Hypermethylation of the *IGF2* differentially methylated region 2 is a specific event in insulinomas leading to loss-of-imprinting and overexpression

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Abstract

Prediction of the evolution of endocrine pancreatic tumors remains difficult based on histological criteria alone. We have previously demonstrated that epigenetic changes are an early event in a mouse model developing insulinomas. Particularly, overexpression of the imprinted *IGF2* was caused by the hypermethylation of CpGs in the differentially methylated region 2 (DMR2). Here, we investigated whether *IGF2* hypermethylation is also observed in human insulinomas and whether this alteration is common to other human endocrine tumors of the pancreas and the digestive tract. We analyzed the methylation status of 40 CpGs located in the DMR0 and DMR2 of the *IGF2* as well as in the *H19* DMR by pyrosequencing in a cohort of 62 patients with pancreatic or small intestine endocrine tumors. Altered methylation patterns were observed in all tumor types for the different regions of *IGF2*, but not for *H19*. However, hypermethylation of the *IGF2* DMR2 was specific for insulinomas and did not occur in any of the other types of tumors which were characterized by a loss of methylation in this region. Gain of methylation in the *IGF2* DMR2 in insulinomas correlated with loss-of-imprinting and promoter 4 mediated overexpression of *IGF2* at the RNA and protein level. Furthermore, a decreasing degree of methylation in the different regions of *IGF2* correlated well with increasing degree of malignancy according to the WHO classification of pancreatic endocrine tumors (PETs), suggesting that methylation of *IGF2* might be a useful biomarker for classification and staging of PETs.

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Introduction

Pancreatic endocrine tumors (PETs) represent 1–2% of pancreatic neoplasms, but are of great clinical interest due to their heterogeneous evolutionary potential (Halfdanarson *et al.* 2008). They are classified into functioning or non-functioning tumors based on their clinical manifestations. Functioning tumors are associated with clinical syndromes caused

by inappropriate secretion of hormones such as gastrinomas and insulinomas, the latter being the most frequent islet cell tumor of the pancreas (Grant 2005, Halfdanarson *et al.* 2008). PETs occur sporadically or in association with genetic syndromes such as the multiple endocrine neoplasia type 1 (MEN1) syndrome caused by mutations in *MEN1* and characterized by multifocal endocrine tumors affecting the endocrine pancreas, parathyroids, anterior

pituitary, adrenal, and thyroid glands (Zikusoka *et al.* 2005). Different genetic and chromosomal alterations have been implicated in the pathogenesis of endocrine tumors (reviewed in Zikusoka *et al.* (2005)), and it has recently been shown that epigenetic alterations might play an equally important part during tumorigenesis. DNA methylation plays a key role in the regulation of gene expression, and altered DNA methylation patterns have been observed in several diseases, particularly cancer where genome-wide hypomethylation coincides with gene-specific hypermethylation (Jones & Baylin 2007). Hypermethylation of the promoter region of tumor-suppressor genes including *CDKN2A* (*p14*, *p16*), *COX2*, *RASSF1A*, *hMLH1*, *MGMT*, *ESR1*, and *RAR-β* has been observed in several studies investigating pancreatic and ileal endocrine tumors (Serrano *et al.* 2000, Chan *et al.* 2003, House *et al.* 2003b, Liu *et al.* 2005, Choi *et al.* 2007). Methylation changes including gene-specific hypermethylation and global hypomethylation are generally more pronounced in tumors of the digestive tract than in PETs (Chan *et al.* 2003, Liu *et al.* 2005, Choi *et al.* 2007). Presence of methylation at gene promoters in general and at the *CDKN2A* and *RASSF1A* promoters, in particular, has been associated with tumor recurrence, more advanced tumor stage, presence of metastases, and shortened survival (House *et al.* 2003b, Liu *et al.* 2005), while *hMLH1* methylation associated with microsatellite instability and prolonged survival (House *et al.* 2003a).

Imprinted genes are a subgroup of genes that might be particularly sensitive to DNA methylation alterations during tumorigenesis. Their parent-of-origin-dependent mono-allelic expression pattern is at least partially controlled by CpG-rich differentially methylated regions (DMRs; Reik *et al.* 2001). The insulin-like growth factor 2 (*IGF2*)–*H19* locus at chromosome 11p15.5 is a paradigm of human imprinting (Lewis & Reik 2006). The *IGF2* is expressed from the paternally inherited allele only and plays a key role in mammalian growth and apoptosis influencing fetal cell division and differentiation (Jones & Clemmons 1995). The *H19* gene, located 135 kb towards the telomere, is oppositely imprinted and is expressed only from the maternal allele. The human *IGF2*–*H19* locus is controlled by three DMRs, the *IGF2* DMR0 which is located between exons 2 and 3, the DMR2 (between exons 8 and 9), and the *H19* DMR located 4 kb upstream of the transcription start of *H19*, which is the imprinting control region (ICR) of the *IGF2*–*H19* locus (Fig. 1A). The *H19* ICR contains seven binding sites for the zinc finger protein CTCF, which acts as methylation-sensitive insulator. On the unmethylated

