Insulin-like growth factor 2 expression in prostate cancer is regulated by promoter-specific methylation

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Deregulation of the insulin-like growth factor (IGF) axis and dysbalance of components of the IGF system as potential therapeutic targets have been described in different tumor types. IGF2 is a major embryonic growth factor and an important activator of IGF signaling. It is regulated by imprinting in a development- and tissue-dependent manner and has been implicated in a broad range of malignancies including prostate cancer (PCa). Loss of imprinting (LOI) usually results in bi-allelic gene expression and increased levels of IGF2. However, the regulatory mechanisms and the pathophysiological impact of altered IGF2 expression in PCa remain elusive. Here, we show that in contrast to many other tumors, IGF2 mRNA and protein levels were decreased in 80% of PCa in comparison with non-neoplastic adjacent prostate and were independent of LOI status. Instead, IGF2 expression in both tumors and adjacent prostate depended on preferential usage of the IGF2 promoters P3 and P4. Decreased IGF2 expression in tumors was strongly related to hypermethylation of these two promoters. Methylation of the A region in promoter P4 correlated specifically with IGF2 expression in the 20% of PCa where IGF2 was higher in tumors than in adjacent prostate. We conclude that IGF2 is downregulated in most PCa and may be particularly relevant during early stages of tumor development or during chemotherapy and androgen deprivation. PCa differs from other tumors in that IGF2 expression is mainly regulated through methylation of promoter-specific and not by imprinting. Targeting of promoter-specific regions may have relevance for the adjuvant treatment of PCa.

1. Introduction

Prostate cancer (PCa) is the most common malignancy in men in the United States and Europe. Nevertheless, knowledge about its pathogenesis and progression is still limited. Age is one of the most important risk factors in PCa. About 75% of all cases occur in men over the age of 65. Therefore, age-dependent accumulation
of DNA damage and epigenetic changes have been proposed to cause this age-dependent increase (Damashke et al., 2013; Malins et al., 2003). Epigenetic changes such as DNA methylation, loss of imprinting (LOI), and histone modification often result in aberrant gene expression that alters cell physiology and may predispose cells to malignant transformation. LOI (i.e., loss of monoallelic expression of specific genes) has been associated with several cancer types and has been suggested to be an early driver of tumorigenesis (Damashke et al., 2013; Hubertus et al., 2011; Uribe-Lewis et al., 2011; Xu and Taylor, 2014).

Among the 90 imprinted genes in humans, the IGF2/H19 (microRNA-675) gene locus (Fig. 1) is probably the best studied and LOI of IGF2 is a frequent event in the aging prostate (Fu et al., 2008; Kwabi-Addo et al., 2007). Deregulation of the IGF axis is of relevance in a variety of cancers including PCa (Heidegger et al., 2016). IGF1 and IGF2 are growth factors that promote cell proliferation, protect from apoptosis, and induce resistance to anticancer therapies through activation of the PI3K-AKT pathway (Belharazem et al., 2016; Hamamura et al., 2008).

Several mechanistic models for LOI of IGF2 have been proposed, among which the most popular (and probably too simplistic) is called the ‘enhancer competition model’ (Nordin et al., 2014). According to this model, CCCTC-binding factor (CTCF), a multizinc finger protein and transcriptional repressor, can bind to an unmethylated imprinting control region (ICR) within the maternal allele of the IGF2/H19 gene locus and thereby prevents transcription of IGF2. On the paternal allele, in contrast, hypermethylation of the ICR prevents CTCF binding and thus allows for IGF2 expression (Hark et al., 2000; Yang et al., 2003). To determine the IGF2 LOI status, we used restriction-fragment length polymorphisms of a single nucleotide polymorphism (SNP) at rs680 (820 A/G), which introduces an ApaI restriction site at IGF2 exon 7. Cases heterozygous for this SNP at the DNA level can be used to determine whether IGF2 mRNA is transcribed from one or two alleles (Belharazem et al., 2012; Ogawa et al., 1993).

