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SDHA mutations in adult and pediatric wild-type gastrointestinal stromal tumors

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Most gastrointestinal stromal tumors (GISTs) harbor oncogenic mutations in *KIT* or platelet-derived growth factor receptor-α. However, a small subset of GISTs lacks such mutations and is termed 'wild-type GISTs'. Germline mutation in any of the subunits of succinate dehydrogenase (SDH) predisposes individuals to hereditary paragangliomas and pheochromocytomas. However, germline mutations of the genes encoding SDH subunits A, B, C or D (*SDHA*, *SDHB*, *SDHC* or *SDHD;* collectively *SDHx*) are also identified in GISTs. SDHA and SDHB immunohistochemistry are reliable techniques to identify pheochromocytomas and paragangliomas with mutations in *SDHA*, *SDHB*, *SDHC* and *SDHD*. In this study, we investigated if SDHA immunohistochemistry could also identify *SDHA*-mutated GISTs. Twenty-four adult wild-type GISTs and nine pediatric/adolescent wild-type GISTs were analyzed with SDHB, and where this was negative, then with SDHA immunohistochemistry. If SDHA immunohistochemistry was negative, sequencing analysis of the entire *SDHA* coding sequence was performed. All nine pediatric/adolescent GISTs and seven adult wild-type GISTs were negative for SDHB immunohistochemistry. One pediatric GIST and three SDHB-immunonegative adult wild-type GISTs were negative for SDHA immunohistochemistry. In all four SDHA-negative GISTs, a germline *SDHA* c.91C>T transition was found leading to a nonsense p.Arg31X mutation. Our results demonstrate that SDHA immunohistochemistry on GISTs can identify the presence of an *SDHA* germline mutation. Identifying GISTs with deficient SDH activity warrants additional genetic testing, evaluation and follow-up for inherited disorders and paragangliomas.
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Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the digestive tract, but accounts for <1% of all gastrointestinal neoplasms.[1–4] GISTs are derived from the interstitial cells of Cajal and up to 95% of these tumors express CD117 (KIT).[1,4–6] Approximately one-third to half of KIT-negative GISTs stain for DOG-1.[3,7,8] The majority of GISTs arise in the stomach (50–60%) and in the small intestine (30%), other tumor sites include the esophagus, large bowel and rectum (10%).[1,2,4]

Most GISTs (75–80%) harbor oncogenic mutations in KIT (receptor for stem cell factor) and an additional 7% in platelet-derived growth factor receptor-α (*PDGFRA*).[2,5,6,9–11] These GISTs respond to targeted imatinib mesylate therapy, which inhibits tyrosine kinase activity.[12,13] A small subset (10%) of GISTs lacking *KIT* or *PDGFRA* mutations are defined as wild-type GISTs.[9] In pediatric patients, 85% of GISTs are KIT/PDGFRA wild-type. These occur mainly in girls, and usually have a clinically indolent course.[4,9,14,15] Unfortunately, wild-type GISTs respond poorly to imatinib.[16] The pathogenetic mechanism in wild-type GISTs is still not clearly understood.

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Succinate dehydrogenase (SDH), an enzyme that is involved in the fundamental processes of energy production, participates in both the citric acid cycle and the electron transport chain.[17] SDH functions not only in mitochondrial energy generation, but the genes encoding this enzyme also act as tumor suppressors. SDH consists of the subunits SDHA, SDHB, SDHC and SDHD. Germline mutation in any subunit predisposes individuals to hereditary paragangliomas and pheochromocytomas.[18] In addition, mutations in these genes can also cause GISTs.[9,10,19] The familial dyad of paraganglioma and GIST is also known as Carney–Stratakis syndrome.[10,15] Carney triad describes the association of paragangliomas with GISTs and pulmonary chondromas.[15] GISTs arising in the setting of Carney triad or Carney–Stratakis syndrome are also 'wild-type GISTs'.[15]

In previous reports, we showed that negative SDHA and SDHB immunohistochemistry reliably identifies pheochromocytomas and paragangliomas caused by germline mutations in *SDHA*, *SDHB*, *SDHC* and *SDHD*.[20,21] In addition, Carney–Stratakis and Carney triad associated and wild-type pediatric GISTs can be recognized by SDHB immunohistochemistry.[17,22]

In this work, we performed SDHA immunohistochemistry on 33 wild-type GISTs, including nine pediatric/adolescent GISTs, in order to investigate whether immunohistochemistry could identify *SDHA*-mutated GISTs.