maternal allele, the insulator protein binds to the ICR and forms – at least in mice – higher order chromatin loops that block the access of the enhancers to the *Igf2* promoters (Murrell *et al.* 2004). On the paternal allele, however, DNA methylation blocks CTCF binding and permits the enhancers to access the *IGF2* promoters resulting in *IGF2* expression. Alterations of the methylation patterns of the *H19* ICR lead to loss-of-imprinting (LOI) and disruption of the allele-specific gene expression patterns of the entire locus. These changes have been observed in growth disorders such as Beckwith–Wiedemann and Silver–Russell syndrome (Gicquel *et al.* 2005, Eggermann *et al.* 2008) and in Wilms tumor, where biallelic *IGF2* expression is accompanied by *de novo* methylation and silencing of the maternal *H19* allele (Bjornsson *et al.* 2007). However, LOI of *IGF2* leading to pathological biallelic expression but without alterations of the *H19* ICR is a common phenomenon in cancer, and has been observed in many human cancers including breast and colorectal cancers (Cui *et al.* 2003, Kaneda & Feinberg 2005, Ito *et al.* 2008). Although changes in *IGF2* methylation and expression have to our knowledge not yet been studied in human PETs, several studies hint at a possible implication of *IGF2* in the pathogenesis of pancreatic endocrine neoplasms and more specifically insulinomas.

Igf2 was biallelically expressed in pancreatic β-cells of a mouse model in which the SV40 large T-antigen was under control of the rat insulin regulatory region, alleles were activated early during tumorigenesis and the level of expression was found to correlate with hyperproliferation and tumor growth (Christofori *et al.* 1994, 1995). We have previously shown that mice with a conditional knockout of *Men1* in pancreatic β-cells develop insulinomas (Fontaniere *et al.* 2006). Six CpGs in the core region of the *Igf2* DMR2 were significantly hypermethylated in these mice leading to overexpression of the gene probably through increased levels of transcription initiation (Murrell *et al.* 2001, Fontaniere *et al.* 2006).

Here, we investigated whether the methylation and expression changes observed in the different mouse models are also present in human insulinomas and/or other human endocrine tumors of the pancreas or the digestive tract. We found hypermethylation of the *IGF2* DMR2 to be a specific event in human insulinomas, which does not occur in any of the other types of endocrine tumors that are characterized by a loss of methylation at this locus. The increase in methylation in insulinomas leads to a LOI of *IGF2* and correlates with *IGF2* overexpression at the RNA and protein level. Most interestingly, the methylation level

Figure 1 (A) Structural characteristics of the human *IGF2*-*H19* locus with nine exons and five exons for *IGF2* and *H19* respectively (grey numbered squares). Transcription start and promoters used are indicated by arrows. Regions of differential methylation (DMRs) are shown with black (methylated allele) and white (unmethylated allele) bars. *IGF2* DMR0 (3 CpGs) and *IGF2* DMR2 (DMR2a=17 CpGs and DMR2b=9 CpGs) were analyzed. For the *H19* DMR, the third CTCF-binding site containing 11 CpGs is represented. For each DMR, the number of CpGs analyzed is indicated and their relative positions are represented by filled circles. The number of bp analyzed is indicated under each region. (p, paternal; m, maternal). Overview of the methylation patterns of the *IGF2*-*H19* locus (expressed in percentage of the mean methylation values for each CpG) in normal adjacent tissue (B) and their corresponding tumors (C). (D) The differential DNA methylation for each tumor type between the average tumoral DNA methylation patterns and the peritumoral methylation profile is shown. Details on the average DNA methylation degree for each region as well as the s.d. and range of methylation values are given in the Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>.

of the *IGF2* DMRs correlated well with the WHO classification for PETs with methylation levels decreasing with increasing grade of malignancy.

Materials and methods

Study group

A total of 62 patients with endocrine tumors of the pancreas or the digestive tract were analyzed to investigate changes in the DNA methylation patterns. All patients of this study were submitted to surgical resections in the Hospital Cochin, Paris, France. Tumor samples were collected in accordance with French law and ethical guidelines and their use approved by the institutional ethics committee. Analyzed samples included 11 insulinomas and 5 normal adjacent

pancreatic tissues, 12 gastrinomas and 7 normal adjacent pancreatic tissues and 19 non-functioning PETs with 7 normal adjacent pancreatic tissues, and 18 small intestine endocrine tumors (SIETs) with 11 normal adjacent ileal mucosa (Table 1). The gastrinomas were either of pancreatic (seven tumors) or duodenal wall (five tumors) origin. PETs were classified and graded as benign, uncertain behavior, and well-differentiated endocrine carcinomas according to the WHO criteria (Klöppel *et al.* 2004). Two of the non-functioning PETs and two gastrinomas had a *MEN1* phenotype and *MEN1* mutations were confirmed by mutation analysis.

For expression analysis, 19 corresponding RNA samples were available for 6 of the insulinomas, 4 of the gastrinomas, 3 of the non-functioning PETs, and

Table 1 The main clinical characteristics of patient cohort

		Insulinomas	Gastrinomas	Non-functioning PETs	SIETs	Total
Number of patients		12	12	20	18	62
Age	Min	20	22	18	44	18
	Max	76	68	75	80	80
	Average	48.25	48.33	50	62.28	52.21
Sex	Male	5	10	11	12	38
	Female	7	2	9	6	24
Tumors		11	12	19	18	60
Peritumoral samples		5	7	7	11	30
Number of patients with matched tumor and peritumoral samples available		4	7	6	11	28
Metastases	Positive	0	10	14	18	42
	Negative	11	2	5	0	18
World Health Organization classification	Benign	5	0	0	–	5
	Uncertain behavior	6	2	5	–	13
	Well-differentiated endocrine carcinoma	0	10	14	–	24

5 SIETs. A pancreatic RNA sample from an individual without signs of tumor development was obtained to determine the expression level of *IGF2* in normal pancreas, and expression levels were further verified using two commercial pancreatic RNA preparations from two different lots originating from Caucasian males aged 35 (Clontech).

