0013-7227/02/$15.00/0
Printed in U.S.A.

The Journal of Clinical Endocrinology & Metabolism 87(3):1170–1176

Association of H19 Promoter Methylation with the Expression of H19 and IGF-II Genes in Adrenocortical Tumors

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Low H19 and abundant IGF-II expression may have a role in the development of adrenocortical carcinomas. In the mouse, the H19 promoter area has been found to be methylated when transcription of the H19 gene is silent and unmethylated when it is active. We used PCR-based methylation analysis and bisulfite genomic sequencing to study the cytosine methylation status of the H19 promoter region in 16 normal adrenals and 30 pathological adrenocortical samples. PCR-based analysis showed higher methylation status at three *Hpa*II-cutting CpG sites of the H19 promoter in adrenocortical carcinomas and in a virilizing adenoma than in their adjacent normal adrenal tissues. Bisulfite genomic sequencing revealed a significantly higher mean degree of methylation at each of 12 CpG sites of the H19 promoter in adrenocortical carcinomas than in normal adrenals ($P < 0.01$ for all sites) or adrenocortical adenomas ($P < 0.01$, except $P < 0.05$ for site 12 and $P > 0.05$ for site 11). The mean methylation degree of the 12 CpG sites was significantly higher in the adrenocortical carcinomas (mean ± SE, 76 ± 7%) than in normal adrenals (41 ± 2%) or adrenocortical adenomas (45 ± 3%; both $P < 0.005$). RNA analysis indicated that the adrenocortical carcinomas expressed less H19 but more IGF-II RNAs than normal adrenal tissues did. The mean methylation degree of the 12 H19 promoter CpG sites correlated negatively with H19 RNA levels ($r = -0.550$; $P < 0.01$), but positively with IGF-II mRNA levels ($r = 0.805$; $P < 0.001$). In the adrenocortical carcinoma cell line NCI-H295R, abundant IGF-II, but minimal H19, RNA expression was detected by Northern blotting. Treatment with a cytosine methylation inhibitor, 5-aza-2′-deoxycytidine, increased H19 RNA expression, whereas it decreased IGF-II mRNA accumulation dose- and time-dependently (both $P < 0.005$) and reduced cell proliferation to 10% in 7 d. Our results suggest that altered DNA methylation of the H19 promoter is involved in the abnormal expression of both H19 and IGF-II genes in human adrenocortical carcinomas. (*J Clin Endocrinol Metab* 87: 1170–1176, 2002)

HUMAN H19 AND IGF-II genes, mapped contiguously on chromosome 11p15.5, are expressed in a parental origin-specific manner, a phenomenon known as genomic imprinting (1). H19, a gene whose transcript is not translated (2), is supposed to be involved in tumor suppression (3). IGF-II is an important mitogen, playing a role in normal fetal and postnatal growth and in tumorigenesis (4). H19 and IGF-II have been found to show coordinate, reciprocal regulation in a tissue-specific and developmentally regulated manner. H19 is not expressed in choroid plexus or leptomeninges, where IGF-II is expressed biallelically in mouse and man (5). IGF-II is also expressed biallelically in H19 knockout mice (6, 7). In methyltransferase-deficient mice the normally silent paternal allele of the H19 gene is activated, whereas the normally active paternal allele of the IGF-II gene is repressed (8).

Aberrant cytosine methylation has been found to be associated with altered gene expression in tumors (9–11). Certain regions (often located at the 5′-ends of genes) rich in CpG dinucleotides are known as CpG islands (12). The promoter CpG islands are usually unmethylated in normal tissues, but the imprinted genes form an exception, with one of the parental alleles being often methylated (9). In the mouse, the H19 promoter has been found to be methylated when transcription is silent and unmethylated when it is active (13). Normal methylation patterns are frequently disrupted in tumor cells with region-specific hypermethylation (11). In Wilms' tumors, DNA hypermethylation in the H19 promoter is associated with remarkable down-regulation of H19 expression and loss of imprinting of the IGF-II gene (14). In addition, aberrant methylation of the differentially methylated region upstream of the H19 gene seems to be necessary, but not sufficient, for loss of imprinting (15). In Wilms' tumorigenesis, H19 inactivation has been considered a preneoplastic event (16, 17). Therefore, hypermethylation of the H19 promoter appears to be an important factor leading to low H19 and high IGF-II expression.

Both H19 and IGF-II genes are supposed to be involved in the normal development and tumorigenesis of human adrenals. Human fetal adrenals express abundantly both H19 and IGF-II genes, and the regulation of these two genes is parallel and multifactorial, suggesting common regulatory mechanisms for these adjacent genes (18). In normal human adult adrenals IGF-II expression is low (19, 20), but H19 expression remains quite high (21). H19 and IGF-II genes are expressed at about the same level in benign adrenocortical neoplasms as in normal adrenal glands, whereas adrenocortical carcinomas show very low H19 and high IGF-II expression (21, 22). Gicquel *et al.* (23) found that about 80% of the adrenocortical tumors with high IGF-II expression exhibited LOH

Abbreviation: Azad, 5-Aza-2′-deoxycytidine.

