplified using the mismatch primer 4F2: CCTTGGACAAGTTGTTACTGCAT and the primer 4R2: AAAAAGGTCAGAGCTTCCACAGCA. Primer 4F2 introduces a restriction site for *Nde*I, which allows the digestion of the wild-type 170-bp allele in two fragments of 148 bp and 22 bp. DNA fragments were visualised on a 6% polyacrylamide gel.

Sequencing of all coding exons of *SDHB* and *SDHC* was performed as described elsewhere (28, 18). Mutation c.647C>T (p.R217C) in exon 7 of *SDHB* was screened using an RFLP in 93 German controls. The primers mutF (ATCCCTGGTTTTCC AGGCCG) and 7r (CTCTTTGTGAG CACATGCTAC) were used to amplify a 211-bp-PCR product. Since the primer mutF introduces a restriction site for *Mbo*I, wild-type alleles can be cut into 188 bp and 23 bp fragments, whereas mutated alleles maintain the size of 211 bp.

Loss of heterozygosity (LOH). Six fluorescent-dye-labelled microsatellite markers (4 dinucleotide and 2 tetranucleotide markers), located on selected regions of chromosome 11, were used for LOH analyses (Table II). Primer sequences were obtained from the Genome Data Base (GDB; http://www.gdb.org). Labelled nucleotides were purchased from MWG, Germany. The PCR was performed using a commercial PCR master-mix (Promega, USA). The PCR conditions and primer sequences can be obtained by contacting the corresponding author. LOH analysis was performed on an automated capillary sequencer CEQ 8000 (Beckman Coulter, USA) and evaluated with the FRAGMENTS software (Beckman Coulter). We assigned a ratio of 0.70 or less to be indicative of LOH (29).

Fluorescence in situ hybridisation (FISH). FISH was performed on paraffin-embedded tumour and normal tissues of each patient to confirm the loss of the entire chromosome 11 using an α-satellite probe specific for the centromere of chromosome 11 (30) and a centromeric probe specific to satellite III DNA on chromosome 1 (31) as reference. Probes for chromosome 11 were labelled with digoxigenin and probes for chromosome 1 with biotin using a NICK-Translation Kit (Roche, Germany). The pre-treatment of the slides and the hybridisation procedure were modified according to Blanco *et al.* to achieve the best results with these tumours (32). Posthybridisation solutions should contain formamide to increase the level of stringency. The slides were washed with 50% formamide/2xSSC at 42˚C, with 2xSSC at 50˚C, then with 0.1xSSC at 55˚C. Fluorescent-labelled antibodies (Sigma, St. Louis, USA) were applied after 30-min blocking with BSA. The slides were counterstained with DAPI/antifade and examined by fluorescence microscopy (Zeiss Axioplan 2 by Zeiss, Germany). Two hundred nuclei were counted per case. All signal counting was performed with a magnification of more than 630.

Results

Mutation screening in candidate genes. All 17 patients were analysed for mutations in the *SDHD* gene representing the gene most frequently involved in PGL tumours. Only two mutations were found in our tumour cohort (Table I). Mutation p.Y114C (c.341A>G) in exon 4, detected in heterozygosity in tumour DNA but not in the normal DNA of patient 8, is a missense mutation that changes tyrosine to cysteine in the protein sequence. This amino acid substitution has been described in only one familial case to date (3) and is now reported in a sporadic case as well. Restriction digest was performed to determine whether the mutation p.Y114C occurs in healthy control individuals of German origin. None of the 114 controls tested positive for this substitution. Furthermore, a nucleotide substitution in exon 3 (c.204C>T) was detected in the tumour DNA of patient 12. This substitution does not result in an amino acid exchange and is to be considered a polymorphism, which was described in one familial and in one sporadic case, respectively (22).