Materials and methods

Twenty-four adult wild-type GISTs diagnosed in, or referred to, the Erasmus University Medical Center (Rotterdam, The Netherlands) between 1999 and 2011 were included in this study. In addition, seven pediatric and two adolescent wild-type cases from the files of Professor M O'Sullivan, Dublin, were included in this study. We considered a GIST as 'pediatric' if the tumor was diagnosed below the age of 18 years and 'adolescent' if the age at diagnosis was 19–25 years. All these GISTs previously resulted negative for *KIT* and *PDGFRA* mutations (*KIT*: exon 8, 9, 11, 13 and 17; *PDGFRA*: exon 12, 14 and 18). Clinicopathological features of all these cases are shown in Table 1. The tissues were used in accordance with the code of conduct *Proper Secondary Use of Human Tissue* established by the Dutch Federation of Medical Scientific Societies (http://www.federa.org). The Medical Ethical Committee of the Erasmus MC approved the study (MEC-2011-519).

First, all GISTs were analyzed with SDHB immunohistochemistry. If the tumors were SDHB negative, SDHA immunohistochemistry was performed. Immunohistochemical analysis was performed on 4–5 μm sections of formalin-fixed paraffin-embedded tumor as previously described.[20,21] Slides were considered suitable if the internal control (granular staining in endothelial cells) was positive.

If a tumor was scored negative with SDHB immunohistochemistry, but stained positive with SDHA, the entire *SDHB*, *SDHC* and *SDHD* coding sequences, including intron-exon boundaries, were analyzed for mutations. If SDHA immunohistochemistry was negative, sequencing analysis of *SDHA* (NM_004168) was performed. The entire *SDHA* coding sequence, including intron–exon boundaries, was analyzed for mutations, taking into account the *SDHA* pseudogenes (NCBI: NR_003263, NR_003264, NR_003265). DNA was isolated according to manufacturer's instructions (Gentra Systems, Minneapolis, MN or AllPrep DNA/RNA Mini Kit, Qiagen).

When a mutation was found in tumor DNA, the presence of the mutation was also investigated in corresponding germline DNA isolated from paraffin-embedded healthy tissue surrounding the tumor. Sequence analysis is a semiquantitative procedure and supplies some information on the relative amount of the mutated—versus the non-mutated—*SDHA* allele. To substantiate the relative presence of the mutated—and non-mutated—*SDHA* allele, loss of heterozygosity (LOH) analysis was performed in *SDHA*-mutated tumors for a polymorphic microsatellite marker at the *SDHA* locus. For this, PCRs were carried out with fluorescence-labeled primers (Invitrogen, Paisley, UK) (primer sequences are available on request) for 35 cycles with an annealing temperature of 60 °C, and amplified products were analyzed, along with LIZ 500 size standard (Applied Biosystems, Foster City, CA, USA), using capillary electrophoresis on an ABI 3130-XL genetic analyzer (Applied Biosystems). Data were analyzed using GeneMarker software (Soft-Genetics LLC, State College, PA, USA).

