Relaxed imprinting of IGF2 in peripheral blood cells of patients with a history of prostate cancer

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Abstract

Background: Insulin-like growth factor 2 (IGF2) is the predominant IGF in adults and regulates cell growth. In contrast to normal tissues, where IGF2 is imprinted and only expressed from the paternal allele, loss of imprinting (LOI) and biallelic IGF2 expression are observed in many cancers including prostate cancer (PCa). We here studied whether LOI of IGF2 in normal circulating peripheral blood lymphocytes can predict increased PCa risk.

Samples and methods: We analyzed IGF2 protein levels, IGF2 820G/A genotype and imprinting status, as well as methylation status of the IGF2 imprinting control region (ICR) in 113 blood samples of patients with a history of radical prostatectomy (RPE) for PCa. Results were compared to 249 male blood donors with unknown prostate specific antigen (PSA) status.

Results: The 820G/A genotype was enriched in the RPE group and was associated with younger age at cancer diagnosis. LOI in patients was only slightly more frequent than in controls, but IGF2 levels were significantly higher and uncoupled from the imprinting status. Analysis of the IGF2/H19 ICR revealed marked hypermethylation.

Conclusions: The IGF 820G/A genotype is associated with PCa diagnosis at younger age. Increased IGF2 in patients with PCa appears to be the result of impaired imprinting in non-neoplastic cells rather than a paracrine tumor product. Uncoupling of IGF2 protein levels from imprinting status (not LOI alone) and hypermethylation of the ICR characterized PCa patients and could have the potential to indicate persons at risk in screening programs.

Key Words
► cancer
► insulin-like growth factor 2
► imprinting
► prostate
► screening

Endocrine Connections (2012) 1, 87–94
Introduction

Prostate cancer (PCa) is the sixth most common cancer worldwide and the most prevalent cancer in men (1) with a lifetime risk of 16.48% (2). Older age is an important PCa risk factor, as about three-quarters of all cases occur in men over 65 years (3). Age dependence in cancers could be the result of accumulation of DNA damage (4) or epigenetic changes (5) in somatic cells. Imprinting is one mechanism of epigenetic gene regulation. Imprinted genes carry epigenetic marks (e.g. methylation) that usually allow expression of only one allele in a parent-of-origin-dependent fashion (6). Loss of imprinting (LOI) is considered one of the earliest (7) and most abundant alterations in cancer (8). Among the 90 imprinted genes known to date, the insulin-like growth factor 2 (IGF2) and H19, a gene for a noncoding RNA, are probably studied best (9). IGF2 is of special interest, as it belongs to a small set of imprinted genes that can be studied in peripheral blood, shows monoallelic expression in normal blood lymphocytes, and has been linked to tumorigenesis (10). As epigenetic changes such as LOI and demethylation occur early in cancer progression, detection of such changes may be relevant for early cancer detection and prevention. LOI of IGF2 has previously been described in normal circulating peripheral blood lymphocytes of individuals with an increased risk to develop colorectal cancer (11, 12, 13).

IGF2 and H19 belong to the same locus and share similar expression patterns throughout most normal tissues. Imprinting of the IGF2/H19 locus involves a so-called differently methylated region (DMR) that acts as a boundary or insulator element. On the maternally inherited allele, the DMR is unmethylated allowing binding of the zinc finger protein CTCF to seven so-called imprinting control regions (ICRs) on the maternal allele and prevents activation of IGF2 through blockade of enhancer elements (E). On the paternal allele, the ICRs are methylated, thereby preventing CTCF binding and leading to activation of IGF2 through the enhancer.

Materials and methods

Patient and control blood samples

Peripheral blood samples from 113 prostatectomized patients with histologically proven PCa (RPE patients) were collected 12–36 months after RPE (age range 47–85 years, median 67 years). Only persons with PSA levels ≤0.2 and without clinical evidence of disease at follow-up were included. As controls, we used 246 blood samples from volunteer blood donors (age range 19–82 years, median 43 years). Samples were not tested for PSA levels. Informed written consent was obtained from all tested individuals after full explanation of the purpose and nature of all procedures used. The study was approved by the Local Ethics Committee (ref no. Wü 59/04), functioning according to the 3rd edition of the Guidelines on the Practice of Ethical Committees in Medical Research issued by the Royal College of Physicians of London.
Isolation of serum and nucleic acids from peripheral blood samples

Blood samples were processed within 1 h. Serum and PBMNCs were obtained after Ficoll–Hypaque density-gradient centrifugation as described (24). DNA and RNA were isolated from PBMNCs using the QIAamp kit (Qiagen) and thepeqGold Blood RNA Kit (PeqLab, Erlangen, Germany) according to the manufacturer’s instructions.

