Copy number variations alter methylation and parallel \textit{IGF2} overexpression in adrenal tumors

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Abstract

Overexpression of \textit{insulin growth factor 2} (\textit{IGF2}) is a hallmark of adrenocortical carcinomas and pheochromocytomas. Previous studies investigating the \textit{IGF2}/\textit{H19} locus have mainly focused on a single molecular level such as genomic alterations or altered DNA methylation levels and the causal changes underlying \textit{IGF2} overexpression are still not fully established. In the current study, we analyzed 62 tumors of the adrenal gland from patients with Conn’s adenoma (CA, \(n = 12\)), pheochromocytomas (PCC, \(n = 10\)), adrenocortical benign tumors (ACBT, \(n = 20\)), and adrenocortical carcinomas (ACC, \(n = 20\)). Gene expression, somatic copy number variation of chr11p15.5, and DNA methylation status of three differential methylated regions of the \textit{IGF2}/\textit{H19} locus including the \textit{H19} imprinting control region were integratively analyzed. \textit{IGF2} overexpression was found in 85\% of the ACCs and 100\% of the PCCs compared to 23\% observed in CAs and ACBTs. Copy number aberrations of chr11p15.5 were abundant in both PCCs and ACCs but while PCCs retained a diploid state, ACCs were frequently tetraploid (7/19). Loss of either a single allele or loss of two alleles of the same parental origin in tetraploid samples resulted in a uniparental disomy-like genotype. These copy number changes correlated with hypermethylation of the \textit{H19} ICR suggesting

Key Words

\begin{itemize}
\item adrenocortical tumors
\item pheochromocytomas
\item \textit{IGF2}
\item \textit{H19}
\item cancer
\item imprinting
\item DNA methylation
\item copy number analysis
\item tetraploidy
\end{itemize}
that the lost alleles were the unmethylated maternal alleles. Our data provide conclusive evidence that loss of the maternal allele correlates with IGF2 overexpression in adrenal tumors and that hypermethylation of the H19 ICR is a consequence thereof.

Introduction

Tumors developing in the adrenal gland include a heterogeneous group of adrenocortical and adrenomedullary malignancies (Brunt & Moley 2001, Grumbach et al. 2003). Most adrenal tumors are benign and non-hormone producing. However, adrenocortical masses require attention as hypersecretion of steroid hormones and metabolic changes may be present, which might lead to hypertension, abdominal pain, gender disorders, weight gain, or increased blood sugar levels depending on which adrenal hormone is dysregulated (Low & Sahi 2012, Hodin et al. 2014). Adrenal tumors with increased aldosterone secretion lead to Conn’s syndrome (CA), whereas pheochromocytomas (PCCs) secrete excess amounts of catecholamines (Low & Sahi 2012, Hodin et al. 2014). Adrenocortical carcinomas (ACCs) can present as either non-functional or functional tumors and represent the most aggressive group of adrenal tumors. ACCs have often metastasized at the time of diagnosis and therefore are associated with poor prognosis (Volante et al. 2008).

Several genes and pathways have been implicated in the pathogenesis of ACCs including the WNT/β-Catenin pathway, and overexpression of the orphan nuclear receptor SF1 (Fassnacht et al. 2011, Simon & Hammer 2012). Recently, integrated large-scale analyses have identified alterations in driver genes including CTNNB1, TP53, CDKN2A, RB1, ZNRF3, and MEN1 (Barzon et al. 2001, Tissier et al. 2005, Assie et al. 2014, Juhlin et al. 2015). The insulin-like growth factor system has attracted much interest (Ribeiro & Latronico 2012) as the Insulin-like Growth Factor 2 (IGF2) is the most frequently overexpressed gene in ACCs (de Fraipont et al. 2005). High levels of IGF2 have been found to differentiate benign adrenocortical tumors from ACCs at both the RNA and the protein level (de Fraipont et al. 2005, Schmitt et al. 2006, Giordano et al. 2009, Ragazzon et al. 2011). Benign adrenal tumors express IGF2 and H19 at a level similar to normal adrenal tissue (Ilvesmaki et al. 1993a, Liu et al. 1995). In contrast, ACCs and PCCs show a downregulation of H19 expression and overexpression of IGF2 (Ilvesmaki et al. 1993a, Gicquel et al. 1997, Margetts et al. 2005, Slater et al. 2006, Soon et al. 2009, Meyer-Rochow et al. 2010, Ragazzon et al. 2011). Although IGF2 on its own is not sufficient for transformation, it has an active role in promoting ACC tumor growth (Guillaud-Bataille et al. 2014) and the IGF family plays an important role for the development of adrenal tumors, and has been proposed as therapeutic target (Ribeiro & Latronico 2012). IGF2 and the neighboring H19 are located on chromosome 11p15.5 and form the paradigmatic imprinted IGF2/H19 locus (Supplementary Fig. 1, see section on supplementary data given at the end of this article). IGF2 is expressed from the paternal allele only and due to its high expression levels in the fetal adrenal gland has been considered as a key mitogen for its early growth and development (Ilvesmaki et al. 1993b, Mesiano et al. 1993, O’Dell & Day 1998). After birth, IGF2 expression levels decrease drastically and its expression is concentrated to the adrenal capsule and the periphery of the cortex (Baguedano et al. 2005). H19 is maternally expressed and functions both as a long non-coding RNA involved in tumor suppression (Hao et al. 1993) and as a trans-regulator of a network of imprinted genes (Gaborzy et al. 2009).