(loss of maternal allele and duplication of the paternal one), which correlated with the abrogation of H19 expression. Thus, this loss of H19 expression in conjunction with high IGF-II expression may be associated with malignant behavior in adrenocortical carcinomas. However, the mechanisms for this phenomenon remain unknown. To determine whether DNA methylation plays a role, we used PCR-based methylation analysis and bisulfite genomic sequencing to assess the methylation status of H19 promoter in different adrenal tumors compared with that in normal adrenals. We then explored the association of the expression of H19 and IGF-II genes with the methylation status of the H19 promoter.

Materials and Methods

Tissues

Normal adrenals (removed during nephrectomy for kidney tumors, n = 7) and pathological adrenal glands were obtained from patients during operations performed at the Department of Surgery, Helsinki University Central Hospital. The pathological adrenocortical tissues included 1) Cushing's (n = 6), virilizing (n = 2), and nonfunctional (hormonally inactive, n = 2) carcinomas; 2) Cushing's (n = 5), Conn's (n = 5), virilizing (n = 2), and nonfunctional (n = 4) adenomas; and 3) hyperplastic adrenals (two patients with bilateral adrenal hyperplasia due to Cushing's disease and two nodularly hyperplastic adrenals). Tumor-adjacent normal adrenal tissues (n = 9) were also used. The tissue specimens were sent to the Department of Pathology, where the diagnoses were established based on both clinical and histopathological data. The study protocol was approved by the local ethical committees.

Cell cultures

Human adrenocortical carcinoma NCI-H295R cells from American Type Culture Collection (Manassas, VA) were grown in a 1:1 mixture of DMEM and Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 1% ITS+1 liquid medium supplement (Sigma), 2% Ultroser SF (Biosepra, Marlborough, MA), 2 mmol/liter L-glutamine (Life Technologies, Inc., Paisley, UK), and antibiotics (125 μg/ml streptomycin and 125 IU/ml penicillin; Orion Pharmaceutical Co., Espoo, Finland) at 37 C in a 5% CO_2 atmosphere. The medium was refreshed every other day, and the cells were split at a ratio of 1:3 with trypsin after reaching confluence. Treatment with a cytosine methylation inhibitor, 5-aza-2′-deoxycytidine (Azad; Sigma), was initiated on the second day after reseeding the cells. Triplicate dishes were used for the experiments, which were repeated at least three times.

RNA analysis

Total RNA was isolated from frozen tissues by ultracentrifugation through a cesium chloride cushion (24). Cytoplasmic RNA was extracted from the cultured cells (25). Northern blotting and hybridizations were performed as previously described (21). The relative intensities of autoradiographic signals were quantified by densitometric scanning. All of the RNA data shown here were normalized with the respective 28S rRNA values.

PCR-based methylation analysis

PCR analysis based on the inability of *Hpa*II to cut methylated CCGG sequence (26) was used to analyze the H19 promoter containing three *Hpa*II recognition sites (Fig. 1, GenBank accession no. AF125183). Genomic DNA was isolated as reported previously (27). DNA (500 ng) was digested for 6 h with 10 U of the *Hpa*II enzyme (Roche Molecular Biochemicals, Mannheim, Germany) following the manufacturer's recommendations. Optimal PCR conditions were found to produce a clean 462-bp PCR product from undigested genomic DNA, but no product from DNA digested with *Msp*I (a methylation-insensitive isoschizomer of *Hpa*II): denaturing at 95 C for 5 min; 30 cycles of 95 C for 60 sec, 57 C (annealing temperature) for 30 sec, and 72 C for 30 sec; and then final extension at 72 C for 8 min. The PCR reaction volume was 50 μl, containing 0.2 mmol/liter of each dNTP, 0.5 μmol/liter of each primer, 1 × reaction buffer, 2.5 mmol/liter $MgCl_2$, 1.5 U *Taq* DNA polymerase (Fermentas Tamro Corp., Vantaa, Finland), and 50 ng template DNA. The primer set was 5′-AGG TGA TGG GGC AAT GCT CA-3′ (sense, P1 in Fig. 1) and 5′-CCT ACT CCA CAC TCC TCA CT-3′ (antisense, P2). Exon 9 of IGF-II gene (GenBank accession no. X07868) was used as the internal control for the DNA amount because this region has no *Hpa*II/*Msp*I cutting site. The primer set for IGF-II was 5′-CTT GGA CTT TGA GTC AAA TTG G-3′ (sense) and 5′-GGT CGT GCC AAT TAC ATT TCA-3′ (antisense). The PCR products were resolved on 1.8% agarose gels. PCR-based analyses were performed at least twice to ensure reproducibility of the results.

