Frequent aberrant methylation of the imprinted *IGF2/H19* locus and *LINE1* hypomethylation in ovarian carcinoma

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Abstract. Epigenetic alteration of tumor-related genes through changes of DNA methylation is a hallmark for carcinogenesis and aberrant DNA methylation modulates the activity of tumor suppressor genes, imprinted genes and repetitive elements. In ovarian carcinoma, frequent loss of imprinting or aberrant methylation of repetitive elements were reported, however, combined analysis were not performed. We analyzed the aberrant methylation of a differentially methylated region (DMR0) and a CTCF binding site of the *IGF2-H19* locus and methylation of *LINE1* and *Satellite 2* in 22 primary ovarian carcinomas (OC) and controls by a quantitative bisulfite restriction analysis (QUBRA). In 91% of OC, a significant hypomethylation of DMR0 was found compared to controls ($p<0.05$). In 77% of OC, a hypermethylation of a CTCF binding site was found ($p<0.05$). A combined hypomethylation of DMR0 and hypermethylation of the CTCF binding was observed in 73% of OC. Hypomethylation of *LINE1* and Satellite 2 was detected in 100 and 23% of OC, respectively. In summary, we found frequent combined aberrant methylation of the *IGF2-H19* locus and *LINE1* in the vast majority of OC, suggesting that these changes are important events in tumorigenesis.

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Abbreviations: IGF2, insulin-like growth factor 2; DMR, differentially methylated region; ICR, imprinting control region; CTCF, CCCTC-binding factor; BS, binding site; LOI, lost of imprinting; *LINE1*, long interspersed nuclear element 1; QUBRA, quantitative bisulfite restriction analysis; 5-aza-dCR, 5-aza-2'-deoxycytidin; OC, ovarian carcinoma

Key words: ovarian carcinoma, DNA methylation, insulin-like growth factor 2, CCCTC-binding factor, long interspersed nuclear element 1

Introduction

Aberrant DNA methylation is almost always detectable in human cancers and may contribute to dysregulated gene expression and chromosomal instability (1). In particular, loss of imprinting of human *insulin-like growth factor 2* gene (*IGF2*) is a common genetic alteration in human malignancies and aberrant methylation of *IGF2/H19* locus has been detected in Wilms' tumors (2,3), ovarian cancers (OC) (4-6), colorectal carcinomas (7-11), prostate cancers (12), osteosarcomas (13), lung adenocarcinomas (14), and head and neck carcinomas (15,16). Loss of imprinting results in an overexpression of the human *IGF2* gene, which encodes a potent mitogenic growth factor. This gene is located together with *H19* within an imprinting domain of chromosome 11p15.5 (17,18). Physiologically, only the paternally inherited *IGF2* allele is transcriptionally active, while *H19* is oppositely imprinted and active only on the maternal allele (17,18). In the *IGF2* gene a differentially methylated region (DMR0) was identified in exon 3 and between *IGF2* and *H19* the imprinting control region (ICR) is located, which is recognized by the insulator CTCF (19,20). Aberrant methylation of the *IGF2*-DMR and the CTCF binding site within the ICR were reported in cancers (2-16).

DNA methylation is an important silencing mechanism for mobile genetic elements, particularly for those elements containing considerable levels of total CpG dinucleotides such as long interspersed element-1 (*LINE1*) and pericentromeric satellite region Satellite 2 (*Sat2*) (21-24). The activation of transposable elements by hypomethylation may result in critical genomic instability by induction of chromosomal recombination and disturbance of transcriptional control mechanism (24-26). Several reports have already analyzed the aberrant methylation of *LINE1* and/or *Sat2* in human cancers (21,24,25,27-29).

In the present study, we determined the association between aberrant methylation of the *IGF2-H19* locus and repetitive elements by application of a new established quantitative bisulfite restriction analysis (QUBRA) PCR protocol to

evaluate quantitatively methylation status of the *IGF2* differentially methylated region (*IGF2-DMR0*), the CTCF binding site 6 in the ICR of the *IGF2-H19* locus (CTCF-BS6), as well as Satellite 2 (*Sat2*) and *LINE1* elements in ovarian carcinoma.

Materials and methods

Tumor probes, cell lines and DNA preparation. Tumor samples and the clinicopathological data were obtained from 22 patients with ovarian carcinoma (Table I). Blood controls were obtained from healthy individuals with no history of cancer. All patients gave a written consent and the study was approved by the local ethics committee of the Medical Faculty. Tumor histology and grading were classified according to the WHO guidelines. Five human ovarian cancer cell lines (SKOV3, OAW42, OVCAR3, CAOV3 and ES2) and U2OS were cultured in RPMI-1640 or DMEM, supplemented with 10% FCS. Normal human mammary epithelial cells MCF10 were grown in MEGM (Cambrex, Brussels, Belgium) and treated with 5 and 10 μM 5-aza-dCR (Sigma, Taufkirchen, Germany) for 4 days. Genomic DNA was extracted from cultured cells and tissues by protein K digestion and a phenol/chloroform procedure.