Loss of imprinting is believed to result from ICR hypermethylation of both alleles and bi-allelic transcription of IGF2. A good correlation between LOI and increased IGF2 expression in tumors and preneoplastic lesions has been shown for many tumors, including Wilms tumor, colorectal cancer, and esophageal adenocarcinomas (Belharazem et al., 2016; Hubertus et al., 2011; Mori et al., 1996; Nakagawa et al., 2001). Bhushari et al. (2011) observed LOI of IGF2 not only in prostatic cancers, but also in adjacent, morphologically normal prostate, suggesting an epigenetic field defect. LOI of IGF2 in colon mucosa has been associated with an increased cancer risk (Sakatani et al., 2005) by enhancing stemness, self-renewal, and resistance against chemo- and radiotherapy (Zhao et al., 2016). Very recently, Damashke et al. (2017) described a mouse model with loss-of-function mutations of the CTCF-binding site at the IGF2-H19 imprint control region, which resulted in bi-allelic IGF2 expression and increased prevalence of prostatic intraepithelial neoplasia (PIN).

However, allelic imprinting is not the only factor that determines IGF2 expression, as its transcription depends also on promoter methylation. The IGF2 gene contains four promoters (P1–P4), of which P2–P4 are methylated on the paternal allele in young individual (Li et al., 1998). The individual transcripts show a tissue-specific expression pattern during different stages of development (Li et al., 1996). While the resulting protein remains unaltered, the distinct transcripts of the four promoters differ in their 5′-untranslated region and in their translational efficiency and stability (von Horn et al., 2002). Previous studies have shown that several regions of these promoters are required for IGF2 activation in a complex tissue- and development-specific manner (Li et al., 1998). In some malignancies, for example, osteosarcoma and hepatocellular carcinoma, promoter hypomethylation was linked to upregulation of IGF2 mRNA (Li et al., 1997, 2009b).

In this study, we investigated the imprinting status, IGF2 promoter usage, and methylation pattern in correlation to the IGF2 expression in clinical human PCa samples and adjacent, morphologically normal prostate.

Fig. 1. Schematic overview of the IGF2/H19 gene locus with protein coding exons (dark gray) (NG_008849.1) and the four promoter regions P1–P4. The P4 region can be further subdivided into CpG regions P4A, P4B1, and P4B2 (not shown) (Qian et al., 2011). The ApaI SNP rs680 is located at the 5′-UTR. The ICR is located 90 kb downstream of the IGF gene and closer to the H19 locus.
2. Materials and methods

2.1. Prostate patient samples

Frozen prostate samples were collected under stringent quality criteria from 141 patients who underwent radical prostatectomy at the Department of Urology, University Medical Center Mannheim, Germany. The use of PCA specimens for this study was approved by the ethics committee of the University Medical Center Mannheim (2008-312N-MA). The mean patient age at surgery was 64.0 years (range: 45–79 years). The mean preresection prostate-specific antigen (PSA) level was 9.1 ng/mL (range: 2.4–58.8 ng/mL). Eleven tumors were in stage pT2a, n = 1 was in stage pT2b, n = 67 were in stage pT2c, n = 38 were in stage pT3a, n = 21 were in stage pT3b, and n = 3 were in stage pT4. n = 22 tumors had a Gleason score < 7, n = 107 tumors had a Gleason score 7 and 8, and n = 12 tumors had a Gleason score of ≥ 9 (Table 1). Cryosections were HE–stained, and regions of interest were marked by a pathologist (PS). Subsequently, frozen tissue was microdissected to enrich for tumor (hereafter termed ‘T’) and morphologically normal glandular tissue (hereafter termed ‘N’).

2.2. Isolation of nucleic acid from fresh-frozen tissue samples

DNA and RNA were isolated from T and adjacent N fresh-frozen tissue. DNA was isolated using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol and stored at −20 °C. RNA was prepared using TRIzol® reagent (Life Technologies, Waltham, MA, USA) according to the manufacturer’s protocol, resuspended in RNase-free water, and stored at −80 °C.