Bisulfite sequencing methylation analysis

A previously described DNA methylation analysis using bisulfite-induced modification and genomic sequencing (28) with minor modifications was used to assess the methylation status of all 14 CpG sites in the H19 promoter region, including the first 2 CpG sites after the transcription start site (Fig. 1). Briefly, 3 μg genomic DNA were denatured with NaOH and deaminated with sodium bisulfite to convert all unmethylated cytosines to uracils. The outer PCR reaction was carried out with primers 5′-TTG GTA GGT AGG GAG TAG TAG GTA TG-3′ (sense) and 5′-AAC CCA TCA/G TCC CCA ACT AAT AT-3′ (antisense), chosen from the area containing as few CpGs as possible. The PCR was performed for 40 cycles of 96 C for 15 sec, 56 C for 30 sec, and 72 C for 90 sec. The inner PCR was carried out with primers 5′-GTAAAAC-GACGGCCAGT-GGG AGG TGA TGG GGT AAT GTT TA-3′ (sense, P3 in Fig. 1) and 5′-ACC TAC TCC ACA CTC CTC ACT AAC CT-3′ (antisense, P4). The antisense primer was biotinylated to separate and isolate the strands of PCR products with the aid of streptavidin-coated magnetic beads, and the sense primer had in its 5′ a universal oligonucleotide for sequencing. The PCR was carried out for 33 cycles of 96 C for 15 sec, 61 C for 20 sec, and 72 C for 90 sec. Sequencing reactions were carried out according to the instructions of the AutoRead 200 DNA sequencing kit (Pharmacia Biotech, Uppsala, Sweden), using fluorescently labeled primers. The methylation status was categorized with 25% accuracy (28) into 0%, 25%, 50%, 75%, and 100%.

IGF-II imprinting analysis

IGF-II *Apa*I polymorphism of all carcinoma DNAs and cDNAs was analyzed by PCR amplification (29) using the primers mentioned above in the description of the PCR-based methylation analysis.

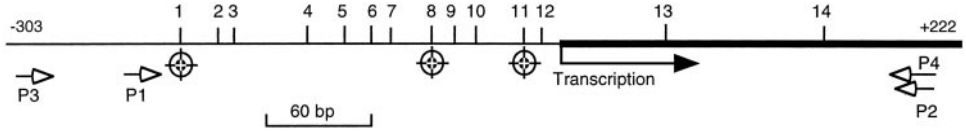

FIG. 1. A diagram of the methylation analysis of the H19 promoter region. The H19 transcription start site (*closed arrow*, nucleotide 10097, GenBank accession no. AF125183), CpG sites (*vertical lines* 1–14), *Hpa*II-cutting sites (*crossed circles*), and primer locations (*open arrows* P1–P4) are depicted. Primers P1 (9857–9876, sense) and P2 (10318–10299, antisense) were used for PCR-based methylation analysis, and P3 (9795–9817, sense) and P4 (10319–10294, antisense) were used for bisulfite genomic sequencing. The nucleotide numbers (−303 and +222) at the ends are counted from the transcription start site.