Quantitative bisulfite restriction analysis (QUBRA). To set up a quantitative bisulfite restriction analysis (QUBRA), unmethylated and completely methylated deaminated DNA sequences were cloned and used as standard to generate calibration curves. Therefore, PCR fragments of *IGF2*-DMR0, CTCF-BS6, *Sat2* and *LINE1* were produced with unmodified genomic DNA isolated from human fibroblast and primers listed in Table II. PCR was performed with 150 ng of genomic DNA in 25 μl reaction, containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each primer and 1.5 units of High Fidelity Taq Polymerase (Roche, Mannheim, Germany). PCR products were cloned in pGEM-T easy (Promega, Mannheim, Germany) and sequenced (Seqlab, Göttingen, Germany). Two micrograms of plasmid DNA was *in vitro* methylated with 10 units of *SSSI* methylase (NEB, Frankfurt, Germany) and 1X SAM at 37˚C for 16 h or mock-methylated. Mock-methylated and methylated plasmid were purified and treated with a standard bisulfite deamination protocol. Briefly, DNA was denatured with 0.3 M NaOH at 37˚C for 15 min. Sodium bisulfite (3.12 M) (Sigma, Taufkirchen, Germany) and 5 mM hydroquinone (Sigma) was added and incubated at 56˚C overnight. DNA was purified with the Wizard DNA Clean Up System (Promega) according to the manufacturer protocol and eluted in 50 μl H_2O. Finally, DNA was treated for 15 min with 0.3 M NaOH at 37˚C, precipitated and resuspended in 50 μl TE buffer. Subsequently, 200 ng of deaminated DNA was amplified with the bisulfite specific primers listed in Table II and PCR conditions described above. PCR fragments were cloned in pGEM-T easy. Completely converted methylated (CpG) and unmethylated (TpG) DNA fragments were identified by sequencing and utilized to generate standard curves for QUBRA. Therefore, different ratio of unmethylated and methylated plasmid were produced (100% = 1 pg plasmid) and amplified with a ^{32}P end labeled primer. Primer (20 pmol) were labeled with 10 units of T4 polynucleotide kinase (NEB, Frankfurt, Germany) and 25 pmol ^{32}P-γ-ATP. DNA was amplified in 25 μl reaction buffer containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 pmol of each primer, 0.2 pmol labeled primer and 2.5 units of Taq polymerase (Invitek, Berlin, Germany) at 95˚C for 30 sec, Ta for 30 sec, and 72˚C for 30 sec for 20 cycles or 40 cycles if no semi-nested PCR was performed. For *IGF2*-DMR0 and CTCF-BS6 a semi-nested PCR was performed using an internal primer with similar conditions as described for the preceding PCR amplification, but for 30 cycles (Table II). For the restriction enzyme analysis, 20 ng of PCR product was digested with 10 units of *TaqI* or *HpyCH4IV* (New England Biolabs; Beverly, MA), according to conditions specified by the manufacturer of the enzyme and separated on 10% polyacrylamid gels with Tris-borate EDTA at 150 V for 45 min. Gels were dried on filter papers and exposed to a phosphor screen and analyzed with Image Quant 5.0 on a Storm 860 (Molecular Dynamics, Sunnyvale, USA). All experiments were done at least three times. Subsequently, standard curves were determined and a polynomial regression was performed. This function was used to calculate the exact amount of methylated DNA in the tissue samples. Therefore, 2 μg of genomic DNA isolated from tissue samples and cell lines was bisulfite modified and QUBRA with 200 ng of bisulfite treated DNA was performed as described above. All experiments were done in triplicates and the amount of methylated DNA was determined utilizing polynomial regression.

Statistical analysis. Statistical analyses were carried out using SPSS14. $P<0.05$ were considered to be significant.

Results

Quantitative methylation analysis of IGF2-DMR, CTCF-BS6, Satellite 2 (Sat2) and LINE1. For several technical reasons conventional methylation specific PCR and combined bisulfite restriction analysis are not useful to measure methylation changes that occur in the process of loss of imprinting and during hypomethylation. To overcome this technical limitation we established a quantitative bisulfite restriction analysis (QUBRA) of the *IGF2*-DMR, CTCF-BS6, *Sat2* and *LINE1* by modifying combined bisulfite restriction analysis (Fig. 1). To adjust for amplification bias during PCR, we utilized distinct amounts of cloned methylated and unmethylated DNA templates and generated calibration curves for QUBRA (Fig. 2). Interestingly for all four analyzed regions, preferential amplification of methylated DNA was observed (Fig. 2). Subsequently, a polynomial regression was performed and the coefficient of determination was obtained (Fig. 2). This polynomial function was utilized to adjust for amplificational bias in our experiments.

Aberrant methylation of the IGF2-H19 locus in primary ovarian carcinoma. Subsequently, we analyzed the methylation status of *IGF2*-DMR0 and of a CTCF binding site (CTCF-BS6) at the *IGF2-H19* locus in 22 primary ovarian carcinomas (OC), five OC cell lines and controls by QUBRA (Fig. 3). In all five controls, an identical methylation pattern was observed (Table III). In 20 out of 22 (91%) primary OC a significant hypomethylation of *IGF2*-DMR0 was found

Table I. Demographic data of the primary ovarial carcinoma.