Results

Case series

Case 1. A 41-year-old woman was diagnosed with a gastric GIST. Microscopy showed an epithelioid morphology and the tumor cells stained positive for CD117 and DOG-1 during routine diagnostic work-up. Sequencing analysis of exons 8, 9, 11, 13 and 17 of the *KIT* proto-oncogene and exons 12, 14 and 18 of the *PDGFR*A gene did not reveal mutations. In previous research, this GIST was tested by SDHB immunohistochemistry.[17] The GIST was immunonegative for SDHB and SDHA (Figure 1). Mutational analysis revealed a germline *SDHA* mutation c.91 C>T leading to p.Arg31X. This patient was homozygous (not informative) for the microsatellite marker, so we could not show LOH, though relative loss of the wild-type allele was seen in the sequencing graph (Figure 2, left panel).

Table 1 Clinicopathological features of adult wild-type and pediatric/adolescent GISTs

Case	*Sex*	*Age at diagnosis*	*Location*	*Cell type*	*Other tumors*	*SDH mutation analysis*
1	F[a]	41	Stomach	Epithelioid	MTC[b]	SDHA c.91 C>T[c]
2	F	53	Stomach	Spindle cell	No	SDHA c.91 C>T[c]
3	F	47	Stomach	Mixed	No	SDHA c.91 C>T[c]
4	M[d]	14	Stomach	Mixed	No	SDHA c.91 C>T[c]
5	F	10	Stomach	Epithelioid	No	
6	M	15	Stomach	Epithelioid	CT[e]	
7	F	21	Stomach	Epithelioid	No	
8	F	12	Stomach	Epithelioid	CT	
9	F	10	Stomach	Mixed	No	
10	F	25	Stomach	Epithelioid	No	
11	F	10	Stomach	Epithelioid	No	
12	F	18	Stomach	Mixed	No	
13	M	51	Stomach	Epithelioid	No	
14	M	57	Stomach	Spindle cell	Adrenal myolipoma	
15	M	54	Duodenum	Mixed	Duodenal lipoma	
16	F	69	Stomach	Epithelioid	No	
17	F	47	Stomach	Epithelioid	No	
18	M	56	Liver (meta)	Mixed	No	
19	F	71	Stomach	Spindle cell	No	
20	F	52	Stomach	Spindle cell	No	
21	M	61	Stomach	Spindle cell	No	
22	F	59	Small intestine	Spindle cell	No	
23	M	48	Small intestine	Spindle cell	No	
24	F	51	Jejunum	Spindle cell	No	
25	M	51	Rectum	Epithelioid	No	
26	M	43	Stomach	Mixed	No	
27	F	48	Peritoneum (meta)	Spindle cell	Uterus leiomyoma	
28	M	52	Jejunum	Mixed	No	
29	F	42	Stomach	Mixed	No	
30	F	52	Meso small intestine	Mixed	No	
31	F	76	Stomach	Mixed	No	
32	F	56	Stomach	Mixed	No	SDHD c.416T>C[c]
33	M	58	Stomach	Mixed	No	

[a]Female.
[b]Medullary thyroid carcinoma.
[c]Germline mutation.
[d]Male.
[e]Carney's triad.

At the age of 45, this patient developed a medullary thyroid carcinoma with local lymph nodes and liver metastases. The medullary thyroid carcinoma was positive for SDHB immunohistochemistry, indicating that the tumor was not caused by mitochondrial complex II disruption. Sequencing analysis of the *RET* gene revealed a mutation p.Met918Thr in the medullary thyroid carcinoma and its metastases, but not in the GIST and the healthy germline tissue of the patient. Somatic mutations are known to occur in up to 40% of sporadic medullary thyroid carcinomas.[23]