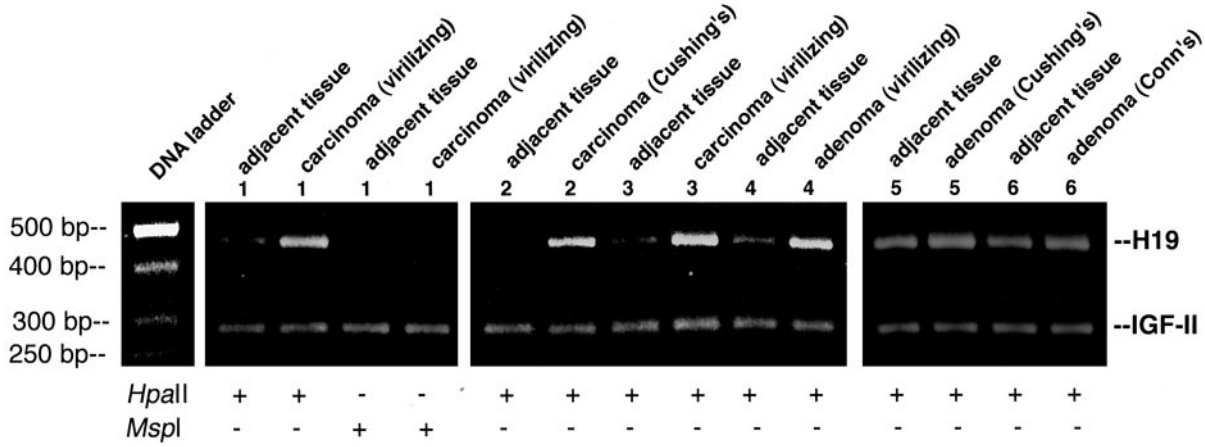

FIG. 2. PCR-based methylation analysis of the H19 promoter of adrenal DNA samples. Each number, 1–6, *above* the lanes represents genomic DNA isolated from a separate patient. After *Hpa*II/*Msp*I digestion (blocked/unblocked by CpG methylation, respectively), only methylated (undigested) genomic DNA produces 462-bp-long H19 PCR products in the *Hpa*II-digested samples; no product is seen in *Msp*I digestions (shown with patient 1). The more abundant H19 PCR products (in *Hpa*II digestion) in three carcinomas and a virilizing adenoma than in their adjacent normal adrenal tissues (patients 1–4) show high H19 promoter methylation in these tumors. The nearly equal intensities of the H19 PCR products in Cushing's and Conn's adenomas to their adjacent normal adrenals suggest unchanged methylation in these tumors (patients 5 and 6). The lower 292-bp band (IGF-II) serves as an internal control of DNA amount (no *Hpa*II/*Msp*I recognition site).

Immunocytochemical staining for cell proliferation analysis

Proliferating cells were demonstrated immunocytochemically in NCI-H295R cell cultures with a commercial cell proliferation kit (Amersham Pharmacia Biotech, Little Chalfont, UK) according to the manufacturer's instructions. In this assay, 5-bromo-2′-deoxyuridine was incorporated into replicating DNA, localized with a specific monoclonal antibody, and stained with a peroxidase-based detection system. The culture area was photographed, and the nuclei were counted from each culture dish. The proliferation percentage was calculated on the basis of positively stained nuclei relative to all cells.

Statistical analyses

Nonparametric Kruskal-Wallis test was used to reveal differences in the methylation status (analyzed by bisulfite sequencing) and in the expression levels of the H19 and IGF-II genes among the different groups of adrenal samples. If significant differences were found, a Mann-Whitney U test was subsequently used for statistical evaluation. A Mann-Whitney U test was also used to evaluate the changes in H19 and IGF-II expression and in the proliferation of the cultured cells. Simple regression analysis was used to determine the correlation of H19 or IGF-II RNA levels with the degree of H19 promoter methylation. The level of significance was chosen as $P < 0.05$.

Results

The primer set P1 and P2 (Fig. 1) amplifies an H19 promoter region across three *Hpa*II sites, located at −210, −69, and −16 from the transcription initiation site. Because of the digestion of unmethylated CCGG sequence, only indigestible methylated DNA will give a PCR product 462 bp in size. As shown in Fig. 2, this PCR product was more abundant in adrenocortical carcinoma DNA samples than in tumor-adjacent adrenal tissues, suggesting a higher degree of methylation in the carcinoma samples. We saw a clear difference in the intensity of the 462-bp product between carcinomas and their adjacent normal tissues (three pairs). No consistent difference was found between adrenocortical adenomas (Conn's and Cushing's syndrome) and their adjacent normal tissues (three pairs). However, PCR-based analysis suggested higher promoter methylation in a virilizing adenoma than in its adjacent normal tissue (Fig. 2), which was verified by sequencing analysis (data not shown).

To clarify the methylation status of the H19 promoter in more detail, we applied automated fluorescent genomic sequencing after bisulfite-induced modification of genomic DNA. Among normal adult adrenal and tumor-adjacent normal adrenal tissues, there was not much variation in the methylation status of each CpG site in the H19 promoter; sites 5, 7, and 8 showed the least variation (mostly 50%, 25%, and 50% methylation, respectively). No significant difference in the average degree of methylation of the first 12 CpG sites in the H19 promoter was found between the normal adrenals (mean ± SE, 41 ± 2%) and hyperplasias (43 ± 5%; $P = 0.962$) or adenomas (45 ± 3%; $P = 0.471$). However, the average degree of methylation was significantly higher in carcinomas (76 ± 7%) than in normal adrenals and adenomas (both $P < 0.005$) or hyperplasias ($P < 0.05$; Fig. 3).

The methylation degree of each 12 CpG site of the H19 promoter in the hyperplastic adrenals and adenomas was similar to that in the normal adrenals, except for site 11, which showed higher methylation in the adenomas than in the normal adrenals ($P < 0.01$). Compared with the normal adrenals, each 12 CpG site showed hypermethylation in the carcinomas ($P < 0.01$; Fig. 4). However, site 11 did not show significantly higher methylation in the carcinomas than in the adenomas ($P = 0.094$), whereas the other sites did ($P < 0.01$ for sites 1–10, $P < 0.05$ for site 12). The methylation status of sites 13 and 14 (the first 2 after the transcription start site, Figs. 1 and 4) was not different in hyperplasias, adenomas, or carcinomas from that in normal adrenals.

As reported previously (21), H19 expression was significantly lower and IGF-II higher in adrenocortical carcinomas than in normal adrenals (a representative Northern blot in Fig. 5A). There was a negative correlation between the H19 RNA levels and the mean methylation degree of the 12 CpG sites of the H19 promoter ($r = -0.550$; $P < 0.01$; Fig. 5B) and,

on the other hand, a positive correlation between the IGF-II mRNA levels and the mean degree of methylation of the H19 promoter (r = 0.805; $P < 0.001$; Fig. 5C).