Three differentially methylated regions (DMRs) are involved in the transcriptional regulation of the IGF2/H19 locus (Supplementary Fig. 1, see section on supplementary data given at the end of this article). IGF2 DMR0 and IGF2 DMR2 are located between exons 2 and 3 and exons 8 and 9 respectively, while the H19 DMR is located 4 kb upstream of the H19 transcription start site. The H19 DMR represents the imprinting control region (ICR) of the IGF2/H19 locus and harbors seven binding sites for the methylation-sensitive insulator CTCF, a multifunctional protein involved in nuclear organization (Kanduri et al. 2000, Holmgren et al. 2001, Ohlsson et al. 2010), which brings downstream enhancers into physical proximity to either the IGF2 or the H19 promoter through parent-of-origin dependent methylation patterns of the H19 ICR (Jinno et al. 1996, Frelvet et al. 1999, Bell & Felsenfeld 2000, Kanduri et al. 2000).

The exact mechanisms underlying IGF2 overexpression in adrenal tumors are poorly understood. DNA
hypermethylation of the H19 promoter region has previously been associated with IGF2 overexpression in ACCs (Gao et al. 2002) as well as somatic copy number changes, where loss of the maternal allele was accompanied by a duplication of the paternal allele (Gicquel et al. 1994, 1997). Maternal loss of chr11p15.5 has also been implicated in the IGF2 overexpression observed in PCCs (Margetts et al. 2005).

While genetic and epigenetic mechanisms are closely intertwined, it is currently unclear if DNA methylation and genetic aberrations are independent events leading to IGF2 overexpression or if DNA methylation patterns reflect only changes at the genetic level. In the current study, we provide the first integrated analysis of gene expression, DNA methylation and genetic variation of the IGF2/H19 locus in adrenal tumor subtypes using high-resolution molecular technologies to unravel the driving force behind IGF2 overexpression in these tumors.

Materials and methods

Study group

Sixty-two adrenal tumor samples from the Timone Hospital (Marseille, France) were analyzed in this study. Clinical and pathological data are summarized in Table 1. Four groups of ACTs were included in the study: 12 aldosterone producing adenomas in the context of Conn’s syndrome (CA), ten pheochromocytomas (PCCs), 20 adrenocortical carcinomas (ACCs) of which two samples (51 and 52) were derived from the two adrenal glands of the same patient, and 20 adrenocortical benign tumors (ACBT). ACBT were either non-secreting cortical tumors or cortisol secreting adrenal tumors with no evidence for malignancy. ACTH dependent bilateral adrenal hyperplasia and ACTH independent adrenal hyperplasia (primary pigmented nodular disease) were not included in this cohort. Adrenocortical tumors were staged using the Weiss score, PCCs were staged using the Pheochromocytoma of the Adrenal gland Scale Score (PASS), which both take histological features such as tumor size, presence of necrosis and mitotic activity including atypical mitoses into account (Weiss 1984, Thompson 2002, Lau & Weiss 2009). An adrenocortical tumor with a Weiss score of three or higher was classified as ACC and PCCs with a PASS score of four or higher were classified as malignant. All pheochromocytomas lacked a positive familial history indicating hereditary disease and had no mutations in the SDHB, SDHD and VHL genes as assessed by Sanger sequencing. Written informed consent was obtained from all patients and the study was approved by the local ethics committee.

Isolation of DNA and RNA

All tumoral tissue samples were carefully evaluated by microscopy to ensure that sampling was from tumoral tissue and not adjacent normal tissue. Genomic DNA was isolated from 20 mg of fresh frozen tumors using the QIAamp DNA Mini Kit with a proteinase K treatment. Total RNA was extracted from 15 to 20 mg of the same tumor tissue using the RNeasy minikit (Qiagen) followed by DNAse treatment according to the manufacturer’s instructions. DNA from whole blood samples of the corresponding patients were extracted using the MagAttract DNA Blood Midi M48 Kit (Qiagen).

Gene expression analysis

According to the manufacturer’s instructions, 500 ng of total RNA was reverse transcribed using Superscript III and oligo(dT) primers (Life Technologies). RNA concentrations

| Table 1 | Clinical and pathological data on the adrenal tumors |
|---------|-----------------------------------------------------|
| Patient subgroups | All | Percentage/ range |
| Conn’s adenoma | | |
| Number of samples | 12 | |
| Female | 6 | 50% |
| Male | 6 | 50% |
| Age at surgery, years (median, range) | 47 | (31–68) |
| Tumor size in mm (median, range) | 14.5 | (10–22) |
| Adrenocortical benign tumors (ACBT) | | |
| Number of samples | 20 | |
| Female | 16 | 80% |
| Male | 4 | 20% |
| Age at surgery, years (median, range) | 55.5 | (23–68) |
| Weiss score (mean, range) | 0.35 | (0–2) |
| Tumor size in mm (median, range) | 40 | (20–60) |
| Pheochromocytomas (PCC) | | |
| Number of samples | 10 | |
| Female | 6 | 60% |
| Male | 4 | 40% |
| Age at surgery, years (median, range) | 50.5 | (41–67) |
| PASS score (mean, range) | 2.7 | (0–9) |
| Tumor size in mm (median, range) | 40 | (26–80) |
| Malignant carcinomas (ACC) | | |
| Number of samples | 20 | |
| Female | 12 | 60% |
| Male | 8 | 40% |
| Age at surgery, years (median, range) | 48 | (28–78) |
| Weiss score (mean, range) | 5.65 | (3–6) |
| No. of patients with metastases | 4 | |
| TNM stage I or II | 7 | |
| TNM stage III or IV | 11 | |
| Tumor size in mm (median, range) | 50 | (33–190) |
were established using a NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE, USA) and the RNA integrity number (RIN) was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France).