Preparation of DNA for methylation analysis

DNAs were isolated from frozen tissue samples using standard phenol–chloroform extraction. DNA concentrations were determined using the Quant-iT dsDNA broad range or high sensitivity assay kit (Molecular Probes, Invitrogen) on a SpectraMAX Gemini XPS microplate spectrofluorometer (Molecular Devices, St Grégoire, France) and normalized to a concentration of 50 ng/μl. Genomic DNA of 1 μg was treated with sodium bisulphite using the EpiTect 96 Bisulfite Kit (Qiagen) according to the manufacturer's instructions.

Methylation assays by pyrosequencing

Quantitative DNA methylation analysis of the bisulphite-treated DNA was performed by pyrosequencing or – in case of several sequencing primers – by serial pyrosequencing (Tost & Gut 2007). Particular

attention was paid to the choice of primer sets since a large number of single nucleotide polymorphisms (SNP) was described in these regions. Regions of interest were amplified using 25 ng of bisulfite-treated human genomic DNA and 5 pmol of forward and reverse primer, one of them being biotinylated. Oligonucleotides for PCR amplification and pyrosequencing (see Supplementary Table 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>) were synthesized by Biotez (Buch, Germany). Analysis of repetitive elements as a surrogate measure of global DNA methylation changes was performed as previously described (Yang *et al.* 2004). Standard reaction conditions were 1 × HotStar Taq buffer supplemented with 1.6 mM MgCl₂, 100 μM dNTPs, and 2.0 U HotStar Taq polymerase (Qiagen) in a 25 μl volume. The PCR program consisted of a denaturing step of 15 min at 95 °C followed by 50 cycles of 30 s at 95 °C, 30 s at the respective annealing temperature (see the Electronic Supplementary Table 1), and 20 s at 72 °C, with a final extension of 5 min at 72 °C. Some assays had particular PCR conditions that are detailed in the Electronic Supplementary Table 1. After verification by standard gel electrophoresis on a 2% agarose gel, 5 μl of PCR product were incubated, for 10 min at room

temperature with shaking, in the presence of 40 μ l of binding buffer (10 mM Tris, 2 M NaCl, 1 mM EDTA, 0.1% Tween 20; pH 7.6; adjusted with 1 M HCl), 2 μ l of streptavidin-coated sepharose beads (GE Healthcare, Uppsala, Sweden), and 33 μ l of ddH₂O. The binding mix was purified and rendered single stranded using the Pyrosequencing Vacuum Prep Workstation (Pyrosequencing AB, Uppsala, Sweden) according to the manufacturer's instructions. Beads were released into 12 μ l annealing buffer (20 mM Tris, 2 mM Mg-acetate; pH 7.6; adjusted with 4 M acetic acid) containing 4 pmol of the respective sequencing primer (see the Electronic Supplementary Table 1). Primers were annealed to the target by incubation at 80 °C for 2 min. Quantitative DNA methylation analysis was carried out on a PSQ 96MD system with the PyroGold SQA Reagent Kit (Pyrosequencing), and results were analyzed using the Q-CpG software (V.1.0.9, Pyrosequencing).

mRNA expression analysis

RNAs were isolated from frozen tissue samples using Trizol. Extracted RNAs were resuspended in 5 μ l ddH₂O and diluted ten times. RNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Labtech, Palaiseau, France) and RNA integrity was assessed with an Agilent 2100 Bioanalyzer (Agilent, Massy, France). The mean RNA integrity number (RIN) was 7.94 ± 0.6 with a coefficient of variation (CV) of 7.5%. Samples with a RIN < 7.5 were excluded from further analysis.

One microgram of total RNA was reverse transcribed using Superscript III and oligo(dT) primers (Invitrogen) according to the manufacturer's instructions. Real-time quantitative PCR was carried out using the SYBR green master mix (Applied Biosystems, Foster City, CA, USA) in a Mx 3005P thermocycler (Agilent Technologies). PCR conditions were 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. At the end of the amplification reaction, melting curve analysis was performed to confirm the specificity as well as the integrity of the PCR product by the presence of a single peak. Optimal primer concentrations were determined for each target gene (see the Electronic Supplementary Table 1). Absence of cross-contamination and primer dimers was checked on genomic DNA and water. Four reference genes were tested for variable expression across the samples (Vandesompele *et al.* 2002). The most stably expressed genes were β -actin and β 2-microglobulin, and the geometric means of these two genes were chosen for normalization. Standard curves were generated from

assays made with serial dilutions of reference cDNA to calculate PCR efficiencies ($100 \pm 15\%$, with $r^2 \geq 0.997$). C_t samples were transformed into quantity values using the formula $(1 + \text{Efficiency})^{C_t}$. Only means of triplicate with a CV of < 10% were analyzed. Interplate variation was determined to be below 7%.

Analysis of the expression of promoter-specific *IGF2* transcripts was performed by semi-quantitative RT-PCR (Claire Louet & Hélène Jammes personal communication). Primers for the P1 were situated in exon 1 and exon 3 to yield a product of 352 bp. Primers for the P3 isoform were placed in exon 5 and on the exon junction 7/8 to yield a 113 bp amplification product, and a 439 bp amplification product was obtained for the analysis of P4-specific transcripts using primers in exon 6 and 9.