Cases 2 and 3. Two sisters (respectively, 47- and 53-year-old) were both diagnosed with a GIST located in the stomach. Microscopy of the GIST of sister 1 mainly showed a mixed epithelioid and spindle cell morphology. Tumor cells stained positive for DOG-1 and CD34 and were partly positive for CD117 on routine diagnostic work-up. The tumor of sister 2 showed a spindle cell CD117- and CD34-positive GIST. In both GISTs, no mutations were found in exons 8, 9, 11, 13 and 17 of the *KIT* gene and exons 12, 14 and 18 of the *PDGFRA* gene. Tumor cells of both tumors were immunonegative for SDHB and SDHA. Immunohistochemical stainings for CD117 and SDHA of both tumors are shown in the two bottom panels of Figure 3. Mutational analysis of tumor and germline DNA revealed in both sisters the same *SDHA* mutation c.91 C>T leading to p.Arg31X. However, relative loss of the wild-type *SDHA* allele was only seen in sister 1 and not in sister 2 in the sequence (Figure 3, middle panel). This was confirmed by the LOH analysis, which showed LOH only in the tumor of sister 1 (two upper panels of Figure 3).

Case 4. A 14-year-old boy was diagnosed with a gastric GIST. Histology showed spindle and epithelioid morphology and CD117 was positive in routine diagnostics. Staining for SDHB and SDHA was negative (not shown). The tumor was defined as wild-type, as no mutations were found in exons 8, 9, 11, 13 and 17 of *KIT* and exons 12, 14 and 18 of *PDGFRA*. Mutational analysis revealed a germline *SDHA* mutation c.91 C>T (p.Arg31X) with no