2.3. Extraction of proteins and IGF2 Enzyme-linked Immunosorbent Assay (ELISA)

Proteins were isolated from fresh-frozen tissue samples using RIPA buffer containing 1 mM PMSF, 1 mM orthovanadate, and cOmplete™ Protease Inhibitor Cocktail (Roche, Mannheim, Germany). Tissue was minced in lysis buffer and lysed for 45 min on ice. The samples were subsequently centrifuged at high speed for 30 min, and supernatant was recovered. Concentrations of total protein were measured with the DC Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) on a microplate reader (Tecan Group Ltd., Switzerland). To measure total IGF2 protein (i.e., free and protein-bound forms) in tissue samples, we used an IGF2 ELISA kit (Mediagnost, Reutlingen, Germany). The assay was performed according to the manufacturer’s protocol; each sample was analyzed in triplicate, and concentrations were calculated according to the standard curve.

2.4. IGF2 rs680G>A single nucleotide polymorphism (SNP) genotyping by restriction-fragment length polymorphism

Analysis of rs680 (G>A) ApaI SNP in the 5′-UTR of the IGF2 gene (NG_008849.1) was determined as described previously (Belharazem et al., 2012; Ogawa et al., 1993). In brief, DNA was amplified using primers F: 5’-CTTGGACTTTGAGTCAAATTGG-3’ and R: 5’-GGTCGTGCCAATTACATTTCA-3’. Digestion products were separated on a 2% agarose gel, and results were visually analyzed.

2.5. Determination of the imprinting status of the IGF2 gene

The IGF2 imprinting status was determined by RT-PCR amplification of RNA from rs680 SNP heterozygous cases and subsequent HinfI and ApaI digestion. Briefly, cDNA was synthetized from 500 ng RNA with the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) after DNase I treatment (Thermo Scientific). cDNA was amplified by 35 cycles of PCR using the primers F: 5’-CTTGGACTTTGAGTCAAATTGG G-3’ and R: 5’-CTTGGACTTTGAGTCAAATTGG G-3’. Digestion products were separated on a 2% agarose gel, and results were visually analyzed. A single band

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Table 1. Patient and tumor characteristics.

| Age       | 64.0 |
|-----------|------|
| Range     | 45–79|
| PSA (ng·mL⁻¹) | |
| Mean (SD) | 8.86 (6.39) |
| Median (range) | 6.73 (2–58.8) |
| Gleason score | |
| 5         | 5 |
| 6         | 17 |
| 7         | 94 |
| 8         | 13 |
| ≥ 9       | 12 |
| pT stage, n (%) | |
| 2a        | 11 (7.8) |
| 2b        | 1 (0.7) |
| 2c        | 67 (47.5) |
| 3a        | 38 (27) |
| 3b        | 21 (14.9) |
| 4         | 3 (2.1) |
after digestion was considered retention of imprinting (ROI), and two bands were considered LOI (Davies, 1993) (Fig. S6).

### 2.6. Relative Quantification of IGF2 and promoter-specific transcription

Quantitative analyses of IGF2 mRNA and promoter-specific transcripts were performed on the Step One Plus Real-Time PCR-System (Life Technologies) using SYBR Green PCR Master Mix (Life Technologies). cDNA was generated as described above. Each sample was analyzed in duplicate. Each run was performed with negative controls using published primers for P1–P4 (Table S1) (Hartmann et al., 2001). Relative quantification was computed using the 2^(-ΔΔCT) method (Livak and Schmittgen, 2001). Promoter-specific transcripts from each of the IGF2 promoters were analyzed by qPCR and statistically tested as described below. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene (Table S1).

