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Genetics

Switch from monoallelic to biallelic human *IGF2* promoter methylation during aging and carcinogenesis

(DNA methylation/cancer/imprinting)

JEAN-PIERRE J. ISSA*, PAULA M. VERTINO*, CORINNE D. BOEHM†, IRENE F. NEWSHAM‡, AND STEPHEN B. BAYLIN*§

*The Oncology Center, Departments of §Medicine and †Pediatrics, The Johns Hopkins University Medical Institutions, Baltimore, MD 21231; and ‡The Gildred Cancer Center, University of California at San Diego, La Jolla, CA 92093

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ABSTRACT **We have previously linked aging, carcinogenesis, and *de novo* methylation within the promoter of the estrogen receptor (ER) gene in human colon. We now examine the dynamics of this process for the imprinted gene for insulin-like growth factor II (*IGF2*). In young individuals, the P2–4 promoters of *IGF2* are methylated exclusively on the silenced maternal allele. During aging, this promoter methylation becomes more extensive and involves the originally unmethylated allele. Most adult human tumors, including colon, breast, lung, and leukemias, exhibit increased methylation at the P2–4 *IGF2* promoters, suggesting further spreading during the neoplastic process. In tumors, this methylation is associated with diminished or absent *IGF2* expression from the methylated P3 promoter but maintained expression from P1, an upstream promoter that is not contained within the *IGF2* CpG island. Our results demonstrate a remarkable evolution of methylation patterns in the imprinted promoter of the *IGF2* gene during aging and carcinogenesis, and provide further evidence for a potential link between aberrant methylation and diseases of aging.**

In humans, about 60% of genes contain a CpG-rich stretch of DNA in their promoters. These CpG-rich areas, which are called CpG islands, are normally devoid of DNA methylation regardless of the expression status of the gene (1). When present, DNA methylation within promoter-associated CpG islands is accompanied by irreversible inhibition of gene expression, as can be seen in the inactive X chromosome in women (2) and in the transcriptionally silent copy of parentally imprinted genes (3). *De novo* promoter methylation has also recently been identified as an alternate mechanism of inactivating tumor-suppressor genes during the development of neoplasia (4–7). In addition, we have recently observed *de novo* methylation of the estrogen receptor (ER) promoter during cellular aging *in vitro* (8) and physiologic aging *in vivo* (9). In the colon, this age-dependent methylation appears to precede, and possibly predispose to, neoplasia because all colonic tumors examined showed dense ER CpG island methylation (9).

The mechanism and dynamics of age and neoplasia-related *de novo* methylation remain undefined. Although increases in the activity of DNA-methyltransferase, the enzyme responsible for maintaining methylation patterns in mammalian DNA, have been described in tumors (10–12), other factors must be operative because only a minority of genes display age- and neoplasia-related hypermethylation (J.-P.J.I., unpublished observations). In this report, we describe the dynamics of hypermethylation during aging and neoplasia at a CpG island in the promoter of the *IGF2* gene.

IGF2 is a fetal growth promoter that is transcribed from four distinct promoters, P1–P4 (13). P2–P4 are contained in a CpG island, and transcription from these promoters is subject to parental imprinting, such that only the paternal copy of the gene is expressed (14, 15). P1 is located more than 20 kb upstream of this CpG island, and it appears to be regulated differently than P2–P4 in that it escapes imprinting in several adult tissues, including the liver (16). We now show promoter methylation at the *IGF2* P2–P4 CpG island that starts on the silenced allele and, with age, appears to spread to the opposite allele. Tumors display biallelic increased methylation at this CpG island, which is associated with low or absent expression from the P2–P4 promoters, but unaffected expression from P1. Our results highlight the importance of age-related spreading of methylation in the establishment of aberrant promoter hypermethylation at selected loci in human neoplasms.

MATERIALS AND METHODS

Tissue Samples. Samples of normal colon were obtained at surgical resection from a group of patients with nonneoplastic diseases of the colon. Normal lymphocytes and bone marrow cells were obtained from healthy donors of bone marrow for transplantation. All other samples, including tumor/normal pairs were obtained at surgical excision. All samples were frozen in liquid nitrogen immediately after resection and stored at −70°C until processing. All patients gave informed consent for the use of their tissue samples.

Cell Culture. All cell lines were obtained from American Type Culture Collection except for normal human foreskin fibroblasts that were a gift from Thea Tlsty (University of North Carolina, Chapel Hill).