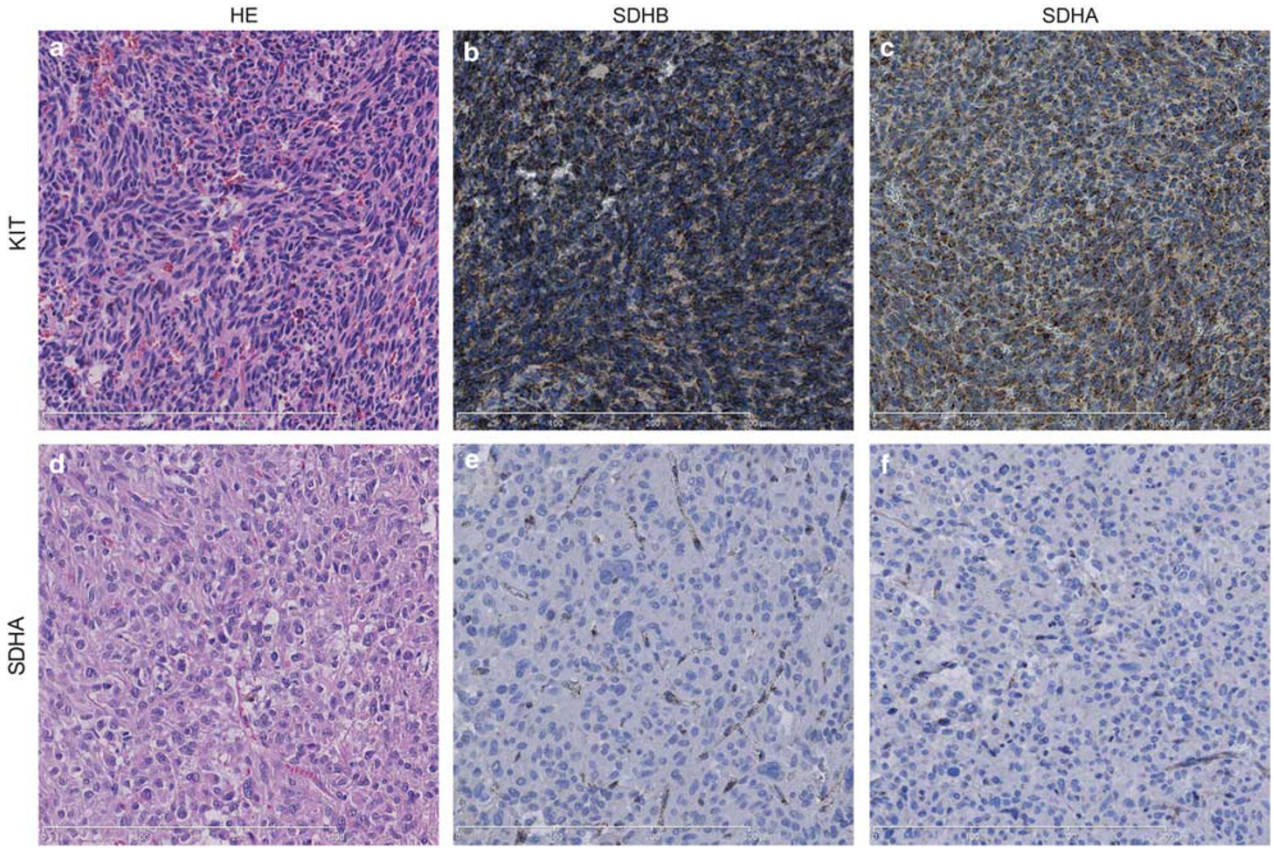

Figure 1 H&E staining and SDHB/SDHA immunohistochemistry. (**a–c**) *KIT*-mutated GIST. (**d–f**) *SDHA*-mutated GIST. (**b**) Strong granular cytoplasmic staining for SDHB and (**c**) SDHA. (**e**) Absent cytoplasmic staining for SDHB and (**f**) SDHA of tumor cells, with positive staining of normal (endothelial) cells.

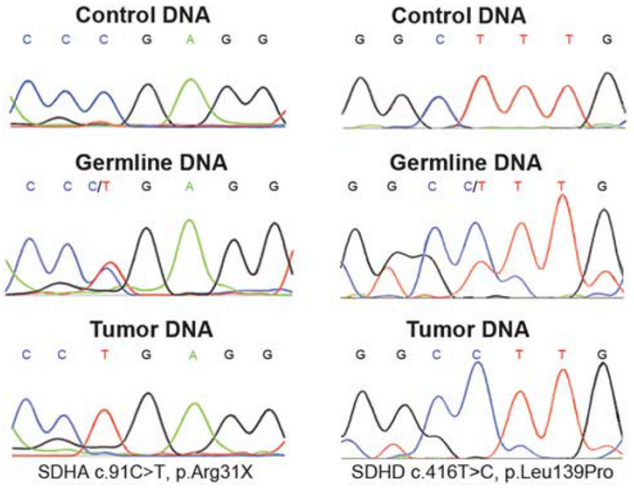

Figure 2 Left panel: chromatogram of *SDHA*-mutated GIST. *SDHA* mutant allele and wild-type allele are shown in germline DNA, and c.91C>T transition leading to a nonsense p.Arg31X mutation in tumor DNA. In addition, tumor DNA only shows the mutant allele, demonstrating loss of the wild-type allele and indicating bi-allelic *SDHA* inactivation (bona fide tumor-suppressor gene). Right panel: chromatogram of *SDHD*-mutated GIST. *SDHD*-mutant allele and wild-type allele are shown in germline DNA and c.416T>C transition leading to a missense p.Leu139Pro mutation in tumor DNA.

relative loss of the wild-type allele. Indeed, LOH analysis showed no LOH in the tumor (Figure 3).

General findings. All nine pediatric/adolescent GISTs were negative for SDHB by immunohistochemistry. Of the 24 adult wild-type GISTs, 7 resulted immunonegative for SDHB (29%). SDHA immunohistochemistry was performed on all SDHB-immunonegative GISTs. One out of nine pediatric/adolescent GISTs (11%) and three out of twenty-four adult wild-type GISTs (13%) were negative for SDHA immunohistochemistry.

Four adult GISTs and eight pediatric/adolescent GISTs were negative for SDHB, but positive for SDHA, by immunohistochemistry. Sequence analysis of these GISTs revealed a germline *SDHD* missense mutation c.416T>C in one adult tumor leading to a p.Leu139Pro. Figure 2 (right panel) shows the sequencing chromatograms of this *SDHD* mutation. Sequencing analysis of the remaining 11 GISTs revealed neither mutations nor LOH in *SDHB*, *SDHC* or *SDHD*.