Expression analyses were performed following the MIQE guidelines to ensure accurate quantification of gene expression (Bustin et al. 2009). The IGF2 and H19 expression assays used in this study are both spanning exon-exon boundaries and have previously been published (Dejeux et al. 2009, Koukoura et al. 2011) (Supplementary Table 1, see section on supplementary data given at the end of this article). Experiments were performed in duplicate for each sample using a cut-off of 10% coefficient of variance (CV). Two samples (9 and 16) were excluded from the expression analysis due to a CV value above the threshold and low RIN respectively. The average RIN among the remaining 60 RNA samples was 8.5. A dilution series of commercial cDNA (10–0.001 ng/µl) was prepared to determine PCR efficiency (E). For each primer set, E was >90%.

The geNorm reference gene selection kit (Primer-Design, Southampton, UK) was used to identify the most stably expressed reference genes across the different adrenocortical tumors using the qbasePLUS Software (Biogazelle, Zwijnaarde, Belgium). The expression of IGF2 and H19 was normalized to the geometric mean of the three reference genes (SDHA, ATPSB, and CYC1). The final RT-qPCRs included 5 µl of cDNA (1 ng/µl), 5 µl primer (300 nM), and 10 µl of SYBR Green I Master mix (Roche). The real-time PCR program was initiated with 10 min at 95 °C followed by 45 cycles of 15 s at 95 °C and 60 s at 60 °C using a LightCycler 480 II (Roche). Melting curve analysis was performed after each run initiated with 5 s at 95 °C, 60 s at 65 °C and finally with a temperature ramping from 65 °C to 95 °C (0.1 °C/s) to exclude amplification of unspecific products.

DNA methylation analysis by pyrosequencing
Quantitative DNA methylation values were obtained using pyrosequencing as previously described (Tost & Gut 2007). Briefly, 500 ng of genomic DNA was treated with sodium bisulfite using the EpiTect Bisulfite Kit (Qiagen) according to the manufacturer’s instructions. The final PCR contained 10 ng of bisulfite treated DNA, 1× PCR buffer (Qiagen), assay specific MgCl2 concentrations, 100 µM of each nucleotide, 200 nM of each primer, and 2 U of HotStarTaq (Qiagen). The PCR program was initiated with an initial denaturation step of 15 min at 95 °C followed by 50 cycles of 30 s denaturation at 95 °C, 30 s at the assay specific annealing temperature and 15 s elongation at 72 °C. The final extension was performed for 5 min at 72 °C. Primer sequences and assay-specific modifications to the general protocol are given in Supplementary Table 1, see section on supplementary data given at the end of this article.

Single nucleotide polymorphism analysis
 Fifty-seven tumor samples had sufficient high-quality material available for genome-wide single nucleotide polymorphism (SNP) analysis using the Illumina-Omni2.5M array (Illumina, Inc., San Diego, CA, USA) (Gunderson et al. 2005, Peiffer et al. 2006)). Arrays were imaged using iScan scanners and the BeadStudio software (Illumina) was used to call genotypes and extract data for downstream analysis.

The log-transformed ratio between measured and expected SNP signal intensity (LogR) and B-allele frequencies (BAF) were visualized using tools implemented in the ‘copynumber’ R library (Gentleman et al. 2004, Nilsen et al. 2012). Inspection of raw data profiles demonstrated a previously described asymmetry in the intensity of the two alleles for each SNP remaining after the normalization steps implemented in Illumina’s Genome Studio Software (Staal et al. 2008). This affected both the allelic proportions and the copy number estimates. Data were corrected for this bias using a quantile normalization approach implemented in the tQN method (Staal et al. 2008). Bias in the estimated copy number related to GC binding artifacts was corrected for using the method by (Cheng et al. 2011). Allele specific copy number estimates corrected for ploidy and infiltration of normal cells were identified using ASCAT (Van Loo et al. 2010). For the correct estimation of the percentage of the tumor cell fraction ASCAT is dependent on genomic aberrations. Therefore ASCAT cannot estimate the tumor percentage of diploid tumor samples without aberrations correctly. As such, we have excluded the tumor percentage estimation for diploid tumors without genetic aberrations in Table 2. Copy number changes were verified by genotyping rs680, which is located in IGF2 (chr11:2,153,634) for paired tumor and blood samples heterozygous for rs680.