Of the 10 available carcinomas, 3 were heterozygous at the IGF-II *Apa*I site. Of these informative tumors, 1 demonstrated loss of imprinting, *i.e.* biallelic IGF-II expression (data not shown).

To confirm the association of DNA methylation with the H19 and IGF-II gene expression levels, we modified DNA methylation of the adrenocortical carcinoma cell line NCI-H295R with Azad, a demethylating agent. Northern blot analysis showed hardly any H19 RNA expression in these cells before treatment. Azad treatment induced a remarkable increase in the H19 RNA content (Fig. 6; $P < 0.005$, pooled data from six experiments). H19 induction was detectable at 1 μmol/liter Azad after 3 or 7 d, and the maximal increase appeared at 3 μmol/liter. Time-course experiments demonstrated that H19 RNA increased within 2 d, and the augmentation continued until 7 d of treatment (Fig. 6). Bisulfite sequencing methylation analysis showed that the methylation of CpG sites 9, 10, and 11 in the H19 promoter was indeed decreased by Azad treatment (Table 1) consistent with PCR-based methylation analysis (data not shown). Interestingly, the induction of H19 gene expression was accompanied by a clear decrease in IGF-II expression ($P <$ 0.005, pooled data from six experiments). This reduction of IGF-II mRNA accumulation during Azad treatment was also dose and time dependent (Fig. 6). In addition, treatment with Azad inhibited proliferation of the cells. The proliferation rate of the cells treated with Azad (measured at 30 μmol/liter Azad for 7 d) was less than 10% of the control ($P < 0.05$, three separate experiments; Fig. 7). Total DNA amount harvested from Azad-treated cells (from two independent experiments) was about 30% of that from the control cultures. Azad treatment also induced a morphological change in the cells; the cells and the nuclei enlarged, and the cells flattened (Fig. 7). The cells treated with Azad looked healthy, with cell viability more than 98%, measured by trypan blue exclusion (data not shown). In addition, the functional viability of the cells was preserved during Azad treatment on the basis of well maintained cortisol secretion (data not shown).

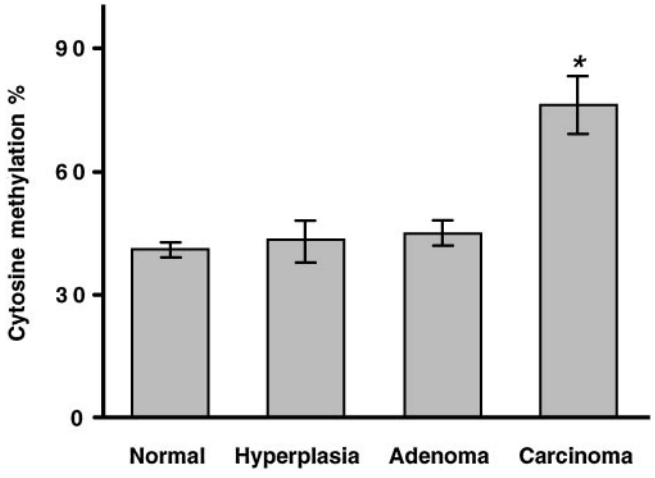

FIG. 3. Cytosine methylation percentage of the 12 CpG sites of the H19 promoter region in normal adrenals (adult normal and tumor-adjacent normal; n = 16), hyperplastic adrenals (n = 4), adrenocortical adenomas (Conn's, Cushing's, virilizing, and nonfunctional; n = 16), and carcinomas (Cushing's, virilizing, and nonfunctional; n = 10). Each column represents the mean percent methylation (±SE, *vertical bars*) of the all 12 CpG sites of the H19 promoter region, analyzed by genomic sequencing after bisulfite modification (*, $P < 0.005$ compared with normal adrenals).

Discussion

Molecular mechanisms leading to adrenocortical tumorigenesis are not yet fully understood, partly due to the difficulty in obtaining human adrenal samples (30, 31). It has been suggested that the loss of the putative H19 tumor suppressor activity and the gain of the IGF-II mitotic effect may be involved in the pathogenesis of adrenocortical neoplasms (21–23). If an epigenetic error is the cause of the H19 silencing in adrenocortical carcinomas, this error will most likely occur in the regions that control H19 gene expression. Our methylation analysis revealed that the CpG sites in the H19 promoter are hypermethylated in most carcinomas, whereas the two CpG sites after the transcription start site are not. Given the negative correlation of the H19 promoter methylation