Sequencing analysis of *SDHA* performed on the four GISTs negative for SDHA immunohistochemistry showed the same *SDHA* nonsense c.91C>T mutation (p.Arg31X) in all four. Powerplex16

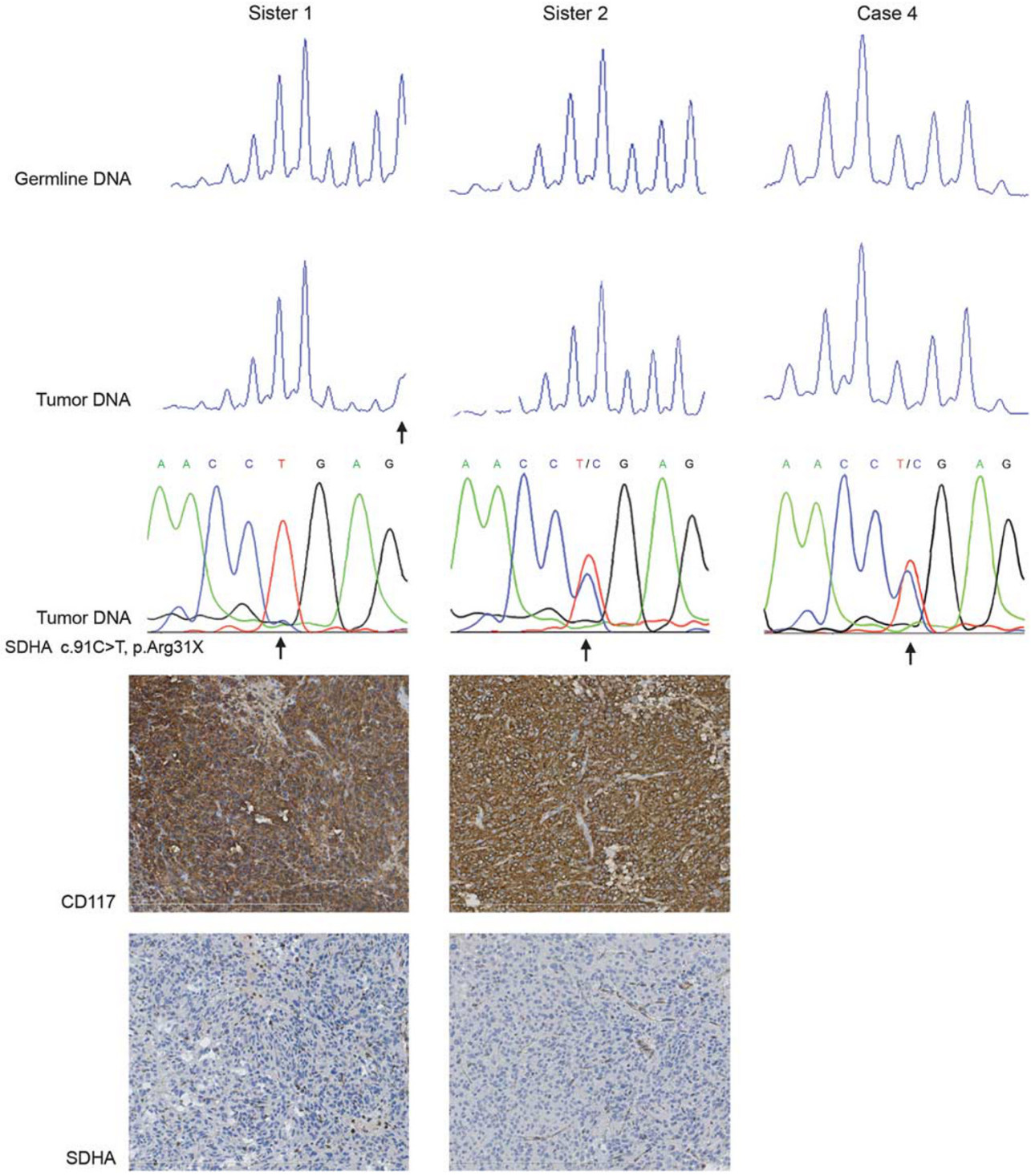

Figure 3 Three upper panels: LOH electropherograms. Sister 1 shows LOH for a microsatellite marker on the centromeric side of *SDHA*. The arrow indicates the allele with relative loss. Sister 2 and case 4 show no LOH. Middle panel: sequencing chromatograms of tumor DNA. *SDHA* p.Arg31X owing to c.91C>T. Arrows indicate the mutation. The chromatogram of sister 1 reveals predominantly the mutant allele, while there is no relative loss of the wild-type *SDHA* allele in sister 2 and in case 4. Two bottom panels: CD117 and SDHA immunohistochemistry in the tumors of both sisters. Strong positive staining for CD117 and absent staining for SDHA of tumor cells, with positive SDHA staining of normal (endothelial) cells.

analysis showed that our four *SDHA*-mutated patients did not share the same alleles (except from the two sisters who showed an overlap of some alleles), excluding contamination (data not shown).

Discussion

The precise role of *SDHA* as a tumor-suppressor gene in oncogenesis is poorly understood.[24] Oncogenic

SDHA mutations have been described in paragangliomas and recently in GISTs.[9,21,24] Burnichon *et al*[24] detected LOH at the *SDHA* locus in 4.5% of a large series of paragangliomas and pheochromocytomas. Loss of the wild-type allele of *SDHA* in a tumor from a patient with a germline-inactivating mutation in *SDHA* indicates that complete loss of SDHA function accompanies tumor formation. In the present study, we found the same *SDHA* (p.Arg31X) mutation in 1 of 9 (11%) pediatric/adolescent wild-type GISTs and in 3 of 24 (13%) adult wild-type GISTs. This inactivating *SDHA* mutation can be detected by SDHA immunohistochemistry, as the tumor cells show absent SDHA staining in the presence of positive staining of internal control normal (endothelial) cells. All four identified *SDHA* mutations were demonstrated to be present in the germline.