Case	Stage (FIGO)	Grade	Age	Disease-free survival (month)	Overall survival (month)
OC2	Ic	G1	67	22.0	120.2
OC3	Ic	G2	65	49.8	65.8
OC4	IIIc	G2	67	12.0	46.9
OC6	Ic	G1	64	31.8	64.4
OC13	IIIc	G2	55	16.9	16.9
OC14	IIIb	G2	50	11.5	11.5
OC15	IIIb	G3	76	-	0.1
OC22	Ia	G1	81	-	55.1
OC30	IIIc	G3	49	5.5	22.8
OC32	IIIc	G3	61	13.6	17.4
OC37	Ic	G2	59	43.0	70.5
OC39	IIIc	G2	62	4.8	20.6
OC41	IIIb	G3	57	42.6	68.2
OC43	IIIc	G3	73	7.9	62.9
OC45	Ia	G2	64	28.2	55.1
OC46	Ib	G3	61	25.4	69.1
OC49	IIIc	G3	66	-	0.0
OC50	IIIc	G2	59	7.5	19.4
OC51	IIIc	G3	72	14.1	36.4
OC55	IIb	G3	58	64.6	64.6
OC56	IIIc	G3	68	4.1	4.1
OC57	IIIc	G3	72	34.6	43.2

Table II. Primer sequence, annealing temperature (Ta) and product length.

Primer	Sequence (5'-3')	Ta product
*LINE12*UN	GGGGGGAGGAGCCAAGATGG	60°C
*LINE12*LN	CGAGCTTCCCGGCTGCTTTG	493 bp
CBS6UN	TGAAGGTTGGGGAGATGGGAGGA	61°C
CBS6LN	CGTGACTTGAGTCCCAGGCCATG	461 bp
*IGF2*UN	CACCCTGGGGCCAAGGCAGT	61°C
*IGF2*LN	CTTGAGGGGTCATGGCACGGAAT	279 bp
*SAT2*UN2	CGAACGGACCCGAATGGAATC	59°C
*SAT2*LN2	TTTGATGTTGATTCCATTCGATTCCA	251 bp
*SAT2*UBS2[a]	TGGAATGGAATGGAATAATTTATTGGA	54°C
*SAT2*LBS2[a]	TCCATTAAATAATAACTCCTTTCATTTCCA	176 bp
*LINE1*U1[a]	GGGGGGAGGAGTTAAGATGGT	54°C
LINE1L2[a]	ACTACTTTATTTACCTAAACAAACCTAAACAA	482 bp
UCTCFA[a]	GTGTTTTAGTTTTATGGATGATGGGGAT	54°C
LCTCFA[a]	ATCCCAAACCATAACACTAAAACCCTC	408 bp
UCTCFB[a]	GGGGGTTTTTGTATAGTATATGGGTATTTT	327 bp
U*IGF2*A[a]	AAGGTAGTTTTTTTGGGAATGTTTATTT	50°C
L*IGF2*B[a]	CTCCAAACACCCCCACCTTAA	227 bp
U*IGF2*B[a]	TTTTTTGTTGTATTTTGGATTTAGATTTTTT	185 bp

[a]Primer for bisulfite treated DNA.