Genotyping rs680
The PCR for genotyping contained 10 ng of genomic DNA, 1× PCR buffer (Qiagen), 1 mM MgSO4, 100 µM of each nucleotide, 200 nM of each primer, and 3 U of Platinum
### Table 2  IGF2 and H19 expression levels compared to DNA methylation and genetic status of 11p15.5

| Conn's adenoma ID | IGF2 exp. | H19 exp. | Genomic status of 11p15.5 | Ploidy | H19 ICR meth. | DMR2 meth. | DMR0 meth. | Tumor cell in % |
|-------------------|-----------|----------|--------------------------|--------|---------------|------------|------------|----------------|
| 1                 | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | NA             |
| 2                 | Overexp.  | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 3                 | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | NA             |
| 4                 | Normal    | Normal   | Normal                   | 2      | Normal        | Normal     | Hypermeth. | NA             |
| 5                 | Normal    | Downreg. | Normal                   | 2      | Normal        | Normal     | Hypermeth. | NA             |
| 6                 | Overexp.  | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 7                 | Normal    | Downreg. | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 8                 | Normal    | Downreg. | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 9                 | NA        | NA       | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 10                | Normal    | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 11                | Normal    | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |
| 12                | Overexp.  | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | NA             |

| Adrenocortical Benign Tumours ID | IGF2 exp. | H19 exp. | Genomic status of 11p15.5 | Ploidy | H19 ICR meth. | DMR2 meth. | DMR0 meth. | Weiss | Tumor cell in % |
|---------------------------------|-----------|----------|--------------------------|--------|---------------|------------|------------|-------|----------------|
| 13                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 14                              | Overexp.  | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Hypermeth. | 0     | NA             |
| 15                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 16                              | NA        | NA       | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |
| 17                              | Normal    | Downreg. | NA                       | NA     | High          | Hypometh.  | Normal     | 0     | NA             |
| 18                              | Normal    | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |
| 19                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 20                              | Overexp.  | Downreg. | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 21                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 22                              | Normal    | Downreg. | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |
| 23                              | Normal    | Downreg. | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |
| 24                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 25                              | Normal    | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |
| 26                              | Normal    | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |
| 27                              | Normal    | Downreg. | NA                       | NA     | High          | Hypometh.  | Hypermeth. | 0     | NA             |
| 28                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 29                              | Normal    | Normal   | Normal                   | 2      | Normal        | Hypometh.  | Normal     | 0     | NA             |
| 30                              | Overexp.  | NA       | Normal                   | NA     | High          | Hypometh.  | Hypermeth. | 0     | NA             |
| 31                              | Normal    | Downreg. | Normal                   | 2      | High          | Hypometh.  | Normal     | 0     | NA             |
| 32                              | Overexp.  | Normal   | Normal                   | 2      | Normal        | Normal     | Normal     | 0     | NA             |

| Pheochromocytomas ID | IGF2 exp. | H19 exp. | Copy number status of 11p15.5 | Ploidy | H19 ICR meth. | DMR2 meth. | DMR0 meth. | PASS | Tumor cell in % |
|----------------------|-----------|----------|-----------------------------|--------|---------------|------------|------------|------|----------------|
| 33                   | Overexp.  | Downreg. | Loss of one allele          | 2      | High          | Normal     | Normal     | 2    | 38%            |
| 34                   | Overexp.  | Downreg. | Loss of one allele          | 2      | High          | Normal     | Normal     | 6    | 53%            |
| 35                   | Overexp.  | Downreg. | Normal                     | 2      | High          | Normal     | Hypermeth. | 6    | 38%            |
| 36                   | Overexp.  | Downreg. | Loss of one allele          | 2      | Normal        | Normal     | Hypermeth. | 1    | 53%            |
| 37                   | Overexp.  | Downreg. | Normal                     | 2      | Normal        | Hypermeth. | Normal     | 0    | 71%            |
| 38                   | Overexp.  | Downreg. | Loss of one allele          | 2      | High          | Hypermeth. | Hypermeth. | 9    | 60%            |
| 39                   | Overexp.  | Downreg. | UPD                        | 2      | High          | Hypermeth. | Hypermeth. | 0    | 79%            |
| 40                   | Overexp.  | Normal   | Normal                     | 2      | Normal        | Hypometh.  | Normal     | 0    | 65%            |
| 41                   | Overexp.  | Downreg. | Normal                     | 2      | Normal        | Hypometh.  | Normal     | 0    | 81%            |
| 42                   | Overexp.  | Downreg. | Loss of one allele          | 2      | High          | Hypometh.  | Normal     | 3    | NA             |

| Adrenocortical Carcinoma ID | IGF2 exp. | H19 exp. | Copy number status of 11p15.5 | Ploidy | H19 ICR meth. | DMR2 meth. | DMR0 meth. | Weiss | Tumor cell in % |
|----------------------------|-----------|----------|-----------------------------|--------|---------------|------------|------------|-------|----------------|
| 43                        | Overexp.  | Downreg. | Loss of one allele          | 2      | High          | Hypometh.  | Hypermeth. | 3    | 54%            |
| 44                        | Overexp.  | Downreg. | UPD genotype               | 4      | High          | Hypometh.  | Hypermeth. | 7    | 72%            |
| 45                        | Overexp.  | Downreg. | UPD                        | 2      | High          | Hypometh.  | Normal     | 3    | 70%            |
| 46                        | Overexp.  | Downreg. | Loss of one allele          | 2      | High          | Hypometh.  | Hypermeth. | 5    | 86%            |
| 47                        | Overexp.  | Downreg. | UPD genotype               | 4      | High          | Hypometh.  | Normal     | 4    | 84%            |
| 48                        | Overexp.  | Normal   | Normal                     | 2      | Normal        | Normal     | Normal     | 3    | 59%            |
Taq DNA Polymerase High Fidelity (Life Technologies) in a final volume of 25 μl. The PCR program was initiated with a denaturation step of 4 min at 95 °C followed by 50 cycles of 30 s at 95 °C, an annealing temperature at 56 °C for 30 s, and elongation at 72 °C for 15 s. The final extension was performed for 4 min at 72 °C. Quantitative genotyping was performed on 10 μl of the PCR product using pyrosequencing. Primer sequences are given in Supplementary Table 1, see section on supplementary data given at the end of this article.