### 2.7. Analysis of miRNA-675

TaqMan® MicroRNA Assays (hsa-miR-675 and RNU24; Life Technologies) were used to identify specific mature miRNA levels. Briefly, the RT reaction was performed with 10 ng total RNA per reaction. The RT reaction was incubated for 30 min at 16 °C, 30 min at 42 °C, and 5 min at 85 °C. 4 μL of 6× diluted RT product was added to a 10 μL miR-675/RNU24 assay and Taqman® PCR Mastermix (Life Technologies) reaction. PCRs were performed in technical duplicate on a Step One Plus Real-Time PCR-System (Life Technologies) (95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s, and 60 °C for 1 min). After normalization for RNU24, the differences in miR-675 expression were computed as described above (Livak and Schmittgen, 2001).

### 2.8. Methylation analysis of promoter regions and CTCF-binding site 6 (BS6)

Methylation of DNA was quantified using the PyroMark™ Q24 system (Qiagen, Hilden, Germany) with corresponding reagents. Bisulfite conversion was performed with the EpiTect Fast Bisulfite Conversion Kit (Qiagen) according to the manufacturer’s protocols. In brief, 400 ng of DNA was treated with bisulfite and 10–20 ng of converted DNA was used for the transcript-specific PCR with SuperHotTaq DNA Polymerase (Bioron, Ludwigshafen, Germany). Primers for promoter methylation analysis were adapted from published methylation-specific primers (MSP) for P2, P3, and P4 (Qian et al., 2011) resulting in primers ‘P2A’, ‘P2B’, ‘P2C1’, ‘P2C2’, ‘P2C3’, for promoter 2, ‘P3’ for promoter 3 and ‘P4A’, ‘P4B1’, ‘P4B2’ for promoter 4 (Table S2). The nine different promoter assays cover 100 CpGs in total. CTCF BS6 (GenBank accession no. AF087017) spans over 227 nucleotides including 17 CpG islands (Paradowska et al., 2009). To cover all CpGs, the BS6 was split into two methylation assays (Table S2). All primers for methylation analysis were designed using PyroMark Assay Design SW 2.0.

### 2.9. Statistical analysis

Correlation analysis between IGF2, H19, and CTCF expression were performed using GraphPad Prism (San Diego, CA, USA) using Spearman’s correlation. Fisher’s exact test was used to analyze clinical parameters. A P value < 0.05 derived from two-tailed t-test was considered statistically significant. Gaussian distribution was checked using D’Agostino–Pearson normality test.

### 3. Results

#### 3.1. IGF2 LOI/ROI is equally distributed in tumors and adjacent prostate

As T and N tissue was not available from all 141 patients. 122 N and 128 T (109 paired) samples were screened for IGF2 rs680; 32 N and 32 T (22 paired) samples were heterozygous (i.e., informative) for the ApaI SNP and could be further analyzed for LOI/ROI status (Table 2). Overall, LOI and ROI were equally prevalent in N and T. Among the 64 tested samples, 29 (45%); 16 T [25%] and 13 N [20%] showed LOI and 35 (55%); 16 T [25%] and 19 N [30%] showed ROI (Fig. 2A and Table 2). Within the 22 paired samples, 16 (71%) showed concordance with respect to ROI/LOI, six cases (29%) were discordant (five cases LOI in T vs. ROI in N and one case ROI in T and LOI in N) (Fig. 2B).

#### 3.2. IGF2 mRNA and protein expression are generally lower in tumors than in normal prostate

Overall, the expression of IGF2 mRNA was significantly higher in N than in T (Fig. S1A). Nevertheless, in 12 cases (20%), the IGF2 mRNA level was higher in T than in N (Fig. 2C). Within this group, paired specimens showed a large variance with fold changes ranging from 1.2 to 26. A similar observation was also found for IGF2 protein using ELISA in 17 paired T and N samples. In 13 paired cases, IGF2...
concentrations were significantly lower in T than in N ($P < 0.0012$) (Fig. 2D). Four cases (24%) had higher IGF2 protein concentrations in T than in N, and three of those four also had higher IGF2 mRNA levels.