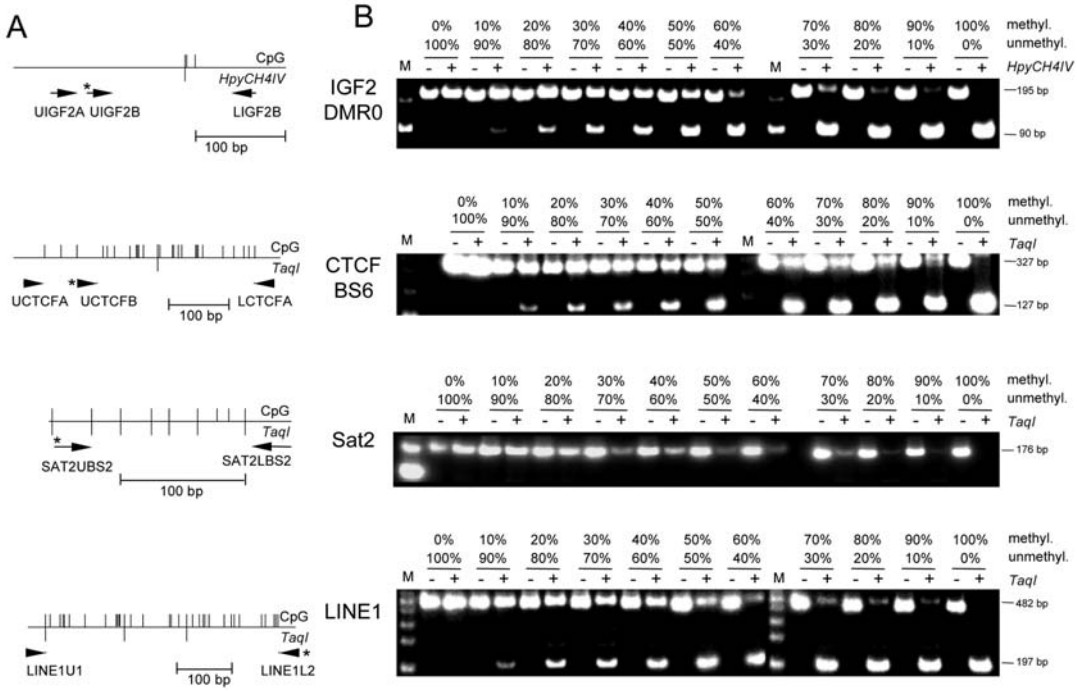

Figure 1. Methylation analysis of *IGF2*-DMR0, CTCF-BS6, *Sat2* and *LINE1* by a quantitative bisulfite restiction (QUBRA). (A) Maps of *IGF2*-DMR, CTCF-BS6, Satellite2 (*Sat2*) and *LINE1*. Positions of CpGs and of restriction enzyme recognition sites (*HpyCh4IV* and *TaqI*) are shown. Arrows indicate utilized primers and labelled primers are marked by asterisks. (B) QUBRA. The indicated amounts of methylated (methyl.) and unmethylated (unmethyl.) DNA standards were mixed and amplified with a labeled primer by PCR. Products were digested (+) or mock-digested (-) with the indicated enzymes and analyzed by PAGE and gels were exposed to phosphors screens. A labeled 100 bp ladder (M) was utilized as a length marker.

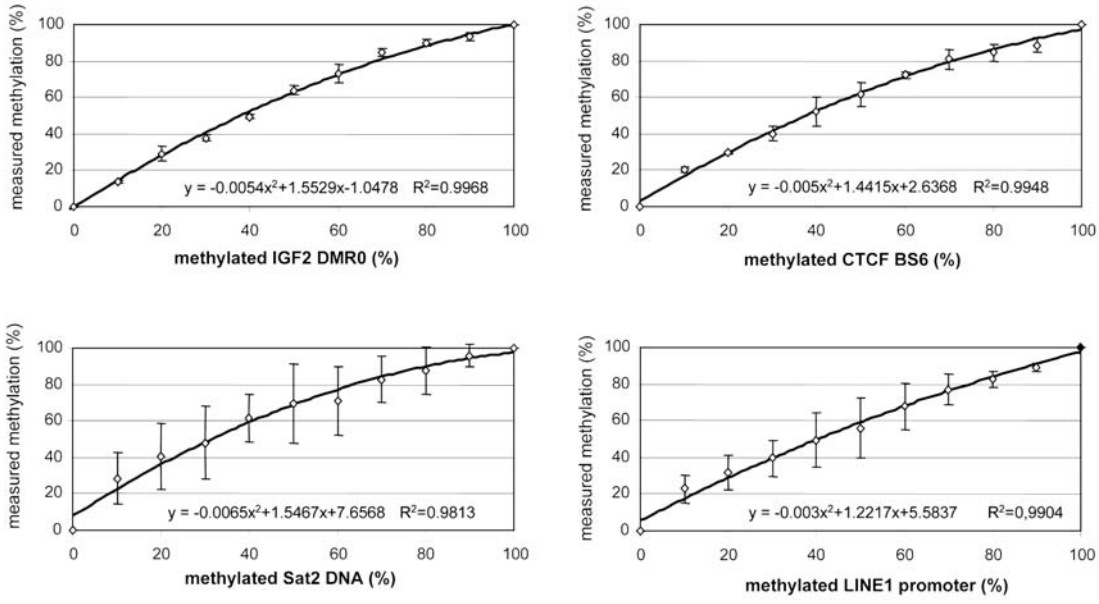

Figure 2. Calibration curves of QUBRA. Utilized and measured percentage of methylated *IGF2*-DMR0, CTCF-BS6, *Sat2* and *LINE1* was determined from three independent experiments and plotted on a graph. Standard deviations are indicated. A polynomial regression was performed and for each regression the coefficient of determination was calculated.

compared to controls ($p<0.05$; Fig. 3A and B; Table III). Moreover, hypermethylation of CTCF-BS6 was observed in 17 out of 22 (77%) OC compared to the controls ($p<0.05$; Fig. 3C and D; and Table III). A combined hypomethylation of *IGF2*-DMR0 and hypermethylation of CTCF-BS6 was detected in 73%. In the OC cell lines the methylation pattern at the *IGF2-H19* locus was different compared to primary OC and controls. Three cell lines (SKOV3, OVCAR3 and ES2) showed hypomethylation of both regions and OAW42 and CAOV3 exhibited a hypermethylation of *IGF2*-DMR0