Statistical analysis

The non-parametric Mann–Whitney test was used to assess potential differences between groups for the methylation and the expression analysis. A two-sided t-test was used to test for associations between categorized molecular alterations and categorical clinical parameters. Correlation analyses were made using the Spearman’s rank correlation coefficient. A nominal $P$ value $<0.05$ was considered significant.

Results

To provide a comprehensive analysis of the mechanisms underlying IGF2 overexpression in adrenal tumors, gene expression levels were integrated with copy number and ploidy analysis, and the DNA methylation status of three DMRs including the ICR upstream of H19 (H19 DMR) and two secondary DMRs (DMR0 and DMR2) within IGF2 (Supplementary Fig. 1, see section on supplementary data given at the end of this article).

**Table 2 Continued**

|   | Overexp. | Normal | Loss of one allele | High | Hypometh. | Hypermeth. |   |   |
|---|---------|--------|--------------------|------|-----------|-----------|---|---|
| 49 | Overexp. | Normal | Loss of one allele | 2    | High      | Hypometh. | 5 | 68%|
| 50 | Overexp. | Downreg. | Normal | 2    | High      | Normal    | 8 | NA |
| 51 | Overexp. | Downreg. | Triploid | 4    | High      | Hypometh. | 8 | NA |
| 52 | Overexp. | Downreg. | NA | NA | High | Hypometh. | Hypermeth. | 8 | NA |
| 53 | Overexp. | Downreg. | Triploid | 4    | High      | Normal    | 6 | 32%|
| 54 | Overexp. | Downreg. | Normal | 2    | High      | Normal    | 6 | 95%|
| 55 | Overexp. | Downreg. | Loss of one allele | 2    | High      | Hypometh. | 8 | 88%|
| 56 | Overexp. | Downreg. | Loss of one allele | 2    | High      | Hypometh. | 8 | 88%|
| 57 | Normal | Downreg. | UPD genotype | 4    | Normal    | Normal    | 7 | 39%|
| 58 | Normal | Downreg. | Normal | 2    | High      | Hypometh. | 6 | 70%|
| 59 | Overexp. | Downreg. | Loss of one allele | 2    | High      | Hypometh. | 7 | 67%|
| 60 | Overexp. | Downreg. | Loss of two alleles | 4    | Normal    | Hypometh. | 5 | 59%|
| 61 | Overexp. | Downreg. | Tetraploid | 4    | Normal    | Hypometh. | 4 | 81%|

NA, no aberrations; for correct estimation of the tumor percentage, ASCAT is dependent on genomic aberrations. For diploid tumors without aberrations, the tumor percentage can therefore not be calculated.

**Differential IGF2 and H19 expression characterize PCCs and ACCs**

Gene expression changes of IGF2 and H19 were analyzed in 60 fresh frozen adrenal tumors (Fig. 1). IGF2 was considered overexpressed when the expression level was three times above the S.D. of the mean IGF2 expression value observed for the CAs, which express IGF2 and H19 at levels comparable to normal adrenal tissue (Ilvesmaki et al. 1993a, Liu et al. 1995). H19 was likewise considered downregulated when the expression level of individual samples was below three times the S.D. of the mean H19 expression level observed for CAs. IGF2 showed overexpression in 85% (17/20) of the ACCs and 100% (10/10) of the PCCs, whereas only 4/19 ACBTs and 3/11 CAs showed overexpression of IGF2 ($P$% 0.0003 for both ACC and PCC vs CAs; Mann–Whitney test) (Fig. 1). Expression levels did not correlate with clinical parameters such as presence of metastases, TNM stage. Only a tendency for a positive correlation between IGF2 expression and tumor size for the ACCs was observed, but did not reach statistical significance ($R^2=0.273, P=0.067$, Spearman’s correlation). Decreased expression levels of H19 also characterized both ACCs (18/20) and PCCs (7/10) when compared to the CA samples ($P=0.0001$ and $P=0.01$ respectively; Mann–Whitney test). However, the level of H19 down regulation was less pronounced for PCCs compared to ACCs ($P=0.0053$; Mann–Whitney test) (Fig. 1). Concomitant IGF2 overexpression and H19 downregulation characterized the majority of the ACCs (15/20), PCCs (7/10), and a single ACBT, but was not present in all cases (Table 2).
The DNA methylation levels of the three CTCF binding sites analyzed were highly correlated ($\rho>0.75$, $P=2.2\times10^{-16}$, Spearman’s correlation). PCCs and ACCs showed hypermethylation of the $H19$ ICR compared to the samples from patients with CA and ACBTs ($P<0.0001$ for both the ACCs and PCCs, Mann–Whitney test). The DNA methylation level of the three CTCF binding sites was not as high for PCC samples as for ACCs ($P=0.022$, Mann–Whitney test) and this difference could neither be explained by copy number changes nor content of tumor cells in the samples. Of note, the $H19$ ICR DNA methylation level correlated positively with the $IGF2$ expression for the PCCs ($\rho=0.93$, $P=0.00013$, Spearman’s correlation), while no correlation between $IGF2$ expression and $H19$ ICR methylation was observed for the ACCs ($\rho=0.34$, $P=0.14$, Spearman’s correlation). Increased DNA methylation levels of the $H19$ ICR were observed for four adenomas (samples 17, 19, 30, and 31). However, their DNA methylation levels only exceeded our chosen threshold by 1–5% and only one of these (sample 30) had concomitant $IGF2$ overexpression.