### 3.3. Correlation of IGF2 with clinicopathological parameters

IGF2 expression (either high or low) was not directly correlated with tumor stage, Gleason grade, or PSA level.

| Table 2. Screened patient samples |
|----------------------------------|
| Patients screened                | 141 |
| Normal                           | 122 |
| Tumor                            | 128 |
| Paired                           | 109 |
| rs680G>A SNP                     |     |
| Homozygous (T/N)                 | 76/75|
| Heterozygous (T/N)               | 32/32|
| LOI/ROI (T)                      | 32  |
| LOI/ROI (% LOI)                  | 16/16(50.0)|
| LOI/ROI (N)                      | 32  |
| LOI/ROI (% LOI)                  | 13/19(40.6)|
| N/T pairs                        | 22  |
| ROI (%)                          | 12 (54.5)|
| LOI (%)                          | 4 (18.2)|
| LOI T/ROI N (%)                  | 5 (22.7)|
| LOI N/ROI T (%)                  | 1 (4.5) |

(IGF2high). However, there was an age-dependent decrease in IGF2 levels in T compared to N over two decades ($P < 0.028$) (Fig. 1F). In addition, we observed a statistical correlation between IGF2 imprinting and lymphovascular (LVI) and perineural invasion (PNI): 57% of the cases (four of seven) with LOI showed LVI (vs. 14% in ROI, $P < 0.025$), while PNI was more frequently seen in ROI cases (29%, four of 14 cases with ROI vs. 0%, 0 of 15 cases with LOI, n.s.; Fig. S1G,H).

### 3.4. IGF2 expression does not correlate with imprinting status and ICR methylation

LOI is thought to result in bi-allelic IGF2 transcription and increased IGF2 protein levels. In addition, according to the enhancer competition model, activation of IGF2 through LOI is accompanied by repression of H19 (miR-675). We therefore compared the relative IGF2 mRNA expression to LOI and ROI status and to miR-675 expression (Fig. S2A–C, D–G, respectively). In N, there was a trend toward higher IGF2 expression levels in cases with LOI compared to ROI, which was not observed in T. There was no correlation between mRNA expression of IGF2 and miR-675/H19 (Fig. S2H,I).

In humans, six CTCF-binding sites within the ICR of the IGF2/H19 locus have been described with only one
of them being relevant for imprinting regulation (Paradowska et al., 2009). This region is called the differentially methylated region (DMR). According to the open-access database JASPAR, (Portales-Casamar et al., 2010) this DMR is coincident with CTCF BS6. To test whether changes in the DMR are relevant for IGF2 regulation, we investigated methylation at the CTCF BS6 locus. There was no difference in the methylation pattern of any of the 17 CpGs within BS6 when comparing N and T in relation to LOI or ROI (Fig. S3).

3.5. P3 and P4 promoter usage correlates with IGF2 expression

As we could not find any clear correlation between LOI and IGF2 expression, we investigated if IGF2 promoter usage is altered in PCa. IGF2 is transcribed from four different promoters (P1–P4) in a development- and tissue-specific manner (Qian et al., 2011). Therefore, we analyzed the four promoter-specific transcripts of IGF2 by qRT-PCR. In both T and adjacent N, the percentage of transcripts from P3 (68.3%/63.3%) was highest, followed by P4 (24.6%/21.7%), whereas transcripts from P2 (5.3%/8.3%) and P1 (1.8%/6.8%) were much less abundant (Fig. 3A). The relative expression of promoter transcripts P1 and P2 in T and N transcripts was not statistically different, whereas transcripts from P3 and P4 were 2.5- to 3-fold lower in T than in N (Fig. 3B). Overall, the level of P3 and P4 transcripts correlated significantly with IGF2 mRNA levels (Fig. S3E,F). However, when blotting IGF2 mRNA levels against P3 and P4 expression in T

![Graphs showing promoter-specific IGF2 transcripts (P1–P4) in T and N.](image)