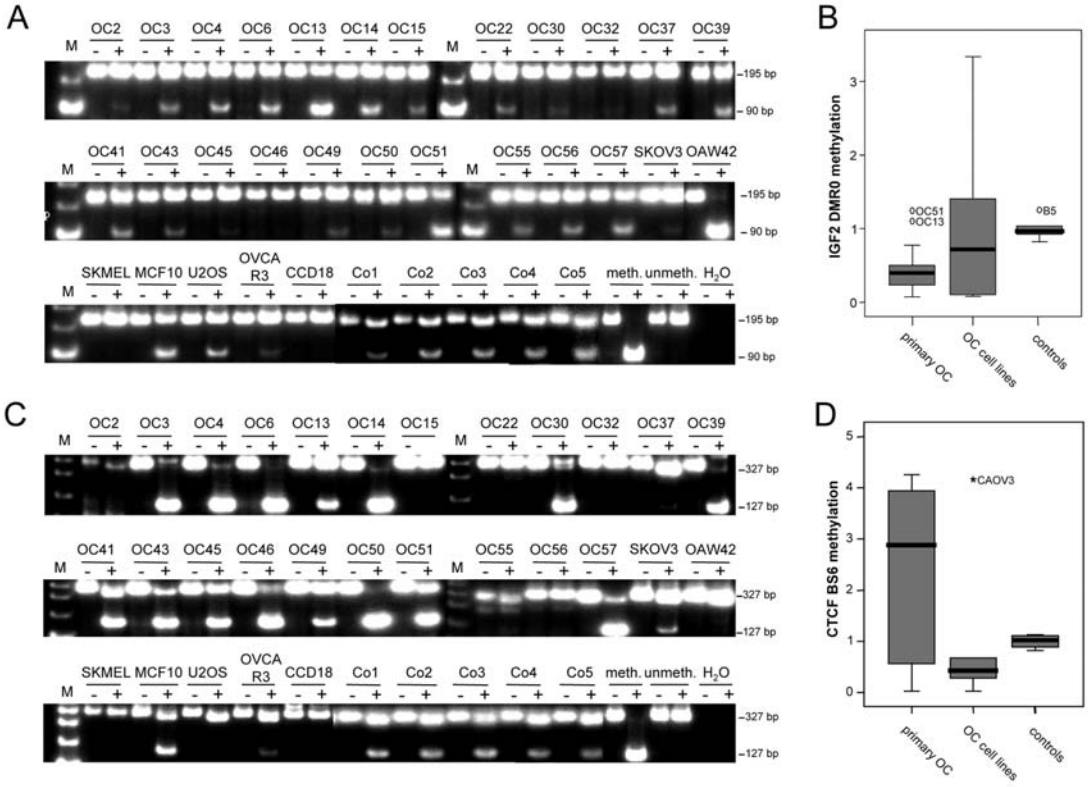

Figure 3. Methylation analysis of *IGF2*-DMR0 and CTCF-BS in ovarian carcinoma. (A) Methylation of the *IGF2*-DMR0 in primary ovarian carcinoma (OC) samples, cancer cell lines (SKOV3, OAW42, SKMEL, U2OS and OVCAR3), normal cell lines (MCF10 and CCD18) and controls (Co) was analyzed by QUBRA with restrictions enzyme (+) or mock digest (-). Methylated (meth.) and unmethylated (unmeth.) standards were separated together with a labelled 100 bp ladder (M). (B) Whisker-box-plot of the *IGF2*-DMR0 methylation status of primary ovarian carcinomas (OC), OC cell lines (SKOV3, OAW42, OVCAR3, CAOV3 and ES2) and five controls (Co). All methylation levels are relative to controls (=1). (C) Methylation analysis of the CTCF-BS6 in primary ovarian carcinoma (OC) samples, cancer cell lines and controls. For details see Fig. 3A. (D) Whisker-box-plot of the CTCF-BS6 methylation in primary ovarian carcinomas (OC), OC cell lines and controls. For details see Fig. 3B.

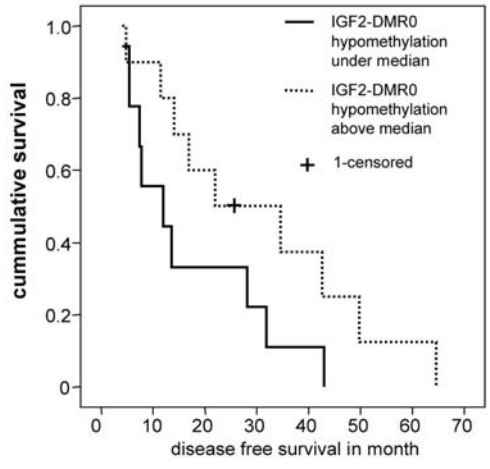

Figure 4. Kaplan-Meier survival curve. Patients were divided in those with a tumor that harboured *IGF2*-DMR0 hypomethylation under median and tumors with *IGF2*-DMR0 hypomethylation above median and the disease free survival was plotted in a Kaplan-Meier survival graph.

(Fig. 3 and Table III). CAOV3 was the only cell line with a hypermethylation of CTCF-BS6. Interestingly, we observed that patients (n=9) with a hypomethylation under median of *IGF2*-DMR had a trend for a shorter disease free survival compared to patients (n=10) with hypomethylation above median (p=0.086; Fig. 4).

LINE1 and Satellite 2 hypomethylation. To analyze the methylation status of repetitive sequences in OC, the methylation of the pericentromeric heterochromatin associated Satellite 2 DNA (*Sat2*) and of the retrotransposon *LINE1* was investigated by QUBRA (Fig. 5A). Only five of 22 (23%) cases showed hypomethylation of *Sat2* (Fig. 5B and Table III). In contrast, hypomethylation of *LINE1* was detected in all analyzed cases (Fig. 5C and Table III) and was much more pronounced in poorly differentiated OC compared to well differentiated cases (Fig. 5D).