A high correlation was also observed for the DNA methylation levels for the two regions analyzed within the $IGF2$ DMR2 (DMR2a and DMR2b, $\rho=0.63$, $P=1.43\times10^{-7}$, Spearman’s correlation). A gradient of hypomethylation of $IGF2$ DMR2 was observed with CA samples presenting the highest DNA methylation level followed by the ACBTs with a significant difference between the two groups ($P=0.0076$, Mann–Whitney test). The PCCs and ACCs had the lowest DNA methylation levels when compared to CA samples ($P=0.014$ and $P=0.019$ respectively, Mann–Whitney test) (Fig. 2). The observed hypomethylation of the $IGF2$ DMR2 was independent of the somatic copy number changes of the region (see the following section) and associated with neither Weiss nor PASS score nor $IGF2$ expression levels (Table 2). DNA methylation changes did not correlate with any clinical parameters in the tumor groups.

The ACCs further displayed a slight, though significant, higher DNA methylation level for three CpG sites analyzed in the $IGF2$ DMR0 compared to all other groups ($P=0.0067$, Kruskal–Wallis test), which correlated positively with the Weiss score ($\rho=0.60$, $P=0.039$, Spearman’s correlation).

**Somatic copy number changes of chr11p15.5 are abundant in ACCs and PCCs**

Genome-wide SNP analysis was used to establish the chromosomal status of chr11:1 986 296–2 223 233...
harboring the IGF2/H19 locus as this technology allows the detection of copy number changes as well as copy number neutral events. The chromosomal and allelic status was established using the BAF and Log R ratios, estimated by ASCAT (Van Loo et al. 2010), allowing discrimination between different forms of events: polyplody, allelic loss and gain, and uniparental disomy (UPD). ASCAT results were confirmed by genotyping rs680 using pyrosequencing, which confirmed loss of heterozygosity for 8/8 informative samples (Supplementary Table 2, see section on supplementary data given at the end of this article).

Somatic copy number changes of the IGF2/H19 locus were restricted to PCC and ACC samples (Table 2). PCC samples remained in the diploid state, while ACCs were often tetraploid (7/19 analyzed) (Table 2). Representative samples showing different somatic copy number changes of the chr11p15.5 locus are shown in Fig. 3. Figure 3A represents a diploid sample with no genetic alterations accounting for 36/57 of the samples analyzed within this study including 12/12 CA samples, 16/16 ACBTs, 4/10 PCCs and 4/19 ACCs. Seven out of 12 diploid ACCs and half of the PCCs presented with loss of a single allele (Fig. 3B). Seven ACC samples were found to be tetraploid of which two samples (51 and 53) had lost a single allele of chr11p15.5 resulting in triploidy of this region (Fig. 3C). Another three tetraploid ACC samples (44, 46, and 57) presented with an UPD-like genotype as a consequence of loss of two alleles of the same parental origin (Fig. 3D). Of the two remaining tetraploid samples, ACC sample 61 had lost one of each allele, thus having normal allelic dosage, whereas the other (sample 62) did not show any loss of chr11p15.5. UPD is characterized by copy number neutral variation and occurred in a single ACC sample (45) and a single PCC sample (39), both of which were diploid (Fig. 3E).

**IGF2** overexpression in PCCs and ACCs correlates with somatic copy number changes of chr11p15.5

Thirty-six diploid samples did not show any copy number changes at chr11p15.5 (Fig. 4), of which 13 (36%) overexpressed **IGF2** (Table 2). Of these 13 diploid samples,
Figure 3
ASCAT profiles of adrenal tumors. (A) A Conn’s adenoma sample (1) being diploid and with no somatic copy number changes at chr11p15.5 thus presenting a normal sample. (B) A tetraploid ACC sample (51), for which loss of a single allele resulted in triploidy of chr11p15. (C) An UPD-like genotype (57) being tetraploid with loss of two alleles of the same parental origin. (D) A diploid sample (39) with a copy number neutral variation presenting an UPD. (E) The PCC 33 represents samples being diploid and with loss of a single allele of chr11p15.
11 samples had normal DNA methylation levels of the \textit{H19} ICR (three CAs (2, 6, and 12), three ACBTs (14, 20, and 32), four PCCs (35, 38, 40, and 41), and a single ACC sample (48)). The two remaining samples with no copy number alterations and \textit{IGF2} overexpression were diploid ACCs (50 and 54) and they both showed hypermethylation of the \textit{H19} ICR. In contrast all PCCs showed overexpression of \textit{IGF2}, where all samples with somatic copy number changes showed hypermethylation of the \textit{H19} ICR. Seventeen out of 20 ACCs presented with overexpression of \textit{IGF2} and this mainly correlated with somatic copy number changes of chr11p15.5 and hypermethylation of the \textit{H19} ICR. Somatic copy number changes of chr11p15.5 were not exclusively associated with high IGF2 expression levels as an ACC sample (57) with an UPD-like genotype had IGF2 expression levels comparable to the samples from patients with Conn’s adenoma.