Analysis of LOI

LOI was assessed by genotyping tumor DNA samples to identify informative samples for the *ApaI* polymorphism in exon 9 of *IGF2* (rs680). The ratio of the expressed alleles was quantitatively analyzed in the corresponding cDNA samples by pyrosequencing. Sequences of primers and modifications to the universal amplification protocol (see above) are given in the Electronic Supplementary Table 1. Genotyping and quantitative determination of allele ratios were carried out on the PSQ 96MD system with the PyroGold SNP Reagent Kit (Pyrosequencing), and results were analyzed using the PyroMark software (V1.0, Pyrosequencing AB).

Immunohistochemistry

We carried out immunohistochemical studies on paraffin sections using a tissue microarray (TMA) containing 28 insulinomas, 50 non-functioning PETs, and 10 peritumoral pancreatic specimens. TMAs were produced using a microarrayer (Beecher Instrument, Woodland, CA, USA). For each tumor, three cores of 0.8 mm in diameter were arrayed. For optimization of the assay and to avoid any confusion through the potential absence of islets of Langerhans on the small TMA sections, five normal pancreatic parenchymas were analyzed on standard paraffin sections. Sections were incubated with an antibody against IGF2 (clone W2H1, dilution: 1/50; Millipore SAS, Molsheim, France).

Statistical analysis

Differences in the distribution of methylation between two groups were assessed by the non-parametric Mann–Whitney or Kruskal–Wallis tests with $P < 0.05$ considered as significant.

Results

To investigate whether the hypermethylation of the *IGF2* DMR2 that we recently reported in a mouse model of insulinomas (Fontaniere *et al.* 2006) is also present in human endocrine tumors, we analyzed the DNA methylation patterns of 40 CpGs localized in the DMR0 (3 CpGs) and DMR2 (26 CpGs) of *IGF2* and at the third CTCF-binding site (11 CpGs) of the *H19* DMR (Fig. 1A) by pyrosequencing in a cohort of 62 patients with pancreatic and digestive tract endocrine tumors.

Tissue-specific differences of *IGF2* methylation

The methylation patterns in the normal adjacent pancreatic tissues ($n=19$) of the three different types of PETs (insulinomas, gastrinomas, and non-functioning PETs) showed on average the expected allele-specific methylation levels of 40–50% for the *IGF2* DMR0 and the two regions analyzed in the DMR2 (Fig. 1B). However, the methylation degree of individual CpG positions showed strong but very reproducible variations (20–70%). No differences were detected between the normal adjacent tissues of the three tumor types. In contrast, the normal adjacent tissue of the SIETs ($n=11$) displayed a significant higher methylation ($\sim 10\%$) compared with the pancreatic tissues for both regions of the *IGF2* DMR2 analyzed (Fig. 1B; Table 2).

Changes of *IGF2* methylation in tumors

Tumors were globally hypomethylated compared with their peritumoral tissues with the exception of the insulinomas (Fig. 1C and D, Table 2; Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Gastrinomas and non-functioning PETs showed a significant hypomethylation for the DMR0 (gastrinomas: 27.3%, compared with the adjacent tissue 44.8%, $P=0.0208$; non-functioning PETs 23.6 vs 47.7%, $P=0.0005$; Fig. 2A). A certain degree of hypomethylation was also found in the DMR2, but this did not reach statistical significance (gastrinomas: DMR2a 34.6 vs 40.1%, DMR2b 32.2 vs 37.2%; non-functioning PETs: DMR2a 33.1 vs 40.6%, DMR2b 32.5 vs 39.6%). The hypomethylation was more pronounced in the SIETs (Fig. 2B). Statistically significant hypomethylation was observed for all DMRs in these tumors compared with the peritumoral tissues (DMR0 25.9 vs 53.6%, $P=0.0003$; DMR2a 35.4 vs 52.5%, $P<0.0001$; DMR2b 29.2 vs 47.8%, $P=0.0008$). The methylation level found in the SIETs was similar to those observed in the gastrinomas and non-functioning PETs.

In contrast to these three tumor types that exhibited different degrees of hypomethylation in the two *IGF2* DMRs, the insulinomas were characterized by a hypermethylation compared with the normal adjacent tissue (Figs 2C and 3A; see the Electronic Supplementary Table 2). A trend towards higher methylation levels was observed in the DMR0 for the insulinomas (59.3 vs 47.4%). The hypermethylation was statistically significant for the two regions of the DMR2 (DMR2a 52.5 vs 41.7%, $P=0.0188$; DMR2b 56.3 vs 39.9%, $P=0.0018$).

Thus, high levels of methylation in the *IGF2* DMR2 and to a lesser extent DMR0 are a specific molecular alteration of insulinomas, which is not observed in any of the other tumor types analyzed.