Southern Blots. DNA was extracted using standard methods. Five micrograms of genomic DNA was digested with 100 units of the appropriate restriction enzyme as specified by the manufacturer (New England Biolabs). DNA was then precipitated, electrophoresed on a 1% agarose gel, transferred to a nylon membrane (Zetaprobe, Bio-Rad), and hybridized with the appropriate probe. All blots were exposed in a PhosphorImager cassette (Molecular Dynamics) for 2–4 days before imaging. All image analysis was carried out using the IMAGEQUANT software from Molecular Dynamics. Probes used included a 0.6-kb 5′ *IGF2* probe obtained by digesting plasmid phins311 obtained from American Type Culture Collection with *Hin*dIII; a 0.4-kb 5′ *THBS1* probe obtained by digesting the insert from plasmid pTS33 (American Type Culture Collection) with *Sty*I; a 0.65-kb 5′ *c-abl* probe obtained by PCR amplification of genomic DNA using primers 5′-CAAACTTCCCTGATGGTGCCCTCTTG (upper) and 5′-TGACGTGTATTGTGCTCTTCCTATGT (lower); and a 0.5-kb 5′ *CNP* probe obtained by PCR amplification of genomic DNA using primers 5′-TTGTGGGAGCAGAGAAGGAAGGTTAC (upper) and 5′-GAGAGGAGGGAAAGAGGAGAGGTGAG (lower).

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Abbreviation: ER, estrogen receptor.

Allele-Specific Methylation Assay. For identification of individuals polymorphic for a *Bam*HI restriction fragment length polymorphism 5′ of *IGF2*, 100 ng of genomic DNA was amplified by PCR using primers 5′-AGCTCACCGC-GAAGCTGGAGGCTG (upper) and 5′-AGGTCCCTG-GAGCACACTGAGCAG (lower). One-third of the PCR product was digested with 10 units of *Bam*HI (New England Biolabs), electrophoresed on a 2% agarose gel containing ethidium bromide, and visualized by UV exposure in an Eagle eye I imager (Pharmacia). Densitometric analysis of the gels revealed equal amplification of both alleles in heterozygotes. Standardization experiments were carried out; in these experiments, various quantities of DNA from individuals who are homozygous for different *IGF2* alleles were mixed and amplified. These studies revealed equal and linear amplification of both alleles under the PCR conditions used.

To determine allele-specific methylation, 2 μg of genomic DNA was digested with 40 units of *Tha*I (New England Biolabs) according to the manufacturer's instructions. Digested DNA (150 ng) was then amplified as described above. One-third of the PCR product was digested with 10 units of *Bam*HI, electrophoresed on a 2% agarose gel, transferred to a nylon membrane (Zetaprobe, Bio-Rad), and probed with the 5′ *IGF2* probe. We found Southern transfer and radioactive probing necessary to increase the sensitivity of the reaction when amplifying *Tha*I-digested DNA. As above, all blots were imaged and analyzed using a PhosphorImager. All PCR-methylation assays were done in duplicate, and independent determinations gave consistent results.

Reverse Transcription–PCR. RNA was extracted using standard methods. Six micrograms of RNA was reverse transcribed using random hexamers (Boehringer Mannheim) and Moloney murine leukemia virus reverse transcriptase (BRL) according to the manufacturer's instructions. The cDNA was amplified by PCR using standard buffer conditions. For *IGF2* P1 promoter-specific PCR, primers were 5′-GGCCAGAGAGC-CCAGTCCTGAGGTGA (upper primer, located in *IGF2* exon 2, which is exclusively transcribed from P1) and 5′-TGGAAGAACTTGCCCACGGGGTATCT (lower primer, located in *IGF2* exon 9, which is common to all *IGF2* alternate transcripts). For *IGF2* P3 promoter-specific PCR, primers were 5′-TCCTCCCTGGACAATCAGACGAATTC (upper primer, located in *IGF2* exon 5, which is exclusively transcribed from P3) and the same exon 9 lower primer as for P1. To confirm integrity of the RNA, the same amount of cDNA was amplified by PCR using primers located in the first and third exons of the actin gene (upper primer, 5′-ACCATGGATGAT-GATATCGC; lower primer, 5′-ACATGGCTGGGGTGTT-GAAG). A total of 300 ng of RNA was used for each reaction, and one-third of the amplified product was electrophoresed on a 2% agarose gel and visualized by UV exposure after ethidium bromide staining. All reactions were done at least twice, and all were controlled with reverse transcription samples in which the reverse transcriptase enzyme was omitted.

RESULTS

Partial Methylation of the Human *IGF2* CpG Island. The *IGF2* gene has a 4-kb CpG island surrounding promoters P2–P4 of the gene (Fig. 1*A*). We studied the methylation state of this CpG island by restriction digestion of genomic DNA using methylation-sensitive restriction enzymes followed by Southern blotting and probing with a 5′ *IGF2* probe. Using *Hin*dIII as a flanking cut and *Eag*I as the methylation-sensitive enzyme, we found that, besides the expected band at 2.0 kb, additional bands of higher molecular weight were present, indicating partial methylation of the most 5′ *Eag*I sites (Fig. 1*B*). This partial methylation was present in all normal tissues examined, including adult colon, small intestine, lung, liver, breast, kidney, fibroblasts, arteries, brain, lymphocytes, and