**Fig. 3.** Promoter-specific IGF2 transcripts (P1–P4) in T and N. (A) Expression of promoter-specific transcripts P1–P4 in T and N. P3 and P4 were dominant in both T and N. (B) P3- and P4-specific transcripts were significantly higher expressed in N than in T (P < 0.004). (C+F) Significant correlation between total IGF2 mRNA and promoter transcripts P3 in T (r = 0.42, P < 0.009) and N (r = 0.45, P < 0.002). (D+F) The correlation between total IGF2 mRNA and promoter transcripts P4 was statistically significant only in N (r = 0.71, P < 0.0001), but not in T (r = 0.05295, P > 0.05). **P > 0.005, ***P > 0.0005.
and N separately, we found a significant correlation between IGF2 and both P3 \((r = 0.41, \ P = 0.0054)\) and P4 \((r = 0.75, \ P < 0.0001)\) in N (Fig. 3C,D), and between IGF2 and P3 \((r = 0.41, \ P = 0.009)\), but not P4 \((r = 0.13)\) in T (Fig. 3E,F).

3.6. Methylation of P3 and P4 is increased in PCa and P4 methylation correlates with high IGF2 expression

As only P3 and P4 were found significantly different, we further investigated their methylation status in 16 T and 16 N. The promoter methylation assay was adapted from Qian et al. (2011), and CpG methylation of the promoter-specific regions was quantified by pyrosequencing. P3 and P4 showed a significantly higher methylation in T than in N (Fig. 4A,B), which correlated well with the overall lower IGF2 expression in T.

To investigate if the promoter methylation of P3 and/or P4 is involved in IGF2 regulation, we then separated the T samples into IGF2\(_{\text{low}}\) cases with elevated and into IGF2\(_{\text{high}}\) cases with low IGF2 expression. As expected, in IGF2\(_{\text{low}}\) cases, both promoters P3 and P4 showed higher methylation in T than in paired N samples (Fig. 4C,D). In IGF2\(_{\text{high}}\) cases, global analysis of the methylation of P3 and P4 promoters revealed no differences between T and N samples. However, P4 methylation in IGF2\(_{\text{high}}\) was lower than in IGF2\(_{\text{low}}\).

![Graphs showing methylation levels](image)

**Fig. 4.** Methylation of promoter P3 and P4 correlates with IGF2 mRNA expression in PCa and normal prostate. (A) Overall higher methylation of P3 \((P < 0.05)\) and (B) P4 \((P < 0.05)\) in T \((n = 21)\) compared to N \((n = 22)\). (C+D) However, when cases were separated into IGF2\(_{\text{low}}\) and IGF2\(_{\text{high}}\) tumors, this difference was found only in IGF2\(_{\text{low}}\) tumors for P3 \((P < 0.05)\) and P4 \((P < 0.05)\). (E) In IGF2\(_{\text{high}}\) tumors, there was a significant correlation between IGF2 expression and methylation of P4 \((r = 0.64, \ P < 0.05)\). (F) The methylation of the CpG in P4A correlated significantly with the P4 promoter-specific transcript \((r = 0.80, \ P < 0.05)\). *\(P > 0.05\).
cases. A closer analysis revealed that this difference was mainly due to differential methylation of the first three CPGs within the P4 subregion P4A (Fig. S4). We then analyzed whether P3 and P4 methylation correlates with IGF2 mRNA expression in T and in the corresponding N of IGF2high. IGF2 expression significantly correlated only with the methylation of P4 in T (Fig. 4E and Fig. S5A–C). To corroborate that the P4 methylation is regulating the P4-specific transcription, we correlated the P3 and mean P4 methylation with its corresponding promoter-specific transcripts, but no significance was detected (Fig. S5D, E, H, I). However, a more detailed analysis of the P4 promoter subregions (P4A, P4B1, P4B2) revealed that the CpG methylation of P4A correlated significantly with P4-specific transcripts of IGF2 (Fig. 4F, Fig. S5F, G, J–L). This indicates that differential methylation of P4 is responsible for the IGF2 regulation in T.