ELISA

ELISA assays to detect serum IGF2 and IGFBP3 were purchased from Diagnostic System Laboratories (Webster, TX, USA) and performed according to the manufacturer’s instructions. All serum samples were assayed in triplicates. The mean of the three values was used for further statistical evaluation.

Single nucleotide polymorphism genotyping by restriction-fragment length polymorphism

Heterozygosity at the Apal-sensitive 820G>A locus on exon 7 of the IGF2 gene (GenBank sequence: X07868) was determined as described previously (25). In brief, DNA was amplified using primers F: 5’-CTTGGACCTTGGACATCA-AATTGG-3’ and R: 5’-GGTCGTGCAATTACATTACA-3’, followed by HinfI and ApaI digestion. One microliter of the amplification product was used as a template for another 40 cycles using primers

Analysis for LOI of IGF2

LOI or retention of imprinting (ROI) was determined by cDNA amplification from heterozygous cases by a nested RT-PCR method. In brief, total RNA was treated with DNaseI before RT. cDNA (1 μl) was amplified by 40 cycles of PCR using F: 5’-TCTCTGGAGACGTACTGTGCTA-3’ and R: 5’-CGGGGATGCATAAAGTATGAG-3’ as primers. One micromolar of the amplification product was used as a template for another 40 cycles using primers F: 5’-ACCGTGCTTCCGAGCAAC-3’ (exon spanning) and R: 5’-GGTCGTGCAATTACATTACA-3’. The obtained PCR products were ethanol precipitated before direct DNA sequencing as described previously (26). For PCR amplification of bisulfite-treated DNA, primers specific for methylated and unmethylated sequences spanning the seven CpG-rich IGF2 ICRs located 2 kb upstream of the H19 transcription site were designed after published sequences (GenBank ID: 283120; primers available upon request). The obtained PCR products were sequenced and the results were analyzed using CHROMAS Software version 2.0. The relative percentage of methylated vs unmethylated bases at a given CG site was calculated by dividing the amplitudes for C’s (i.e. methylated bases) by the amplitudes for T’s (i.e. unmethylated bases).

Statistical analyses

The allelic frequencies were compared with χ²-test calculated on 2×2 contingency tables. The odds ratio (OR) was calculated to demonstrate the strength of difference between groups of subjects. χ²-Test was also used to test for deviation from Hardy–Weinberg equilibrium. Mann–Whitney U test was used for statistical comparisons between unrelated groups. Spearman’s test was used to test for correlations between variables. A P value <0.05 was considered statistically significant.

Table 1 820G/A (Apal) genotype frequencies and Hardy–Weinberg (HW) equilibrium test in peripheral blood mononuclear cells (PBMNCs) in the blood of patients with a history of radical prostatectomy (RPE) for prostate cancer.

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>G/G</th>
<th>A/G</th>
<th>A/A</th>
<th>P value for HW</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBMNC RPE</td>
<td>113</td>
<td>40 (43%)</td>
<td>67 (50.5%)</td>
<td>6 (6.5%)</td>
<td>0.001</td>
</tr>
<tr>
<td>PBMNC controls</td>
<td>246</td>
<td>119 (48.5%)</td>
<td>106 (43%)</td>
<td>21 (8.5%)</td>
<td>0.702</td>
</tr>
</tbody>
</table>

The allelic frequencies between RPE patients and controls were statistically different (P=0.02).

http://www.endocrineconnections.org
DOI: 10.1530/EC-12-0054
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Results

Biased distribution of 820G/A alleles in prostate carcinoma patients

The distribution of the 820G/A genotypes in RPE patients and control persons is summarized in Table 1. Among men with a history of PCa, the 820G/A genotype was significantly more frequent than among healthy control persons (50.5% in RPE patients vs 43% in controls; OR 1.92, 95% confidence interval 1.22–3.02, \( P<0.005 \)), resulting in a deviation from expected Hardy–Weinberg proportions (\( P<0.001 \)). Of note, among RPE patients, men with 820G/A were significantly younger (median age at diagnosis 65 years, range 49–83) at PCa diagnosis than men with the G/G (median age at diagnosis 69 years, range 47–85) or the A/A genotype (median age at diagnosis 73 years, range 65–85) (\( P<0.0001 \); Fig. 2). 820G/A was previously found to be associated with body mass index (BMI) (27). To test whether the observed age difference was linked to differences in body weight, we compared BMI of 820G/A patients to those with other genotypes but found no statistical difference (\( P>0.17 \), data not shown).