Correlation between methylation frequency. All cases with aberrant methylation of the *IGF2/H19* locus also exhibited hypomethylation of *LINE1* (Table III). There was no association between methylation rates (data not shown). To analyze the effect of an inhibitor of DNA methyltransferase on the methylation status of *Sat2*, *LINE1*, *IGF2*-DMR0 and CTCF-BS6 in normal human epithelial cells, we treated the cell line MCF10 with 5-aza-2'-deoxycytidine (5-aza-dCR) for four days and analyzed the methylation by QUBRA (Fig. 6 and Table IV). This treatment induced a hypomethylation of *Sat2*, *LINE1* and *IGF2*-DMR0, however, CTCF-BS6 was not demethylated.

Table III. Summary of methylation analysis in primary ovarial carcinoma.

	Case	*IGF2* DMR0 methylation (±SD)	CTCF BS6 methylation (±SD)	*Sat2* methylation (±SD)	*LINE1* methylation (±SD)
Primary ovarial carcinoma (OC)	OC2	0.68 (0.42)	2.31 (1.97)	1.00 (0.00)	nd
	OC3	0.41 (0.08)	3.63 (0.58)	0.23 (0.10)	0.22 (0.04)
	OC4	0.32 (0.02)	4.28 (0.02)	1.00 (0.00)	0.39 (0.09)
	OC6	0.40 (0.16)	4.29 (0.00)	0.82 (0.06)	0.44 (0.06)
	OC13	1.10 (0.26)	0.56 (0.39)	1.00 (0.00)	nd
	OC14	0.46 (0.14)	3.03 (1.72)	1.00 (0.00)	0.47 (0.05)
	OC15	0.37 (0.06)	0.53 (0.63)	1.00 (0.00)	0.63 (0.13)
	OC22	0.23 (0.00)	0.03 (0.03)	1.00 (0.00)	0.83 (nd)
	OC30	0.24 (0.10)	3.97 (0.05)	1.00 (0.00)	nd
	OC32	0.07 (0.02)	0.04 (0.01)	1.00 (0.00)	0.52 (nd)
	OC37	0.19 (0.08)	0.42 (0.57)	1.00 (0.00)	0.76 (0.03)
	OC39	0.72 (0.16)	4.23 (0.04)	0.98 (0.01)	0.62 (0.04)
	OC41	0.42 (0.06)	2.89 (0.10)	1.00 (0.00)	0.39 (0.13)
	OC43	0.21 (0.00)	3.63 (0.24)	0.84 (0.12)	0.11 (0.12)
	OC45	0.21 (0.11)	2.51 (0.11)	0.80 (0.05)	0.52 (0.12)
	OC46	0.47 (0.33)	4.17 (0.08)	0.94 (0.04)	0.23 (0.03)
	OC49	0.50 (0.24)	1.98 (1.52)	0.94 (0.05)	0.24 (0.14)
	OC50	0.36 (0.15)	4.29 (0.00)	0.99 (0.01)	0.58 (0.39)
	OC51	1.24 (0.09)	2.91 (0.39)	0.99 (0.01)	0.10 (0.09)
	OC55	0.48 (0.04)	1.31 (0.92)	1.00 (0.00)	0.50 (0.04)
	OC56	0.38 (0.07)	0.40 (0.53)	0.68 (0.02)	0.07 (0.06)
	OC57	0.77 (0.08)	3.11 (0.94)	1.00 (0.00)	0.54 (0.13)
OC cell lines	SKOV3	0.08 (0.01)	0.43 (0.14)	1.00 (0.00)	0.48 (0.18)
	OAW42	3.33 (0.02)	0.03 (0.04)	1.00 (0.00)	0.53 (0.16)
	OVCAR3	0.10 (0.01)	0.68 (0.17)	0.40 (0.07)	0.32 (0.11)
	CAOV3	1.41 (0.14)	4.19 (0.10)	1.00 (0.00)	0.39 (0.11)
	ES2	0.72 (0.02)	0.29 (0.03)	0.69 (0.05)	0.48 (0.02)

All methylation levels are relative to controls (=1); SD, standard deviation; nd, not determined.

Table IV. Methylation of *IGF2* DMR0, CTCF BS6, *Sat2* and *LINE1* in MCF10.

5-aza-dCR	*IGF2* DMR0 (±SD)	CTCF BS6 (±SD)	*Sat2* (±SD)	*LINE1* (±SD)
0 μM	1.10 (±0.03)	0.99 (±0.10)	1.00 (±0)	0.84 (±0.00)
5 μM	0.89 (±0.06)	1.08 (±0.05)	0.94 (±0.05)	0.83 (±0.01)
10 μM	0.44 (±0.03)	0.98 (±0.15)	0.72 (±0.02)	0.42 (±0.34)

All methylation levels relative to blood samples (=1); SD, standard deviation.