Methylation of the *H19* DMR

As the DMR upstream of *H19* is the ICR for the entire *IGF2*–*H19* locus, we investigated whether methylation changes were also present in the DMR. The methylation patterns at the third CTCF-binding site were identical in the different peritumoral tissues, showing the expected allele-specific methylation levels (46.4%, Figs 1B and 3B; see the Electronic Supplementary Table 2). The tissue-specific differences observed in the *IGF2* DMRs were thus not present in the *H19* DMR. Peritumoral samples from all tissues showed a reproducible methylation profile with increased methylation levels for CpG positions 2, 3, and 8, whereas the CpG positions underlying directly the CTCF-binding site (CpG positions 4–7) showed a consistent allele-specific methylation (44.6%). Similarly, the methylation patterns were the same for all tumors independent of their origin (42.2%). The same increase in methylation as in the peritumoral tissues was observed at the CpG position 8, but not at the CpG positions 2 and 3. No significant changes were found in the average methylation level between normal and tumors for the different types of PETs. However, SIETs exhibited a significant difference between peritumoral (46.3%) and tumoral tissues (41.9%; $P=0.0134$). When analyzing the difference of methylation at individual CpG positions, CpG position 3 was significantly differentially methylated between peritumoral and tumoral samples in gastrinomas ($P=0.0088$), non-functioning PETs ($P=0.0169$), SIETs ($P=0.0095$), and border line significant for insulinomas ($P=0.0522$; Fig. 2). However, changes were restricted to a single CpG position and it is unclear whether this changes leads to any functional consequences, especially as the

Table 2 Statistical analysis of the methylation differences

	<i>IGF2</i> DMR0	<i>IGF2</i> DMR2a	<i>IGF2</i> DMR2b	<i>H19</i> CTCF3
Insulinomas versus peritumoral tissues	NS	0.0188	0.0018	NS
Gastrinomas versus peritumoral tissues	0.0208	NS	NS	NS
Non-funct. PETs versus peritumoral tissues	0.0005	NS	NS	NS
SIETs versus peritumoral tissues	0.0003	<0.0001	0.0008	0.0134
Insulinomas P versus gastrinomas P	NS	NS	NS	NS
Insulinomas T versus gastrinomas T	0.0012	0.0002	0.0003	NS
Insulinomas P versus non-funct. PETs P	NS	NS	NS	NS
Insulinomas T versus non-funct. PETs T	0.0002	<0.0001	<0.0001	NS
Insulinomas P versus SIETs P	NS	0.009	0.0373	NS
Insulinomas T versus SIETs T	0.0005	0.0002	<0.0001	NS
Gastrinomas P versus non-funct. PETs P	NS	NS	NS	NS
Gastrinomas T versus non-funct. PETs T	NS	NS	NS	NS
Gastrinomas P versus SIETs P	NS	0.0013	0.0248	NS
Gastrinomas T versus SIETs T	NS	NS	NS	NS
Non-funct. PETs P versus SIETs P	NS	0.0043	0.0790	NS
Non-funct. PETs T versus SIETs T	NS	NS	NS	NS

NS, not significant, figures in bold are statistically significant; P, peritumoral tissues; T, tumor tissues; SIETs, small intestine endocrine tumors; Non-funct. PETs, non-functioning pancreatic endocrine tumors.

CpG sites underlying the CTCF-binding site display the expected methylation profile in all tumoral and normal samples.

Expression of *IGF2*

To evaluate potential transcriptional consequences of the methylation changes in the *IGF2* DMRs, expression analysis of *IGF2* was performed in a subset

of the above-described tumors samples by quantitative RT-PCR. *IGF2* was found to be expressed in normal pancreatic tissue. The expression level was significantly different between normal pancreas, insulinomas, and the three other tumor types (Fig. 4A; Kruskal–Wallis test; $P=0.0005$), and insulinomas were also different when compared with the three other tumor types (Mann–Whitney test; $P=0.0016$).

Figure 2 Detailed methylation patterns of the *IGF2*–*H19* locus for tumors and their corresponding normal adjacent tissue. (A) Gastrinomas and non-functioning PETs (data from both groups were regrouped for better visualization), (B) small intestine endocrine tumors, and (C) insulinomas. Data represent the mean of a tumor type or its peritumoral component for each CpG position. Details on the average DNA methylation degree for each region as well as the s.d. and range of methylation values are given in Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>.

Figure 3 Mean methylation level (+s.d.) for *IGF2* (A) and *H19* (B) for each type of tumors and for each region analyzed (gastrinomas and non-functioning PETs are regrouped). The percentage of methylation corresponds to the average methylation level of the specific region. Details on the average DNA methylation degree for each region as well as the s.d. and range of methylation values are given in Supplementary Table 2, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>.

An increased expression level was specifically found in insulinomas, while no difference in the level of expression was found between the other three tumor types (gastrinomas, non-functioning PETs and SIETs). The latter three were found to be less expressed in the tumoral samples compared with normal pancreatic tissue.

LOI of IGF2

As *IGF2* is an imprinted gene, we studied the possible LOI that might explain the increased expression of *IGF2*. Five insulinomas were heterozygous for the *ApaI* polymorphism in exon 9 of *IGF2* and had biallelic expression of *IGF2*. 30–50% of all transcripts could be attributed to the reactivation of the silent allele by the quantification of alleles in the cDNA. All these insulinomas were of uncertain behavior according to the WHO classification. Unfortunately, all other tumors studied (four gastrinomas, three non-functioning PETs, and three SIETs) were all homozygous for this SNP. These did, therefore, not permit to investigate whether hypomethylation is associated with a LOI.