LOI and increased IGF2 levels in the serum of PSA-negative RPE patients

cDNA from 106 informative cases (41 RPE patients and 65 controls with 820G/A genotype) was tested for LOI status. In control persons, IGF2 levels were tightly correlated with IGF2 imprinting status (\( r=0.8, P<0.0001 \); data not shown), indicating that the vast majority of the detected IGF2 was bound to IGFBP3.

Hypermethylation of IGF2 ICRs in blood cells of RPE patients

To further investigate the reasons for the observed uncoupling of IGF2 levels from imprinting status in RPE patients, we examined the methylation status of the IGF2 imprinting control region (ICR). The percentage of cases with LOI in control persons was identical for all age groups and did not increase with age. LOI in RPE patients was significantly more frequent (16/41 cases (39%), \( P=0.03 \)). In contrast to controls, IGF2 levels in all RPE patients were significantly increased and appeared uncoupled from the imprinting status (IGF2 levels in patients with LOI 1377 ± 333.6 ng/ml vs LOI 1310 ± 126.1 ng/ml, \( P=0.9 \); Fig. 3). Interestingly, circulating IGF2 levels in LOI cases in controls and ROI/LOI in RPE patients were similar, suggesting that under normal conditions, ~35% of circulating IGF2 in the serum is regulated by imprinting. ELISA testing showed that IGF2 protein levels both in patients and in controls were tightly correlated with IGFBP3 levels (\( r=0.8, P<0.0001 \); data not shown), indicating that the vast majority of the detected IGF2 was bound to IGFBP3.

Figure 2
Correlation between IGF2 820G/A allele status and age at tumor diagnosis in patients with a history of prostate cancer. Patients with an 820G/A genotype were on average 4 years younger than patients with an 820G/G genotype and 8 years younger than patients with an 820A/A genotype.

Figure 3
Circulating IGF2 protein levels in patients with a history of prostate cancer (RPE patients) and control persons. In controls, IGF2 levels were significantly higher in persons with loss of imprinting (LOI) compared with persons with ROI with an average increase of 35% in persons with LOI (**\( P=0.001 \)). In RPE patients, in contrast, there was no difference between cases with and without LOI (NS, not significant).
patients, we analyzed methylation of the ICRs 1–7 (Fig. 4). Hypermethylation in one or several of these regions is thought to interfere with CTCF binding, to favor loss of IGF2 imprinting and hence to increase IGF2 expression.

In general agreement with the CTCF boundary model, we observed a higher degree of methylation in samples with LOI than in ROI in both RPE patients (LOI vs ROI, 36–100% methylation; mean, 80.9 vs 28–100% methylation; mean value, 68.7%) and controls (LOI vs ROI, 27–91% methylation; mean, 62.7 vs 27–82% methylation; mean, 53.7%). However, all ICRs in samples of RPE patients, irrespective of the imprinting status, showed a higher degree of methylation compared with control samples (percent methylated bases RPE vs controls (ROI samples), ICR1 63 vs 60%; ICR2 75 vs 58%; ICR3 100 vs 82%; ICR4 78 vs 33%; ICR5 73 vs 64%; ICR6 64 vs 52%; and ICR7 28 vs 27%). We hypothesize that IGF2 imprinting in peripheral blood cells of RPE patients may be incomplete due to global ICR hypermethylation.