Discussion

Conventional methylation specific PCR and combined bisulfite restriction analysis does not provide quantitative information on the proportion of methylated sequences among total alleles. For measurement of methylation changes that occur during loss of imprinting and during hypomethylation of repetitive DNA elements, we established a quantitative bisulfite restriction analysis (QUBRA) of the *IGF2*-DMR, CTCF-BS6, *Sat2* and *LINE1*. In contrast to other quantitative assays like Bio-COBRA (30) and pyrosequenzing, which depend on micro fluidics chips and a Bioanalyzer or a Pyrosequenzer, QUBRA is an inexpensive and reliable method and requires only standard laboratory equipment.

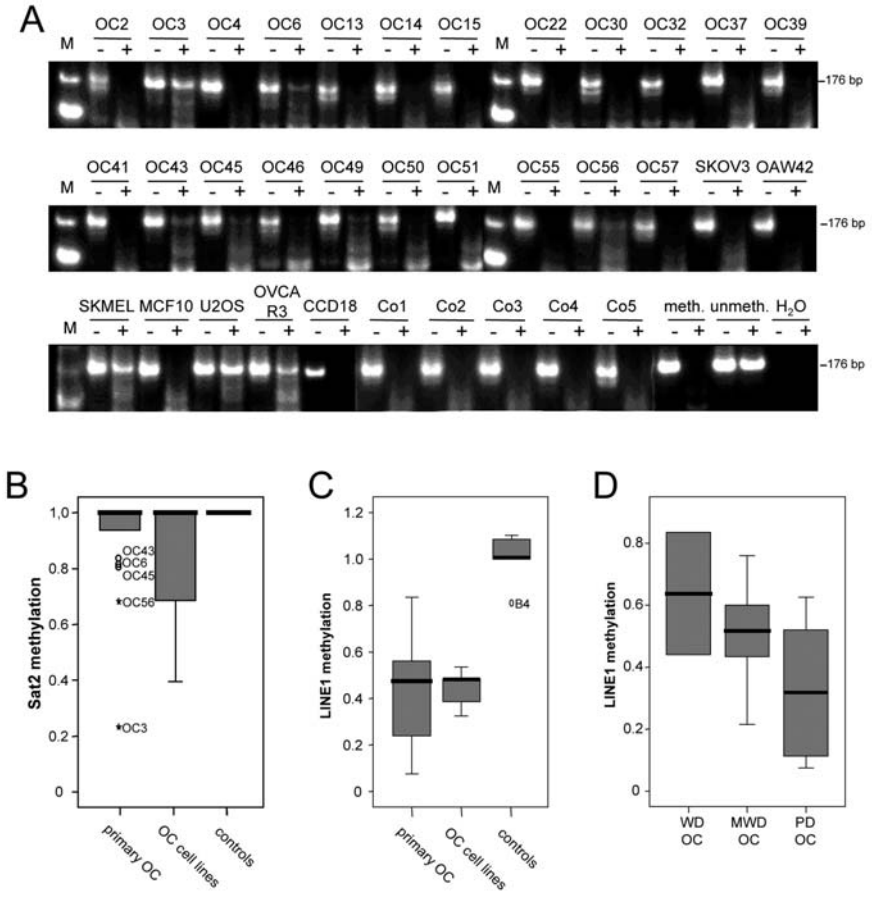

Figure 5. Methylation analysis in ovarian carcinoma. (A) Methylation of the *Sat2* in primary ovarian carcinoma (OC) samples, cancer cell lines (SKOV3, OAW42, SKMEL, U2OS and OVCAR3) normal cell lines (MCF10 and CCD18) and controls (Co) was determined by QUBRA with restrictions enzyme (+) or mock digest (-). Methylated (meth.) and unmethylated (unmeth.) standards were separated together with a 100 bp ladder (M) by PAGE. (B) Whisker-box-plot of the *Sat2* methylation status of primary ovarian carcinomas (OC), OC cell lines (SKOV3, OAW42, OVCAR3, CAOV3 and ES2) and controls. Methylation levels are relative to controls (=1). (C) Whisker-box-plot of *LINE1* methylation in primary ovarian carcinomas (OC), OC cell lines and controls. For details see Fig. 5B. (D) Whisker-box-plot of *LINE1* methylation. Methylation was associated with differentiational stage of the primary ovarian carcinoma (OC) according to well-differentiated (WD), moderately well differentiated (MWD) and poorly-differentiated carcinoma (PD).

To the best of our knowledge, this is the first study, which analyzed combined methylation status of *IGF2-H19*, *LINE1* and *Sat2* in ovarian carcinoma (OC). All primary OC exhibited LINE1 hypomethylation and in 91% of OC a significant hypomethylation of the *IGF2* DMR0 was found. This result shows that hypomethylation of LINE1 is also associated with demethylation of DMR0 in exon 3 within the *IGF2* gene. As already suggested by Cui *et al* this implies a CTCF and enhancer-independent mechanisms of *IGF2* through aberrant methylation of additional regulatory sequences within the *IGF2* gene (9). The DMR0 has been shown to contain promoter activity (31). Thus, hypomethylation of *IGF2*-DMR, leads to *IGF2* overexpression in colon cancer and is associated with up to 5-fold increased risk of colorectal neoplasia (7,8). In ovarian cancers, maternal allele-specific hypomethylation in *IGF2*-DMR were more likely detectable in tumor cells when compared to normal lymphocyte DNA (5) and this is consistent with our results. *IGF2*-DMR0 hypomethylation was also observed in lymphocytes of the normal population (8,19). We observed hypomethylation of CTCF-BS6 in blood controls (Fig. 3C) and therefore four times higher methylation was observed in some of the OCs.