For the PCCs and ACCs, loss of a single allele in diploid samples was observed for 12/29 (41\%) samples and therefore a recurrent event associated with \textit{IGF2} overexpression and high \textit{H19} ICR methylation levels (Fig. 4C and D).

Tetraploidy was a unique feature for ACCs in our study and with a frequency of 37\% (7/19) it was a frequent event often accompanied by loss of either a single or two allele(s) of the same parental origin at chr11p15.5. Both tetraploid
samples, which had lost a single allele and were therefore triploid for chr11p15.5 (51 and 53) had IGF2 overexpressed and high H19 ICR DNA methylation levels (Fig. 4D). Three tetraploid samples (44, 46, and 57) had lost two alleles of same parental origin resulting in an UPD-like genotype. Whereas the samples 44 and 46 showing an UPD-like genotype had IGF2 overexpressed and high H19 ICR methylation levels, sample 57 had IGF2 expression levels equivalent to the CA samples, but still had the H19 ICR hypermethylated (Fig. 4D) (Table 2). The tetraploid ACC sample 61 had lost one of each allele thus having normal allelic dosage accompanied by normal H19 ICR DNA methylation levels and IGF2 overexpression. The remaining tetraploid sample (62) presented with four allelic copies and was associated with normal IGF2 expression levels and normal H19 ICR methylation levels. The ACC samples 61 and 62 were the only tetraploid samples with equal number of parental allelic copies, which could explain the absence of high H19 ICR DNA methylation levels.

Tumor samples with a higher number of paternal alleles compared to maternal ones had an increased probability of being metastatic (P<0.00001, χ²-test), but none of the other clinical parameters was associated to copy number changes or ploidy.

High DNA methylation levels of the imprinting control region were thus mainly observed for samples showing copy number changes of chr11p15.5 as 13/15 ACCs and 6/6 PCCs with copy number changes had increased DNA methylation levels (Fig. 4C and D) and the majority of adrenal tumors (90%) with somatic copy number changes also showed overexpression of IGF2 (Fig. 4D).

Discussion

ACCs and PCCs develop in the adrenal cortex and the adrenal medulla respectively, which are two distinct organs of separate embryonic origin. However, ACCs and PCCs share the characteristic IGF2 overexpression. Unraveling the molecular mechanisms underlying IGF2 overexpression is important as IGF2 contributes to cell proliferation and tumor progression (Livingstone 2013, Guillaud-Bataille et al. 2014). Studies investigating the underlying mechanisms of IGF2 overexpression in adrenal tumors at more than a single molecular level are limited (Gicquel et al. 1994, 1997, Gao et al. 2002) and no study has so far integrated epigenetic and copy number variation obtained with quantitative high-resolution technologies with gene expression. In the present study we therefore integrated IGF2 and H19 expression levels with the DNA methylation levels of three regulatory DMRs located throughout the IGF2/H19 locus and compared this to the copy number status and ploidy of chr11p15.5 in a cohort of 62 adrenal tumor samples.

In concordance with previous findings, we found a correlation between IGF2 overexpression and the presence of malignant adrenocortical tumors (Ilivsmaki et al. 1993a, Gicquel et al. 1997, Slater et al. 2006, Soon et al. 2009, Ragazzon et al. 2011) as 85% of the ACCs overexpressed IGF2. However, similar to recently published data, the degree of overexpression did not correlate with the Weiss score (Guillaud-Bataille et al. 2014) or other clinical parameters. Moreover, IGF2 overexpression was found in all PCCs, but here the degree of malignancy, as given by the PASS score, correlated with the level of overexpression, similar to previously published results (Margetts et al. 2005, Meyer-Rochow et al. 2010, Sandgren et al. 2010). While due to lack of normal tissue from the adrenal medulla, the gene expression level of the PCCs was compared to the expression level of adenoma in the context of Conn’s Syndrome as reference and thus to a different tissue, the degree of the overexpression was probably not overestimated as the expression of IGF2 in adults is restricted to the adrenal capsule and the periphery of the cortex (Baquedano et al. 2005), and the expression of IGF2 in normal adrenal medullary cells is below the expression level in benign tumors (Meyer-Rochow et al. 2010). Furthermore, healthy adrenal medulla did not show IGF2 expression by immunohistochemical staining in human samples (Soon et al. 2009) or mouse models (Drelon et al. 2012), providing conclusive evidence that the detected high levels of IGF2 in the PCCs are a tumor-specific alteration. Our data is further supported by the fact that the magnitude of overexpression detected in our study is very similar to a previously published study demonstrating the overexpression of IGF2 in PCCs when compared to normal adrenal medulla (Waldmann et al. 2010).