**Discussion**

It has previously been reported that LOI for IGF2 is an epigenetic marker of colorectal cancer risk (12). In a similar approach, we studied IGF2 and its regulation in the peripheral blood of patients with a history of PCa who where PSA negative at the time when the blood sample was collected. It is a commonly held view that increased circulating IGF2 is a tumor product (28, 29). We here show that this traditional view may be inaccurate for PCa patients: our findings in non-neoplastic blood cells rather point to a general relaxation of IGF2 imprinting in these patients. We found enrichment of the 820G/A genotype among patients with a history of PCa (OR 1.92, $P = 0.005$), a finding that may merit further consideration in future population studies. However, this retrospective study was not intended nor statistically powered to detect risk alleles in a general population. Thus, the more important finding was the fact that 820G/A genotype was a risk factor among RPE patients, as men with 820G/A were on average 5 years younger at PCa diagnosis than patients with a G/G or A/A genotype. The reasons for this observation are unclear. 820G/A was previously found to be associated with BMI (27, 30). Currently available data suggest a possible correlation between obesity and PCa risk (31, 32, 33). However, we found no correlation between 820G/A alleles and BMI (data not shown). IGF2 lies in a 19 kb genomic PCa risk region on chromosome 11p15.5, together with insulin (INS) and tyrosine hydroxylase (TH) (34). Many of the single nucleotide polymorphisms (SNPs) in this region

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**Figure 4**

Comparison of imprinting status of the imprinting control regions (ICR) 1–7 within the IGF2/H19 locus in six patients with a history of prostate cancer (RPE) and six controls (schematic representation of results: black circles, methylated; gray circles, unmethylated CGs). In both groups, three cases (nos 1–3) with either loss (LOI) or retention (ROI) of IGF2 imprinting were analyzed. In controls with LOI, all ICRs were hypermethylated compared to cases with ROI. In RPE patients, ICRs showed a higher degree of methylation. Note complete methylation of CGs within ICR3.
are in tight linkage disequilibrium with each other. In line with previous reports, we did not observe co-segregation of 820G/A with other published risk alleles (+1127 INS-PstI and −4217 THI-PstI) (35) (data not shown). This strongly suggests that 820G/A is a putative risk allele of its own.

Using the 820G/A SNP to study imprinting of IGF2, we found that 20% of healthy male control persons showed LOI. In line with previous observations (11, 12), the percentage of LOI cases was identical in all age groups, suggesting that LOI in peripheral blood cells is a genetically imposed condition rather than an age-related phenomenon. Cui et al. (12) found LOI in peripheral blood lymphocytes to be predictive of LOI in both normal and cancerous colonic mucosa of patients with colorectal cancer. Interestingly, Fu et al. (22) reported a more pronounced relaxation of imprinting in prostates of men with PCa than in age-matched control patients without PCa. These previous observations together with the data presented here raise the possibility that LOI in peripheral blood cells reflects a general impairment of imprinting rather than a tissue-restricted pre-neoplastic process. LOI in control persons resulted in a 35% increase in circulating IGF2. In stark contrast to control persons, in whom imprinting status and IGF2 expression level were tightly correlated, IGF2 imprinting and expression were uncoupled in RPE patients and IGF2 protein levels were also increased (again by 35%) in patients with ROI. These results indicate that ~35% of circulating IGF2 is controlled by imprinting. One possible explanation for the increased serum IGF2 came from the observation that the ICRs 1–7 in peripheral blood cells of RPE patients were moderately hypermethylated, suggesting impaired CTCF binding and consequent incomplete or ‘leaky’ imprinting. Discordance between methylation and imprinting status at the IGF2/H19 locus has been previously observed (36) pointing to epigenetic mechanisms other than DNA methylation (10). Importantly, increased IGF2 protein levels in spite of apparently retained imprinting at the IGF2/H19 locus were observed not only in virtually 100% RPE patients with ROI but also in a few control persons. As the controls of this study were persons who were anonymized blood donor volunteers that had not been interviewed for a history of prostatic disease nor tested for PSA levels, this raises the possibility that those few control persons with uncoupling of IGF2 from imprinting may have had occult PCa.

In conclusion, our results suggest that assessment of the IGF2 820G/A locus may provide clinically relevant information with respect to PCa risk and age of onset. Uncoupling of IGF2 imprinting from IGF2 levels and hypermethylation of the ICR in circulating peripheral blood lymphocytes may indicate persons at risk warranting increased surveillance. It will be important to test this prediction in a longitudinal prospective cohort in persons at risk to develop PCa and eventually also other cancers.

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.

Author contribution statement
D Belharazem, M Kirchner, F Geissler, P Bugert, C Sauer, B Kneitz, and S Kuffer performed experiments and assisted in the preparation of the manuscript. P Bugert, M Spahn, H Riedmiller, L Trojan, C Bolenz, and M S Michel were in charge of patients, collected patient and control samples, and participated in writing of the manuscript. A Marx and P Ströbel were principal investigators who designed and supervised the study and were involved in writing and editing the manuscript.

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