In addition to *SDHD* sequencing, tumours lacking SDH activity were analysed for alterations in the coding regions of *SDHB* and *SDHC*, respectively. In tumour samples derived from patient 6, a nucleotide change in exon 7 of *SDHB* was detected. Mutation c.647C>T (p.R217C) was found in homozygosity in DNA extracted from the tumour tissue and was not detected in normal tissue of this patient. This non-

Table I. *Tumour characteristics and DNA, cytogenetic and enzyme analysis.*

Patient No.	loc.	type	LOH 11q23.3-q24	FISH* (11cen)	SDH-assay	SDH mutations
1	TYMP	B	+	14.5%	+	-
2	CAR	B	+	17%	+	-
3	TYMP	B	+	5.5%	+	-
4	TYMP	B	+	8.5%	+	-
5	CAR	B	+	11.5%	+	-
6	CAR	B	+	nd	–	p.R217C (*SDHB*)
7	CAR	M	+	39.5%	–	-
8	CAR	M	+	<1%	nd^{X}	p.Y114C (*SDHD*)
9	CAR	B	+	28%	–	-
10	IUG	B	+	39.5%	nd^{X}	-
11	IUG	B	+	20.5%	nd^{X}	-
12	IUG	B	+	33.5%	nd^{X}	c.204C>T (*SDHD*)
13	CAR	B	+	11.5%	nd^{X}	-
14	CAR	B	+	11%	nd^{X}	-
15	IUG	B	+	13%	nd^{X}	-
16	IUG	B	+	24%	nd^{X}	-
17	NK	M	+	9%	nd^{X}	-

*percentage of chromosome 11-diploid cells in the tumour, 200 nuclei were counted
+ positive, – negative, nd: not done, ^{X}no material for analysis available, loc.: location, CAR: caroticum, LAR: larynx, IUG: jugular, TYMP: tympanicum; NK: localisation not known, B: benign tumour, M: malignant tumour.

Table II. *LOH result of all patients analysed.*

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
D11S1309, 11p14.1	–	–	+	+	ni	–	+	X	+	+	+	+	+	X	–	–	+
D11S1313, 11p11.11	X	+	+	+	–	+	+	+	+	+	+	+	+	–	–	ni	–
D11S987, 11q13.1#	–	ni	–	–	–	ni	+	ni	–	+	–	–	–	–	+	–	X
D11S898, 11q22.2	X	–	–	–	–	–	+	+	–	+	+	+	–	–	–	X	–
D11S1299, 11q23.3*	+	ni	–	+	+	+	+	X	–	+	+	+	+	–	+	–	+
D11S975, 11q23.3-24*	+	+	+	+	+	+	+	+	+	+	+	+	ni	+	+	+	+

+: LOH, –: no LOH, X: no result, ni: not informative, #: PGL2 locus, *: PGL1 locus

conservative substitution was also screened in 93 German control individuals and no carrier individual was detected. In summary, these infrequent mutations do not support the notion of SDH inactivation being the major cause in sporadic paragangliomas.

SDH activity assay. Where fresh tumour biopsies were available (8 patients), a SDH assay was performed: 5 samples displayed substantial enzyme activity, 3 were considered negative. Figure 1 shows an example of a patient with positive staining in tumour cells, which were identified by additional HE staining. These results confirmed the fact that SDH inactivation is not a major event in sporadic paragangliomas.
LOH analysis. LOH in chromosome 11 was detected in all examined tumour samples. The results are listed in Table II for every patient. LOH events with all probes in patients 7 and 10 suggested loss of an entire copy of one chromosome 11. All tumours showed a common loss of the marker D11S975 in 11q23.3-24 containing the PGL1 locus. LOH in the PGL2 region was detected by marker D11S987 in only 3 patients.

FISH analysis. Loss of chromosome 11 material was additionally monitored by FISH analysis. In normal tissue of all patients analysed, the centromeric probe displayed two signals; in contrast, in all tumour samples this signal occurred only once in most interphase nuclei (Figure 2, Table I), indicating either monosomy 11 or at least partial loss and/or rearrangements.