4. Discussion

Deregulation of the IGF axis in the development of PCa (and other cancers) makes this pathway an interesting candidate for early detection or prevention strategies (Singh et al., 2014). The main causes for an intensified IGF signaling are deregulated expression of either IGF-1R or IGF2 (Li et al., 2009a). In this study, we focused on the identification of major factors that govern IGF2 expression in clinical prostate carcinoma samples.

A main observation of this study was the finding that IGF2 expression in PCa, in marked contrast to many other cancers, was reduced in most of the tumors compared to adjacent, morphologically normal tissues. Ribarska et al. have reported an overall down-regulation of IGF2 and H19 before and suggested ZAC1 as a nodal regulator of the imprinted gene network and as a specific regulator of IGF2. ZAC1 has been described to be downregulated in several cancer types including PCa (Ribarska et al., 2014) and might add an additional regulatory level for imprinted genes.

Importantly, and in good agreement with observations in normal prostate tissue (Bhusari et al., 2011) and in peripheral blood cells of patients with a history of PCa (Belharazem et al., 2012), there was a tight correlation between IGF2 levels and imprinting status in normal prostate, which was lost in PCa. These findings suggest that (a) the relevance of imprinting for IGF2 expression varies greatly among different tumor types and (b) that IGF2 imprinting has only a modest impact on IGF2 levels in established human PCAs that is overridden by other factors during tumor development. The fact that the prevalence of IGF2 LOI was similar in tumors and normal prostate and that the IGF2 imprinting status was often concordant in T and N supports the previous view that IGF2 LOI is an early event in the development of PCa and indicates an epigenetic field defect in the adjacent ‘normal’ prostatic tissue (Bhusari et al., 2011). It has been proposed that LOI of IGF2 is a common feature of cancer stem cells including those in PCa and that LOI enhances stemness, self-renewal, and resistance against chemotherapeutic or radiation therapy (Zhao et al., 2016). In good agreement with this concept, Damaschke et al. (2017) recently described increased prevalence of PIN in mice with genetically engineered bi-allelic expression of IGF2.

However, in contrast to these early changes in animals, our observations in established human prostate cancers are not easily compatible with the classic (and oversimplistic) enhancer competition model, as we did not observe a consistent correlation between methylation patterns of the IGF2 ICR and LOI status, nor with expression of either IGF2 or miR-675/H19. However, even though CTCF may not be the main factor regulating IGF2 imprinting in PCa, it still remains important by binding recently described regulatory factors like SUZ12 and vigilin (Liu et al., 2014; Wang et al., 2015). In colon cancer cells, the polycomb-repressive complex 2 docking factor SUZ12 has been proposed to regulate imprinting by binding to the IGF2 promoter, connecting CTCF and the ICR over a long range intrachromosomal loop with IGF2. Vigilin has also been shown to build a complex with CTCF that binds to the ICR of H19 and contributes to IGF2/miR-675/H19 regulation. Furthermore, microRNA may also play a role in IGF2 imprinting. The oncogenic miR-483 upregulates IGF2 expression by binding the promoter P2, hereby reducing in histone H3K27 methylation and decreasing chromatin binding of CTCF and SUZ12 (Zhang et al., 2017). In prostate carcinoma samples, we found that IGF2 expression can be best explained by differential IGF2 promoter usage. Among the four promoters, only P3 and P4 were active and showed significant differences between normal prostate and tumors, whereas P1 and P2 transcripts were not associated with PCa and are probably not relevant for IGF2 expression in adult prostate. In line with observations in other tumor entities (Li et al., 2009b; Qian et al., 2011), the reduced expression of IGF2 in most PCa compared to normal prostate correlated well with P3 and P4 hypermethylation and with downregulation of the respective IGF2 transcripts. Interestingly, hypomethylation of a specific small CpG region of P4 (P4A) correlated well with upregulation of IGF2 in some PCa. It was recently supposed that the methylation of a single specific CpG is sufficient for transcription factor binding and transcriptional
activation and that flanking promoter regions are more activator specific than central parts (Mamrut et al., 2013). These findings could indicate an important role of transcription factors in the regulation of IGF2 expression. One of two recently described factors influencing IGF2 expression is the embryonic stem cell transcription factor ZFP57. This factor is overrepresented in cancer and was proposed as a new oncogene (Tada et al., 2015). In addition, Lee et al. proposed STAT3 as an IGF2 regulating factor. They suggested that STAT3 mediates HDAC inhibitor resistance via direct binding to promoter P3 and P4 and thereby upregulating IGF2. These findings connect IGF2 with advanced disease as STAT3 has been described to promote metastatic progression of PCa (Lee et al., 2016). Together with the above-discussed ZAC1, ZFP57 and STAT3 might be responsible for the increased IGF2 levels in a subset of PCa and may represent imprinting independent regulatory factors with potential therapeutic implications.