Promoter-specific expression analysis

As *IGF2* expression is regulated by different promoters, we searched for the promoter responsible for the biallelic overexpression. We therefore analyzed the contribution of the promoters P1 (transcript fusing exons 1, 2, 3, (4), 7, 8, and 9), P3 (exons 5, 7, 8, and 9), and P4 (exons 6, 7, 8, and 9) to *IGF2* expression using semi-quantitative RT-PCR with primers specific for the different isoforms. We did not observe consistent amplification using the P3 primers. However, promoters P1 and P4 gave clear amplification products of the expected size (Fig. 4B). In a commercial liver sample, we found as expected *IGF2* mRNA expressed from the P1 promoter, while in normal pancreas *IGF2* was expressed only from the P4 and not the P1 promoter. Gastrinomas and non-functioning PETs did not display expression from either promoter or low expression from the P4 as expected from the total *IGF2* mRNA measurements. One insulinoma was strongly and exclusively expressed from the P1 promoter, two insulinomas had expression from both promoters with the P4 transcript being largely dominant, and two insulinomas were expressed only from the P4 promoter. Total mRNA levels correlated well with the band intensity from the P4 promoter. No correlations between promoter usage and any molecular characteristics were found.

Figure 4 (A) Expression data are represented as a box plot with logarithmic scale. Data are normalized in function of geometric mean of the expression of two references genes (β -actin and β 2-microglobulin). (B) Semi-quantitative analysis of promoter-specific *IGF2* transcripts. P1 and/or P4 transcripts are highly expressed in insulinomas, but very weakly in non-functioning PETs and gastrinomas.

Immunohistochemistry for IGF2

The antibody against IGF2 was tested in normal pancreatic tissue and stained weakly the normal islets of Langerhans (Fig. 5A). In contrast, high or moderated expression of IGF2 was observed in 50% of insulinomas (14 out of 28 cases tested, Fig. 5B). In non-functioning PETs, IGF2 expression was only detectable in 16% of the tumor specimens (8 out of 50 samples tested, Fig. 5C). Good correlation between the immunohistochemistry (IHC) and RT-qPCR data was found. The insulinomas with the highest protein expression in the tumors also had the highest level of RNA expression. However, detectable protein expression decreased rapidly for the weaker RNA expression values. No differences were observed for the staining of the peritumoral tissues compared with normal pancreatic tissues, indicating that the over-expression of IGF2 in the insulinomas is a tumor-specific event.

Methylation is correlated with tumor prognostic

We then investigated a possible correlation between the methylation patterns and the classification and staging system of PETs according to the WHO (benign, uncertain behavior, and well-differentiated endocrine carcinomas). A significant decrease in methylation level for the different DMRs of *IGF2* was correlating with the WHO classification, with the benign group displaying the highest degree of methylation (Fig. 6, Kruskal–Wallis test; $P=0.0068$ for DMR0; $P=0.0013$ for DMR2a; and $P=0.0015$ for DMR2b). In contrast, the methylation was stable for all three categories for the *H19* DMR. However, when analyzing specifically insulinomas that were classified as either benign or of uncertain behavior according to the WHO classification, a small but not significant loss of methylation was observed for *IGF2* DMR0 in the more advanced tumors, while the two analyzed regions

of the DMR2 displayed the same hypermethylation in both groups of the tumors (see Supplementary Fig. 1, which can be viewed online at <http://erc.endocrinology-journals.org/supplemental/>). Hypermethylation of the DMR2 is thus a stage-independent molecular alteration in insulinomas. To rule out that the stage-dependent loss of methylation observed in the other tumors is not caused by global changes at the methylation level in the tumors, we also studied the methylation of repetitive element (*Alu*) by pyrosequencing as previously published (Yang *et al.* 2004). All PET samples were highly methylated at the *Alu* elements for peritumoral and tumoral samples without any significant differences in function of the origin or the stage of the tumor. Only the SIET samples to which the WHO classification does not apply showed a trend towards hypomethylation in the tumor samples compared with the peritumoral tissues (Mann–Whitney; $P=0.088$).

Other molecular and clinical correlates

We analyzed the methylation patterns for a potential correlation with the presence/absence of metastases. To avoid any confounding effect of the tumor-type-specific DNA methylation patterns as well as the tumor stage on the analysis, we investigated the potential influence only in gastrinomas and non-functioning PETs. No association between methylation patterns and the metastatic status of patients was found for any of the DMRs. As gastrinomas originated from either the pancreas itself or the duodenal wall, we analyzed whether any correlation between the origin of the tumor and the DNA methylation patterns could be observed. Methylation patterns from the two subtypes of gastrinomas were identical for all regions analyzed. Some patients with gastrinomas and non-functioning PETs developed tumors in association with a MEN1 syndrome. We therefore compared their methylation patterns to the sporadically developed

Figure 5 Immunohistochemistry on paraffin section using an IGF2 antibody. (A) Weak staining for IGF2 of the islets of Langerhans in a peritumoral tissue, (B) strong staining in an insulinoma, and (C) absence of staining in a non-functioning PET. Staining of normal pancreatic tissue was indistinguishable from the staining of peritumoral tissue.

tumors of the same type. No significant differences were observed for the non-functioning PETs. The gastrinomas with a *MEN1* background, however, showed significantly elevated methylation levels in the *IGF2* DMR0 and DMR2b ($P=0.0339$ and $P=0.0317$ respectively), while the results were borderline significant for DMR2a ($P=0.0532$). Whereas most tumors lose methylation compared with the peritumoral tissues, the tumors with a *MEN1* syndrome display similar levels as the normal tissues. However, these results should be taken with care due to the low numbers of *MEN1*-associated tumors.