IGF2 overexpression has previously been associated with LOH at the IGF2/H19 locus in adrenocortical tumor samples. 28/38 adrenal tumors with LOH of the IGF2/H19 locus had lost the maternal allele and gained an extra copy of the paternal allele (Gicquel et al. 1994, 1997). In our cohort 15 out of 19 ACC samples analyzed had somatic copy number alterations at the IGF2/H19 locus, with 6/15 samples having an extra copy of a single allele (Fig. 4D). As all six samples with UPD or an UPD-like genotype displayed hypermethylation of the H19 ICR, we can assume that the unmethylated maternal allele is lost and...
accompanied by a duplication of the normally methylated paternal allele. However, it should be noted that no parental DNA was available to confirm this hypothesis. Due to the limitations of the method used in previous studies, the frequency of UPD cannot be easily compared as the ploidy of the tumors was not determined in the study of Gicquel et al. Even though they observed that loss of the maternal allele is accompanied by duplication of the paternal allele, it remains unknown whether this was due to an UPD in a diploid cell or loss of two alleles of the same parental origin in a tetraploid cell leading to an UPD-like genotype. As only a single ACC and a single PCC sample displayed UPD in our cohort, our data suggest that loss of the unmethylated maternal allele rather than UPD is the critical mechanism underlying IGF2 overexpression. The presence of more paternal alleles than maternal alleles was significantly associated with the presence of metastases. However, the low number of metastatic samples in our study \((n=4)\) makes it difficult to draw general conclusions from these results and will require validation in larger cohorts.

Tetraploidy was found to be a unique feature for the ACCs, which is supported by previous findings (Pignatelli et al. 1998, Blanes & Diaz-Cano 2006). In contrast to our study, tetraploidy has also been observed for PCCs using flow cytometry (Shono et al. 2002) and likewise has tetrasomy of single chromosomes been observed in samples of patients with CA (Shono et al. 2002), which also is in contrast to our and others (Lu et al. 1996). However, it cannot be ruled out that some PCCs could be tetraploid as only a limited number of tumors were analyzed in our study. It should be pointed out, that although the polyploidy of ACCs has previously been reported, none of the studies investigating the mechanism of IGF2 overexpression took this information into account.

Gicquel et al. (1997) did not find IGF2 overexpression restricted to samples with allelic imbalance of the IGF2/H19 locus as 7/82 samples had IGF2 overexpressed without any somatic copy number changes of chr11p15.5. This is in concordance with our findings as we found 13/36 diploid tumors overexpressed IGF2, of which 11 had normal H19 ICR methylation levels. This indicates the involvement of an additional so far unknown mechanism leading to the characteristic overexpression of IGF2 for PCCs and ACCs as well as for a subset of the ACBTs, which were found to be in general chromosomally stable in our study. The amplitude of the expression changes was not directly correlated to the different somatic copy number changes. A tetraploid sample with UPD-like genotype could present with a similar high expression as a diploid sample with no genetic alterations supporting again the hypothesis of other changes probably in trans influencing IGF2 overexpression. In contrast to recently reported differences in DNA methylation patterns in the H19 DMR between high and low IGF2 expressing ACCs (Guillaud-Bataille et al. 2014), we did not find any correlation between methylation patterns and expression levels of IGF2 in the ACCs with their complex genetic background. However, a positive correlation was detected in the PCCs, which remained diploid.

One candidate transcription factor that could be interesting to investigate for its potential role in modulating IGF2 expression levels is PLAG1 as its oncogenic function is mediated through the IGF2 pathway by binding to promoter 3 located between exon 4 and 5, resulting in IGF2 overexpression (Hensen et al. 2002, Voz et al. 2004, Akhtar et al. 2012). Upstream enhancers have also been suggested to be involved in IGF2 overexpression (Ulaner et al. 2003). However, whether the broad range from 4 to ~600 fold of IGF2 overexpression observed in the ACC group could be caused by transcription factors, upstream enhancers or other trans acting elements needs to be elucidated.

In conclusion, our data suggest that IGF2 overexpression in adrenal tumors correlates mainly with allelic loss leading to an imbalance of the ratio between paternal and maternal alleles and that the aberrant DNA methylation levels observed for the H19 ICR are a consequence thereof. Whether demethylation of the IGF2 DMR2 contributes to overexpression of a subset of IGF2 transcripts needs further investigation. Our study further underlines the importance to take ploidy into account to accurately discern the mechanism of gene-specific overexpression in tumors.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-15-0086.

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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Author contribution statement
H M Nielsen, A How-Kit, A Daunay and C Guerin performed laboratory experiments. H M Nielsen, A How-Kit, H K M Vollan, V N Kristensen, P V Loo, L L Hansen, J Tost were involved in the data analysis. C Guerin, F Castinetti,
C D Micco, D Taieb, F Sebag and A Barlier were responsible for the patient cohorts, clinical and pathological analyses and sample preparation. H M Nielsen and J Tost provided the first draft of the manuscript. All authors participated in writing of the manuscript and approved the final version of the manuscript. A Barlier and J Tost initiated and designed the study. F Castinetti and C Guerin, and A Barlier and F Sebag contributed equally, and should be considered joint third and second last authors, respectively.

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