Owing to the fact that we found the same *SDHA* germline mutation in four cases, the possibility of a founder mutation was considered. Three of our investigated patients were from the Netherlands and the fourth was from the United Kingdom. In addition, in an Italian patient the same p.Arg31X was described recently[9] Also, Nannini *et al*, and Wagner *et al*, identified the *SDHA* p.Arg31X mutation (among others) in wild-type and SDH-deficient GISTs, respectively.[25,26] The frequency of *SDHA* mutations within the SDH-deficient GISTs (36%) of Wagner *et al,* is slightly higher, but in accordance with our frequency of *SDHA*-mutated GISTs that show a negative staining of SDHB (25%).

The occurrence of the *SDHA* p.Arg31X mutation in three different countries (Italy, United Kingdom and The Netherlands) renders a founder mutation less likely and might suggest a hotspot mutation. The relatively high percentage (12%) of *SDHA* mutations found in the 33 wild-type GISTs in the present study may be owing to the small sample size and owing to patient selection bias, as Erasmus MC is a tertiary referral center.

Interestingly, the *SDHA* p.Arg31X mutation has also been identified in a Dutch healthy control group (0.3%).[21] However, as the mutation causes a truncated protein and three of the four *SDHA*-mutated tumors showed loss of the wild-type allele, according to Knudson's two-hit hypothesis, the p.Arg31X mutation seems to be involved in the pathogenesis of the GISTs. It is possible that the mutation is present in healthy controls because of low penetrance of tumor development in *SDHA*-mutation carriers. In our two *SDHA*-mutated patients without LOH, there is probably a different mechanism responsible for tumor formation, such as inactivation of the wild-type *SDHA* allele by a somatic mutation or promoter methylation of the wild-type *SDHA* gene.