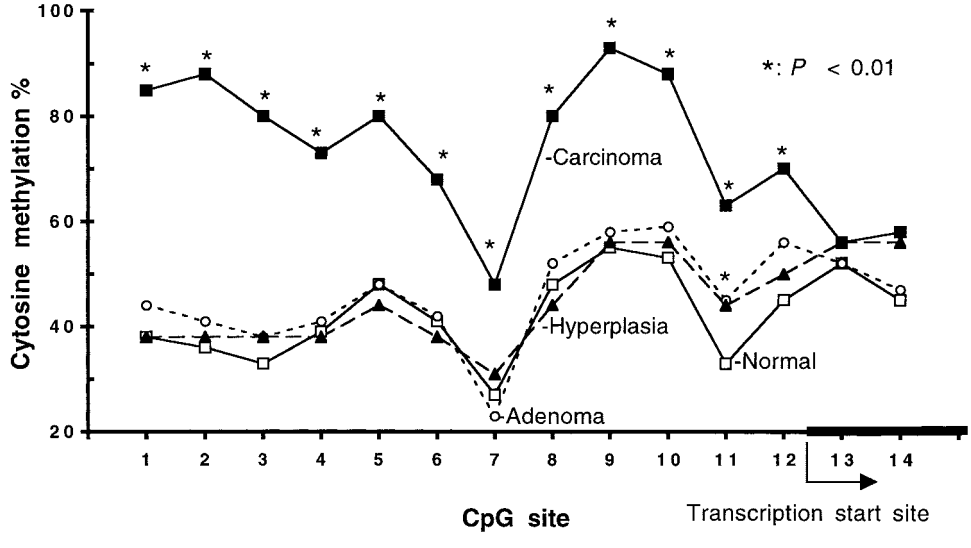

FIG. 4. Mean percent methylation of the individual CpG sites of the H19 promoter region (analyzed by genomic sequencing after bisulfite modification) in normal adrenals (n = 16), hyperplastic adrenals (n = 4), adrenocortical adenomas (n = 16), and carcinomas (n = 10). The distances of the CpG sites in this figure do not correspond to those in the H19 promoter (*, $P < 0.01$ compared with normal adrenals).

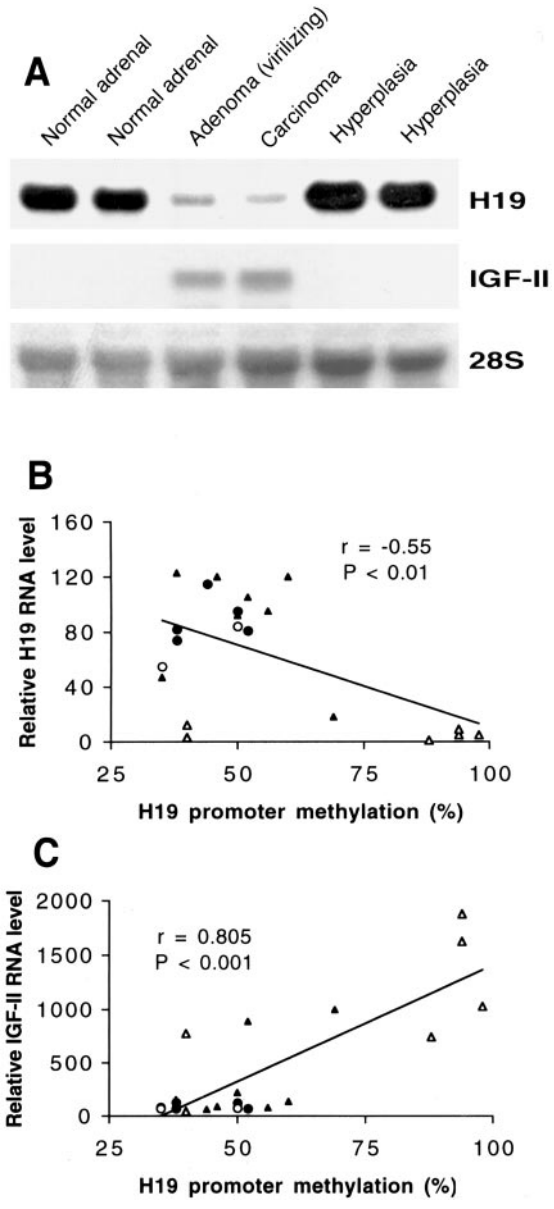

FIG. 5. Expression of H19 and IGF-II RNAs and their correlation with H19 promoter methylation in different adrenal samples. A, A representative Northern blot analysis on H19 and IGF-II RNA expression. Total RNA was extracted from frozen tissues. The Northern blot was prepared with 20 μg RNA on each lane, and the RNA was transferred onto a nylon membrane. The filter was sequentially hybridized with ^{32}P-labeled H19 (*upper panel*), IGF-II (*middle panel*), and 28S ribosomal RNA (*lower panel*) cDNA probes. B and C, The correlation of H19 and IGF-II RNA levels with H19 promoter CpG methylation status. ●, Normal adrenals; ○, hyperplastic adrenals; ▲, adrenocortical adenomas; △, carcinomas. The adenoma with the highest methylation and lowest H19/highest IGF-II expression was a virilizing one. The H19 and IGF-II values were derived from Northern blot autoradiographs with the mean expression level of the normal adrenals adjusted to 100. The H19 promoter methylation status was the mean percent methylation of the 12 CpG sites (based on genomic sequencing after bisulfite modification).

with the H19 RNA expression levels, our results are consistent with previous reports showing the H19 promoter to control H19 expression in mice during development (13) and in other neoplasms, such as Wilms' tumor and hepatoblastoma (14, 16, 32). On the other hand, our present study did not tell much about the significance of the loss of IGF-II imprinting in adrenocortical carcinomas, as only three of the carcinomas were informative.