Discussion

Since the majority of mechanisms underlying PGL tumour development are still unknown, many studies have been undertaken to investigate the molecular basics of sporadic

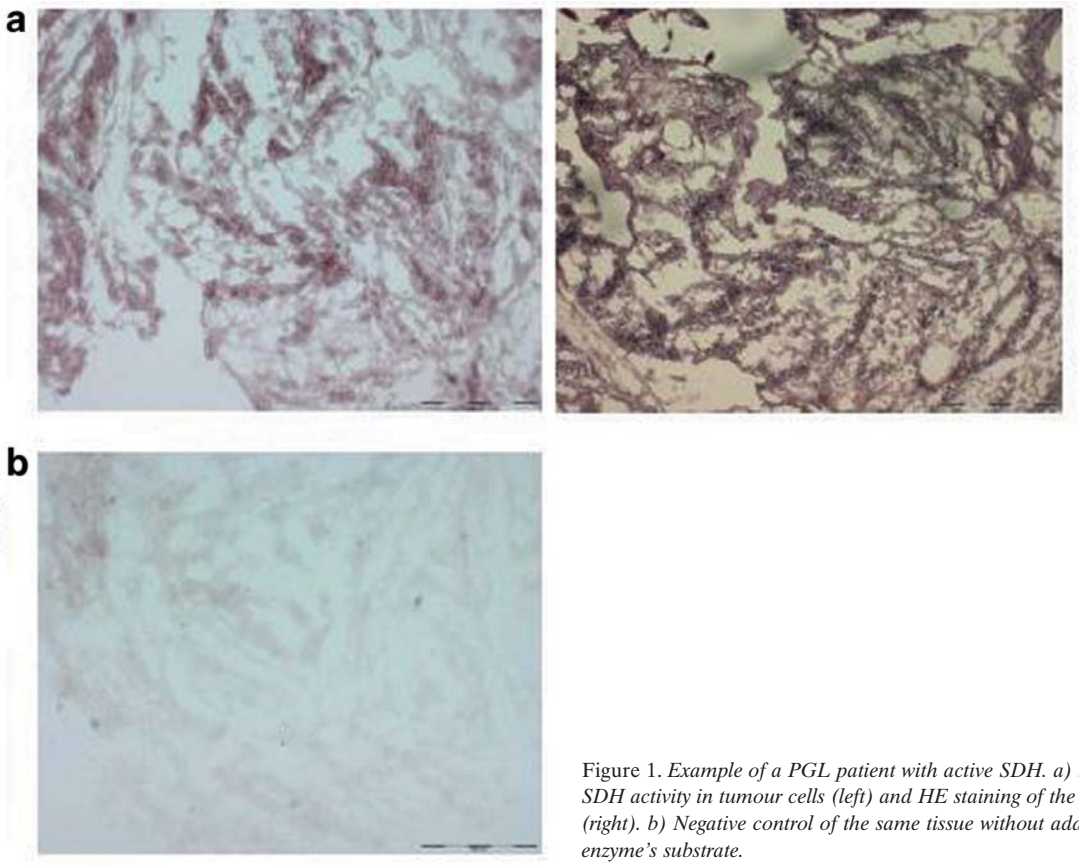

Figure 1. *Example of a PGL patient with active SDH. a) Staining for SDH activity in tumour cells (left) and HE staining of the same tissue (right). b) Negative control of the same tissue without addition of the enzyme's substrate.*

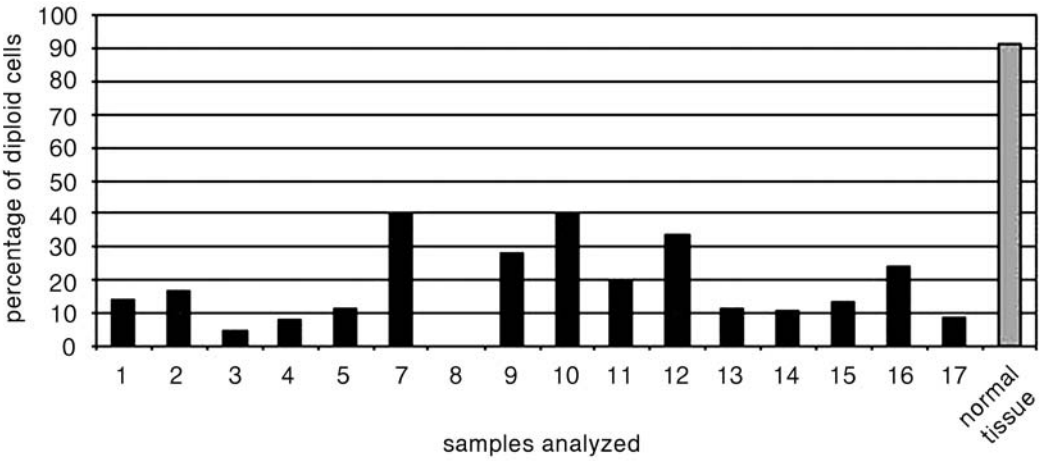

Figure 2. *Results of FISH analysis utilising centromere 11-specific probes on paraffin-embedded tumour samples of PGL patients; 200 nuclei were counted for each case; *mean value of FISH analysis on normal tissue of PGL patients.*