Based on previous findings and our present ones of *SDHA* mutations in wild-type GISTs, we recommend testing for germline mutations of *SDHA* in all patients diagnosed with wild-type GISTs that are negative for SDHA by immunohistochemistry.[9] The link between paragangliomas, pheochromocytomas and GISTs has been established in the Carney–Stratakis syndrome and Carney triad.[10,15] In Carney–Stratakis syndrome, *SDHB*, *SDHC* and *SDHD* mutations have been described.[10] In Carney triad, no mutations in *SDH* have been found.[27] It has been shown that GISTs from patients with Carney–Stratakis syndrome or Carney triad and pediatric GISTs are SDHB negative by SDHB immunohistochemistry.[17,22,28] Janeway *et al*[28] found germline mutations in *SDHB*, *SDHC* and *SDHD* in 6 of 38 wild-type GISTs, but they also found loss of SDHB protein expression in wild-type GISTs without identifiable mutations in *SDHB*, *SDHC* or *SDHD*. This could mean that loss of function of the SDH complex, even without an *SDH* mutation or deletion, contributes to the pathogenesis of wild-type GISTs. However, the absent SDHB expression in their series might be also owing to *SDHA* mutations, for which they did not perform mutational analysis. *SDH* germline mutations were neither found in 66 SDHB-immunonegative wild-type GISTs investigated by Miettinen *et al.*[19] However, the mutational analysis was not performed on all SDH-deficient GISTs in their series, not all the exons of *SDHB*, *SDHC* and *SDHD* were analyzed and again no mutational analysis of *SDHA* was performed.

In accordance with Janeway *et al*[28] and Miettinen *et al*[19], we did not find a mutation in *SDHB*, *SDHC* or *SDHD* in three adult GISTs, which were immunonegative for SDHB, but positive for SDHA, in the present study, but we did find an *SDHD* mutation in one of the cases. Moreover, we did not find any *SDHx* mutations in eight of nine pediatric/adolescent GISTs, even though they were all negative for SDHB immunohistochemistry. Possible explanations for the absence of associated *SDHx* mutations or deletions are mutations in other genes affecting the SDH complex or epigenetic modifications leading to decreased mRNA expression of one of the subunits of the complex. However, we did not compare mRNA expression of *SDHB*, *SDHC* and *SDHD* between the SDHB-immunonegative and -positive cases in our study. In addition, we did not investigate large intragenic deletions of *SDHB*, *SDHC* and *SDHD* in our samples, which can be detected by multiplex ligation-dependent probe amplification analysis. Therefore, large genetic aberrations in the *SDHx* genes cannot be categorically excluded.

As mentioned before, wild-type GISTs respond poorly to the tyrosine kinase inhibitor imatinib. The finding that loss of function of the SDH complex has a role in a subset of wild-type GISTs could be a new focus for treatment. Moreover, identifying GISTs with deficient SDH activity in patients warrants additional genetic testing, evaluation and follow-up for Carney triad, Carney–Stratakis syndrome and paragangliomas.[19] Imatinib targets the constitutively

active tyrosine kinase in GISTs with oncogenic mutations in KIT or PDGFRA.[12] However, the mechanism by which inactivation of one of the subunits of SDH leads to tumorigenesis is still unexplained. Studies suggest that activation of the hypoxic/angiogenic pathway has a role.[29,30] Possibly, pharmacological agents that target the hypoxia pathway or its downstream targets (such as VEGF, GLUT1 and IGF2) could be used as new treatment options.

In conclusion, germline *SDHA* mutations are causal for pediatric/adolescent and adult wild-type GISTs in a subset of patients in our series. SDHA immunohistochemistry can be used to detect GISTs with an *SDHA* mutation and we recommend testing for germline *SDHA* mutations in all patients with SDHA-immunonegative GISTs. Recognition of *SDH*-mutated GISTs by SDHB and SDHA immunohistochemistry is important for prognosis, treatment and follow-up.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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