Age-related methylation

DNA methylation is known to change in response to environmental stimuli as well as with age. Notably, methylation of *IGF2* has been demonstrated to increase with age in epithelial tissues such as the colon (Issa *et al.* 1996). Therefore, we investigated a potential correlation between age and methylation to rule out any confounding effects on our results due to an effect of age. Peritumoral samples displayed a weak correlation of the methylation level of the *IGF2* DMR2a with age ($R^2=0.1452$) (see Supplementary Fig. 2, which can be viewed

Figure 6 Methylation levels of the *IGF2*–*H19* locus (A) *IGF2* DMR0, (B) DMR2a, (C) DMR2b, and (D) *H19* for all pancreatic endocrine tumors classified according to the WHO (benign, uncertain behavior, and well-differentiated endocrine carcinoma (WDEC)).

online at <http://erc.endocrinology-journals.org/supplemental/>). However, no correlation between age and the observed methylation levels was detected for the pancreatic or SIETs. This clearly demonstrates that tumor-specific molecular events rather than aging determine the methylation levels in the different regions of *IGF2*.

Discussion

In the present study, we analyzed the DNA methylation patterns of the *IGF2–H19* locus in endocrine tumors of the pancreas and the digestive tract by pyrosequencing. The methylation profile of *IGF2* displayed tissue-specific differences with the adjacent tissue of the SIETs being more methylated compared with the pancreatic tissue, which was identical for all three tumor types analyzed.

Insulinomas displayed a very particular DNA methylation profile. The increased methylation levels in the *IGF2* DMR2 were found to specifically distinguish this type of tumor from the other PETs, which are characterized by a more or less pronounced loss of methylation in this region. The other three types of tumors analyzed (gastrinomas, non-functioning PETs, and SIETs) showed a significant hypomethylation of the *IGF2* DMR0, which was not found in the insulinomas. Insulinomas of both benign and uncertain behavior displayed this constant hypermethylation of the DMR2 and to a lesser extent DMR0, clearly showing that this alteration is a hallmark of their tumorigenesis. Insulinomas were also the only group of analyzed tumors that consistently overexpressed *IGF2* at the RNA and protein, while all other tumor types had lower expression levels than normal pancreatic tissue. The overexpression of *IGF2* was restricted to the tumoral component of the tissue, as peritumoral tissue exhibited the same immunohistochemistry staining as normal pancreatic tissue. The overexpression is caused by a LOI and reactivation of the normally silent maternal allele. P4-specific *IGF2* transcripts and to a lesser extent P1 transcripts accounted for the observed increase in *IGF2* transcription. The presence of a heterozygous polymorphism, which is located in exon 9 of *IGF2* and thus close to the analyzed DMR in the cDNA, also precludes the loss of the maternal and duplication of the paternal allele as a possible alternative mechanism for the overexpression of *IGF2*. This has, for example, been reported in adrenocortical tumors that are also characterized by *IGF2* overexpression (Gicquel *et al.* 1997).

Gastrinomas, non-functioning PETs, and SIETs displayed a methylation profile, which is comparable with the one observed in many types of sporadic

tumors with a stable methylation of the *H19* DMR, but significant hypomethylation of at least the *IGF2* DMR0 (Murrell *et al.* 2008). The hypomethylation of the DMR0 itself has been proposed as a DNA methylation-based biomarker for risk assessment of cancer development (Cui *et al.* 2003, Kaneda & Feinberg 2005, Ito *et al.* 2008). Hypomethylation of the DMR0 is often – but not necessarily – associated with LOI (Ito *et al.* 2008). Many tumors retain a mono-allelic expression despite a significant hypomethylation. However, all cancers with LOI also displayed hypomethylation of the DMR0 (Ito *et al.* 2008). Unfortunately, none of the tumor samples other than the insulinomas were informative for the assessment of LOI. Our data, in a different tumor context of course, provide further evidence that loss of imprinting and DNA hypomethylation of the DMR0 changes might be two independent events as we observe LOI in the presence of hypermethylation of the DMR0. The DMR0 has been postulated to have promoter activity (Monk *et al.* 2006). Thus, increased methylation could lead to decreased promoter activity. However, we observed no expression of *IGF2* in the hypomethylated tumors and increased expression despite increased methylation in the insulinomas, which might indicate that the changes in the DMR0 methylation might be a surrogate for other molecular changes but without direct functional consequences.

DNA methylation changes in the DMR2 have, to our knowledge, not yet been assessed in human tumors, but preliminary data from our laboratory show a significant hypomethylation of this region in several types of human cancers including breast, liver, and colorectal tumors (J Tost unpublished observations). However, this hypomethylation is not consistently associated with changes at the transcriptional level.

The observed molecular changes of insulinomas at the DNA methylation and mRNA expression level are similar to those we previously described in a mouse model that develops insulinomas due to a pancreatic β -cell-specific knockout of *Men1* (Fontaniere *et al.* 2006). However, the genetic background of the mice did not allow the investigation of a potential LOI as observed in this study. As in the mouse model, the most significant changes of the DNA methylation patterns were located in the *IGF2* DMR2. In the mouse model, the changes were restricted to the CpG positions that constitute the core region of the DMR2, where DNA methylation leads to increased expression through an augmentation of transcription initiation (Murrell *et al.* 2001). Whether the human DMR2 regulates *IGF2* expression through a similar mechanism remains unclear. The organization of the *IGF2–H19* locus is similar between the two species,

but there are some species-specific differences in elements participating in the regulation and establishment of a higher order chromatin structure at this imprinted locus. A DMR corresponding to the mouse DMR1 has not been identified in human. In mouse, the unmethylated maternal allele interacts with *H19* DMR and places thereby *Igf2* in a silent chromatin loop favoring *H19* expression (Murrell *et al.* 2004). The absence of this DMR in human requires therefore other so far unknown mechanisms to establish the long-range chromatin interactions. A second difference is found for the DMR0. This region is only differentially methylated in the mouse placenta, where methylation occurs on the maternal allele, while this region is methylated specifically on the paternal allele in human tissues (Murrell *et al.* 2008). Taking these differences into account, the conserved hypermethylation of the murine and human DMR2 indicates that the upregulation of *IGF2* expression might be a major factor in the tumorigenesis of insulinomas.