PGLs. The gene frequently involved in oncogenesis has been mapped to 11q23.3-q24 (PGL1 locus) in several Dutch families (33) and has been identified as *SDHD* (27). By using a histochemical visualisation of SDH activity in sporadic PGL tumour cells, sequencing, PCR-based LOH and FISH analyses, we confirmed the importance of this locus. Our results indicated that alterations of the PGL1 locus in 11q23.3-24 contribute to the tumour development, however, the significance of the candidate gene *SDHD* for sporadic paragangliomas was not confirmed.

SDH activity was detected in 5/8 patients with sporadic PGL tumours. For these patients, pathogenic mutations in any one of the four genes coding for the SDH subunits can be excluded because the protein is evidently functional. As expected, sequencing of *SDHD*, the most important candidate gene, revealed no DNA change in these tumour samples. Only three patients failed to display SDH activity. For these cases, one may anticipate mutations in *SDHD*, *SDHB* or *SDHC*, respectively. In fact, one of these patients showed a DNA change in *SDHB*. Mutation p.R217C represents a non-conservative amino acid substitution. Screening of a control collective revealed no mutation carrier, thus implying its relevance for this malignancy. Patient 6 showed the p.R217C mutation only in the tumour but not in normal tissue; moreover, LOH in the PGL1 region was noted. Hence, the two-hit model inactivating both alleles can be assumed for this case. The other two patients without SDH enzyme activity displayed no DNA change, however, the use of freshly frozen tumour material of excellent quality represents one critical factor for the SDH enzyme activity assay. Although alterations in non-coding and promoter regions as well as epigenetic factors were not excluded in this study, it is most likely that the lack of enzyme activity was due to an inactive protein resulting from inferior storage.

Sequencing of *SDHD* revealed only one patient with a mutation. Patient 8, who developed a malignant carotid body tumour, carries a missense mutation in exon 4 leading to the substitution p.Y114C (c.341A>G). A change from tyrosine to cysteine may alter the conformation of the protein, since this is a non-conservative substitution from a polar to a non-polar, sulphur-containing amino acid. A restriction test for mutation p.Y114C was established. Among the control individuals, no carrier of this mutation was detectable. For this reason, p.Y114C is possibly a pathogenic mutation. Recently, we reported three additional PGL cases bearing the mutation c.1A>G, resulting in a destruction of the translation initiation codon (34). Along with the 17 patients analysed here, a *SDHD* mutation frequency of merely 20% (4/20 PGL patients) can be calculated for German patients with sporadic paraganglioma.

LOH in 11q23.3-24 (D11S975) was detected in all analysed PGLs, strongly suggesting that loss of the PGL1 region (beyond *SDHD*) contributes to the formation of these tumours. A second common consensus region was identified next to and reaching into the centromere, indicated by LOH of marker D11S1313, a situation confirmed by FISH analyses applying centromere-specific probes for chromosome 11. Two patients (7 and 10) displayed LOH of all markers tested, which may represent the loss of one copy of chromosome 11, a fact supported by FISH analysis. The phenomenon of monosomy 11 has already been described by performing comparative genomic hybridisation (CGH) on familial and sporadic paragangliomas (4).

Recently, absence of mutations in *SDHD* and *SDHB* in patients with a lack in SDH activity was reported (35). Here, frequent SDH activity and rare mutations in genes coding for SDH subunits suggest that, for our collection of sporadic paragangliomas, additional components were involved in their tumour genesis.

Acknowledgements

This study was supported in part by grants of the *Else-Kröner-Fresenius-Stiftung*, the *fortüne*-program Tübingen no. 1003-1-0 and the Dr. Karl-Kuhn-Stiftung. SB and PL acknowledge a fellowship from the Graduiertenkolleg Onkologie, DFG 686-1, Germany.

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Received November 9, 2004
Accepted April 11, 2005