Similar H19 promoter methylation in hyperplastic and normal adrenals suggests that the methylation status in this area has no role in the development of benign hyperplastic changes in human adrenals. No single 1 of the 12 CpG sites in the H19 promoter seems to be more important than the others in determining H19 expression in adrenocortical carcinomas. However, site 11 may become initially methylated in adrenal tumorigenesis, because its degree of methylation in adenomas was between normal adrenals and carcinomas. It has previously been suggested that H19 hypermethylation might be an early epigenetic error that occurs at the onset of Wilms' tumor development (16). All adrenocortical carcinomas are monoclonal in tissue composition, whereas adrenocortical adenomas can be either monoclonal or polyclonal with various intermediate forms (33). Therefore, heterogeneous H19 promoter methylation may occur in benign adrenocortical tumors. The difference in the methylation status between a virilizing adenoma and its adjacent normal adrenal supports the hypothesis that the development mechanism of this type of adenoma may be different from that of other adrenal adenomas (34, 35).

The NCI-H295R cell line is a unique model for studying the clustered imprinted genes on chromosome 11p15, as the cells have a deletion of 11p in one allele (36, 37). On the basis of the absent H19 and high IGF-II expression together with hypermethylation of the H19 promoter, the deleted allele is probably the maternal one. Inhibition of CpG methylation of the H19 promoter with Azad treatment indeed activated H19 expression in these cells, confirming the effect of the methylation status of the H19 gene on its expression (38). As the inhibition of methylation by Azad treatment occurs only on *de novo* synthesized DNA (39), the decrease in DNA methylation of the proliferating cells is probably more significant than that detected in the DNA isolated from all cells in this study. Considering both the reduced methylation of CpG sites 9, 10, and 11 in the cells caused by Azad treatment and the increasing methylation of site 11 from normal adrenals to adenomas (and further to carcinomas), site 11 may be a sensitive point for epigenetic errors. In contrast to the induced H19 expression due to the relief of the suppressed allele, IGF-II mRNA expression decreased with Azad treatment, fitting to the general opposite expression pattern of these two genes from a single allele (13, 40). NCI-H295R cells express IGF type 1 and 2 receptor genes as well as IGF-binding proteins, which allows the cells to respond to endogenous and/or exogenous IGFs (41). The reduced proliferation rate after Azad treatment is thus probably explained by the altered expression of the H19 and IGF-II genes.

In this study we did not analyze methylation of the IGF-II gene, which has been shown to be associated with IGF-II expression in some tumors. The methylation status of an IGF-II upstream repressor element plays a role in IGF-II imprinting in mouse (42). A significantly increased IGF-II mRNA content in pediatric adrenocortical tumors was associated with significant IGF-II gene (exon 7) demethylation