While the IGF pathway as a system has recently been shown to be significantly associated with PCa mortality (Cao et al., 2014), there was no specific correlation between elevated IGF2 and tumor stage, Gleason score, or PSA levels. However, IGF2 has recently regained considerable interest by the finding that GATA2, a zinc finger transcription factor, mediates chemoresistance via direct upregulation of IGF2 in advanced PCa (Vidal et al., 2015). In addition, it has been shown that insulin can upregulate steroid synthesis in cell lines and xenograft tumors in mice (Lubik et al., 2011) and that IGF2 can do the same in PCa cells (Lubik et al., 2013). Together, these observations could indicate that the relative importance of IGF2 may vary during different stages of a tumor. In PCa, IGF2 seems to be particularly important during early phases of tumorigenesis, or when an advanced tumor is challenged through chemotherapy or androgen deprivation, while it may be less relevant in established tumors under ‘steady-state’ conditions. A recent publication has linked LOI of IGF2 through increased NFkB signaling as a mechanism to cope with oxidative stress in PCa cells (Yang et al., 2014). Thus, it may be relevant to study the regulatory mechanisms (e.g., transcription factor binding) and clinical behavior (e.g., response to androgen deprivation or chemotherapy) in those 20 percentage of PCa with increased IGF2 expression in more detail.

5. Conclusions

In summary, we found that IGF2 expression in PCa is independent of the imprinting status of the IGF2-H19 locus, but rather depends on differential methylation and usage of promoter-specific. We identified differential methylation of the region 4A within the P4 IGF2 promoter as particularly important for IGF2 levels in PCa. This finding may have relevance for targeting IGF2 to reduce tumor resistance during chemotherapy or androgen deprivation.

Author contributions

SK, PS contributed to conception and design; PS, AM, CS, MSM, and LT contributed to acquisition of data (acquired and managed patients, provided facilities, etc.); TG, SK, and DB contributed to analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis); SK, PS, and TG wrote the manuscript; SK, TG, DB, AM, MSM, LT, and CS contributed to administrative, technical, or material support (i.e., reporting or organizing data, constructing databases); SK and PS supervised study supervision.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Fig. S1. Correlation of IGF2 expression and imprinting status with clinicopathologic parameters.

Fig. S2. Expression and correlation of IGF2 and miR-675 (H19) in the context of T and N and LOI/ROI.

Fig. S3. Methylation analysis of 17 CpGs in binding site 6 (BS6) of CTCF in the ICR.

Fig. S4. Differential methylation patterns of promoter P4 regions (P4A, P4B1 and P4B2) in T and N with high and low IGF2 expression.

Fig. S5. Comparison of promoter P3- and P4-promoter methylation with IGF2 mRNA in IGF2 high samples.

Fig. S6. Apal and Hinf digestion of 14 cDNA amplified PCR-Products for LOI definition.

Table S1. IGF2 and GAPDH Primer.

Table S2. Pyrosequencing Primer.