The transcription of *IGF2* is controlled by five promoters (Fig. 1) with P2–P4 being genomically imprinted and mainly expressed in fetal tissues (Li *et al.* 1996). The P1 promoter regulates biallelic expression of *IGF2* in adult liver and has been shown to be reactivated in some tumors (Issa *et al.* 1996). Promoter-specific expression analysis confirmed a partial expression from P1 specifically in some of the insulinomas. However, most of the total RNA could be attributed to transcripts from the P4 promoter, which were significantly increased in insulinomas compared with normal pancreatic tissue or other PETs. Upregulation of *IGF2* expression through activation of fetal promoters has previously been reported in various cancers including hepatocellular carcinoma (Tang *et al.* 2006). This increased expression has so far not been correlated with DNA methylation changes in the DMR2, but only at the respective promoter sites. Hepatocellular carcinoma is characterized by a loss of methylation in the *IGF2* DMR2 (J Tost unpublished observations), which correlates potentially with the reported hypomethylation of the activated promoters in carcinogenesis (Tang *et al.* 2006). Insulinomas, however, activate P4 expression through a different mechanism. Here, we provide evidence that hypermethylation of the DMR2 leads to – or correlates with – an increased expression of *IGF2* through biallelic activation of the P4 promoter. Thus, in analogy to the mouse model for *Igf2* regulation, hypomethylation of the promoter could directly increase transcription of the P4 promoter, while hypermethylation of the DMR2 might increase transcription initiation at the respective promoter.

Although patients with insulinomas did not display a MEN1 phenotype, we cannot exclude that some of them harbor inactivating point mutations in *MEN1*. However, taking the results presented in this study into account, it seems to be more likely that the observed *Igf2* hypermethylation in the *Men1*^{−/−} models is part of the tumorigenesis of insulinomas rather than being directly induced by the *Men1* inactivation.

IGF2 has been found to be upregulated through different mechanisms in many tumor types. As the activation of a strong oncogene such as *k-ras* or *myc* is not a common event in PETs (Oberg & Eriksson 2005), it might well be that the autocrine growth-promoting properties of genes such as the IGFs enable and stimulate cell proliferation and tumorigenesis. However, insulinomas do apparently have a balancing mechanism compensating the upregulation, as this does not have deleterious consequences for the patient. While in the SV40 large T-antigen-induced insulinoma mouse model (Christofori *et al.* 1994, 1995) as well as in multiple tumor types such as gastrointestinal stromal tumors (Braconi *et al.* 2008), *IGF2* overexpression contributes to tumor growth, increased mitotic indices, higher risk of relapse, and worse prognosis; insulinomas are benign tumors with a good prognosis.

Multiple studies have assessed molecular changes and prognosis of PETs (reviewed in Halfdanarson *et al.* (2008)). However, results are often confounded and skewed as different types of PETs have not been separately analyzed. For example, insulinomas and gastrinomas have often been categorized together as functioning PETs. However, our study clearly shows that they differ in terms of their molecular signature and probably in their mechanism of tumorigenesis. They should therefore be treated as different entities.

The understanding of the pathobiology of endocrine tumors as well as their clinical management is currently complicated by the difficulty to accurately assess tumor prognosis and disease outcome. The WHO classification provides some guidelines for the classification of PETs depending on molecular parameters such as tumor size, proliferative and hormone activity, angioinvasion, and metastases. Understanding the key molecular changes in the different types of PETs is thus a crucial step towards a better tumor classification, an improved diagnosis and prognosis, and a tumor-specific rationalized therapy. Here, we have established a tight correlation between the WHO classification and the methylation levels of different DMRs of *IGF2*. DNA methylation decreases gradually with advanced tumor stage with the exception of the above-discussed methylation patterns of the DMR2 in insulinomas. Most tumors display a

global hypomethylation of the genome, which has been suggested to initiate and propagate oncogenesis by inducing chromosome instabilities and transcriptional activation of oncogenes and pro-metastatic genes (Ehrlich 2002). However, the decrease in DNA methylation in *IGF2* is gene specific and not a surrogate for global DNA methylation changes, as demonstrated by the unaltered DNA methylation levels for *Alu* elements in the analyzed PETs. Our observations are in concordance with another recent study (Choi *et al.* 2007), which also showed that only endocrine tumors of the small intestine showed a trend towards hypomethylation.

The methylation patterns of *IGF2* offer therefore a molecular marker for the better understanding of the heterogeneous evolutionary potential of PETs. Larger prospective studies requiring probably multicenter collaborations due to the rare occurrence of these tumors will be necessary to fully evaluate the value of this novel promising DNA methylation-based biomarker compared with the WHO classification.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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