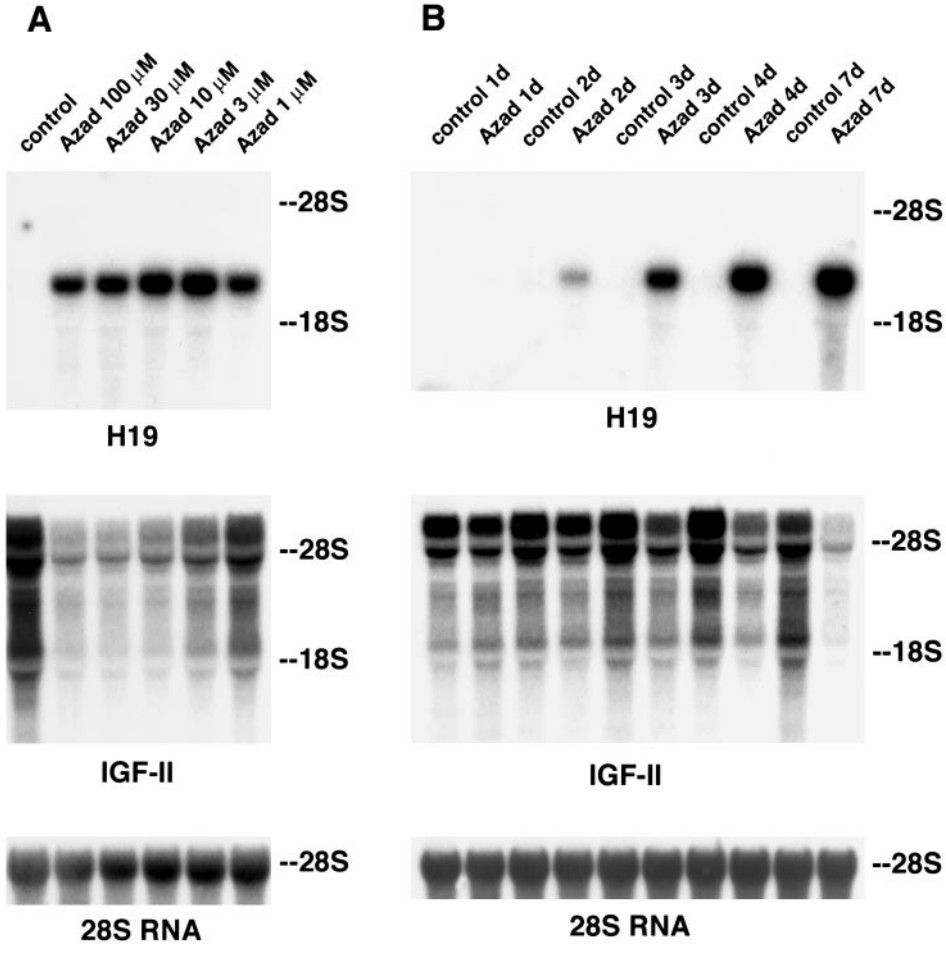

FIG. 6. Dose-dependent (A) and time-dependent (B) effects of Azad on the accumulation of H19 and IGF-II RNAs in cultured NCI-H295R cells. The cells were treated with Azad for 7 d in the dose-response experiment, and the Azad concentration was 30 μmol/liter in the time-course experiment. Cytoplasmic RNA was extracted and Northern blotted with 20 μg total RNA on each lane. The filters were sequentially hybridized with ^{32}P-labeled H19 (*upper panels*), IGF-II (*middle panels*), and 28S rRNA (*lower panels*) cDNA probes. The migration of 28S and 18S ribosomal RNAs is indicated.

TABLE 1. Methylation analysis of the H19 promoter CpG sites in human adrenal carcinoma NCI-H295R cells cultured for 7 d with and without (control) the demethylating agent Azad (30 μmol/liter)

	CpG sites (1–14) and their methylation status (%)													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Control	100	100	75	25	25	50	0	75	100	100	100	100	0	0
Azad	100	100	75	25	25	50	0	75	75	75	75	100	0	0

Genomic sequencing after bisulfite modification of genomic DNA was used to estimate the methylation status as described in *Materials and Methods.*

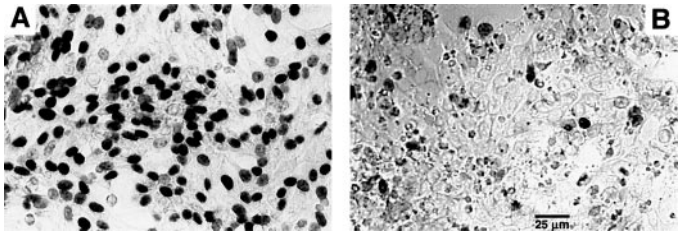

FIG. 7. The effect of Azad on the proliferation of NCI-H295R cells. The cells were cultured for 7 d without (A) or with 30 μmol/liter Azad (B). Immunocytochemical 5-bromo-2′-deoxyuridine staining of nuclei demonstrates the proliferating cells. *Bar*, 25 μM.

compared with normal adrenals (43). However, the changes in IGF-II CpG methylation may be secondary to the inactivation of H19 (44). It has been reported that the presence or absence of the active H19 transcription unit affects IGF-II allelic expression to some extent independently of IGF-II DNA methylation (6, 13, 45). Methylation of the H19 gene regulates the imprinted expression of both H19 and IGF-II genes through mediating methylation-sensitive, enhancer-blocking activity at the H19/IGF-II locus (46–48).

In summary, our results indicate that altered DNA methylation of the H19 promoter may be involved in the abnormal expression of H19 and IGF-II genes in human adrenocortical carcinomas and in the pathogenesis of these neoplasms.

Acknowledgments

Ms. Merja Haukka, Ms. Eija Heiliö, Ms. Arja Korhonen, and Ms. Anne Karppinen are thanked for their technical assistance, and Ms. Sanna Myöhänen, Ph.D., for her methodological advice.

Received August 8, 2001. Accepted December 10, 2001.

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This work was supported by the Emil Aaltonen Foundation, the Jalmari and Rauha Ahokas Foundation (to J.L.), the Academy of Finland, the Sigrid Juselius Foundation, and Kuopio University Hospital (to R.V.).

* Z.-H.G., S.S., and J.L. contributed equally to this paper.

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