We observed that patients with a pronounced hypomethylation of *IGF2*-DMR0 had a trend for a shorter relapse-

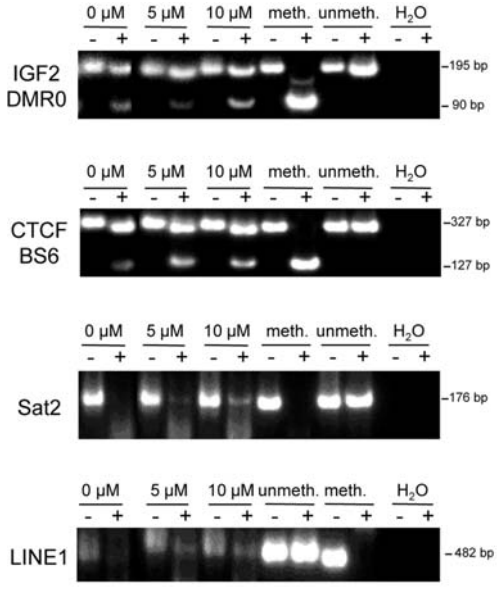

Figure 6. Methylation analysis of MCF10 cell treated with 5-aza-dCR. Human mammary epithelial cells were treated for 4 days with indicated concentrations of 5-aza-dCR and methylation status of *IGF2*-DMR0, CTCF-BS6, *Sat2* and *LINE1* was analyzed by QUBRA with restrictions enzyme (+) or mock digested (-). Methylated (meth.) and unmethylated (unmeth.) standards and a negative control (H_2O) were utilized as controls.

free survival compared to the patients with a weaker hypomethylation (p=0.086). Thus, our finding indicates that methylation status of *IGF2* may have prognostic relevance in OC. However, in a study were only a qualitative methylation status of the *IGF2*-DMR was revealed, no correlation to survival was found (5). Thus the degree of *IGF2*-DMR hypomethylation could be critical for survival analysis, but this has to be validated in a larger cohort of patients.

The hypermethylation of CTCF-BS6 was observed in 77% of OC compared to the controls ($p<0.05$). This finding is in concordance with the enhancer competition model of *IGF2* and *H19* promoters for a shared enhancer. The accessibility of the maternal *IGF2* allele to this enhancer is blocked by CTCF binding to unmethylated CTCF binding sites on *H19* (i.a. CTCF-BS6) (32). Accordingly, relaxation of *IGF2* imprinting could be the result of hypermethylation of CTCF-BS6. It is well known that removal of *IGF2* imprinting in OC may be initiated by methylation and blocking of CTCF binding sites on *H19* (32). In serous epithelial ovarian cancer, a positive correlation between elevated *IGF2* expression and hypermethylation of CTCF-binding site was observed previously (5,33). In our study, we detected frequent combined hypomethylation of *IGF2*-DMR and hypermethylation of CTCF-BS6 indicating a simultaneous or related loss of imprinting at the *IGF2-H19* DMR and ICR.

The methylation of the pericentromeric heterochromatin associated Satellite 2 DNA and of the retrotransposon *LINE1* was investigated by QUBRA. *LINE1* was hypomethylated in all OC. High prevalence of *LINE1* hypomethylation throughout all tumor stages and grades allows the conclusion that hypomethylation of *LINE1* is an early event during ovarian carcinogenesis. Previous studies reported that *LINE1* hypomethylation was associated with FIGO stage (the International Federation of Gynecology and Obstetrics) and tumor grade in OC (34). We observed more pronounced *LINE1* hypomethylation in poorly differentiated OC compared to well differentiated cases. However, only 23% of OC exhibited hypomethylation in *Sat2* region. The low frequence of hypomethylation in *Sat2* DNA might be a consequence of a hypoacetylated heterochromatin and suggests a low degree of pericentromeric rearrangements in the investigated OC (35). Treatment of cells with 5-aza-dCR induced hypomethylation of *Sat2*, *LINE1* and *IGF2*-DMR0, however CTCF-BS6 was not demethylated. This result may indicate a different mechanism to maintain the methylation state at the CTCF binding site and that germ line specific DMR could be resistant to demethylation.

We have generated a quantitative method to investigate aberrant methylation changes at the *IGF2/H19* locus, *LINE1* and *Sat2* in ovarian carcinoma (OC). Combined hypomethylation of the *IGF2* DMR0 and *LINE1* were frequently detected. Interestingly, hypomethylation of *Sat2* was infrequently found and hypermethylation of a CTCF binding site occurred in these OC. These results indicate a specific hypomethylation of distinct elements (*LINE1* and DMR0) and hypermethylation of others (CTCF-BS) rather than global hypomethylation or hypermethylation of genomic DNA. Thus, mechanisms that produce these divergent methylation patterns in the same tumor should be revealed in more detail.

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