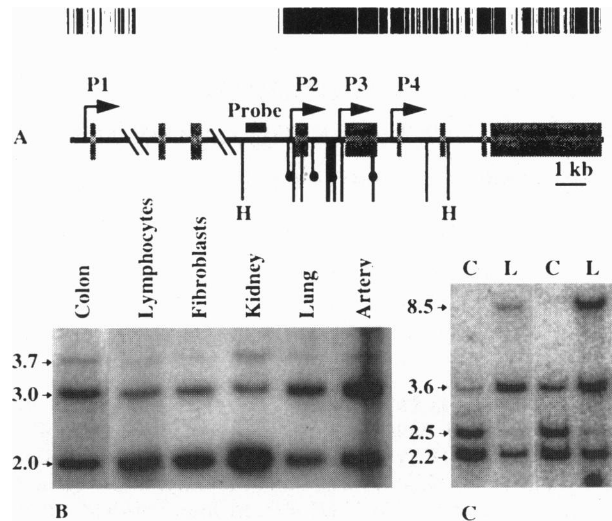

FIG. 1. (*A*) Genomic map of the *IGF2* region. Human *IGF2* has nine known exons represented by filled boxes. The locations of the four known promoters (P1–P4) are indicated by arrows. On top is a CpG map of the known sequences in this region. Each vertical bar represents a single CpG site, and the high density of vertical bars around P2–P4 indicates the presence of a CpG island. A partial restriction map around P2–P4 is represented by vertical bars; straight bars are *Sac*II sites, bars with a rounded edge are *Eag*I sites, and bars with an (H) are *Hin*dIII sites. (*B*) Partial methylation of the *IGF2* CpG island in normal tissues. Shown is a representative Southern blot of normal human adult DNA restricted with *Hin*dIII and *Eag*I and probed with the 5′ *IGF2* probe shown as a box in *A*. The tissue of origin is indicated on top of each lane, and the approximate size of the bands is indicated, in kilobases, on the left of the gels. (*C*) Southern blot of DNA from two paired samples of either normal human colon (C) or liver (L) from the same patient. DNA was restricted with *Hin*dIII and *Sac*II.

bone marrow, as well as colon and kidney from children (Fig. 1*B* and data not shown). A similar pattern of partial methylation was detected at four different methylation-sensitive restriction enzyme sites in the same region (Fig. 1*C* and data not shown). In addition, the same blots were reprobed with several other CpG island probes, including *c-abl*, *THBS1* (thrombospondin), and *CNP* (C-type natriuretic peptide), and a restriction pattern consistent with a complete lack of methylation was observed, establishing that our findings are due to methylation rather than partial enzymatic digestion (data not shown).

Intriguingly, the *IGF2* CpG island methylation was more extensive in the liver than in all other samples examined (Fig. 1*C*), as indicated by the presence of bands of larger molecular weight in all the liver lanes, when compared with colon from the same patient. This is particularly interesting because adult liver expresses relatively high levels of *IGF2* through the P1 promoter (17), and raises the possibility of a link between methylation of this CpG island and expression of the upstream P1 promoter, as discussed further below.

***IGF2* Methylation Increases with Age.** We have previously reported progressive age-related methylation in the human ER CpG island (9), and we sought to determine whether such a phenomenon could also be found at the *IGF2* locus. We started by studying cultured normal human fibroblasts, an *in vitro* model of cellular aging. In these cells, *IGF2* methylation at *Sac*II became progressively more extensive with increasing number of cell passages (data not shown), indicating a cellular aging-related extension of methylation at the restriction sites being examined.

To study this phenomenon *in vivo*, we measured the intensity of the methylation bands shown in Fig. 1*C*, colon lanes, in a panel of colonic tissues from 34 individuals with ages ranging from 8 to 90 years. As shown in Fig. 2, the extent of *IGF2*

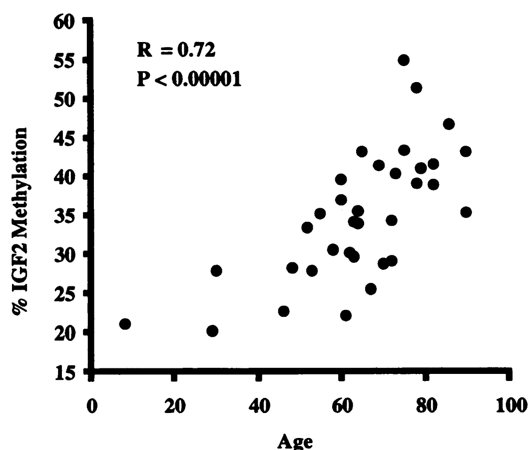

FIG. 2. *IGF2* methylation increases with age. Southern blots of normal human colon DNA restricted and probed as in Fig. 1*C* were exposed in a PhosphorImager, and the density of the higher molecular weight bands (methylation bands) was determined. This density (% *IGF2* methylation), expressed as the percent of the total density in each lane, was plotted against the age of the patient. Each dot represents a separate individual. The data best fit an exponential curve (R = 0.72) and are statistically significant by regression analysis ($P < 10^{-5}$).

methylation in normal colon increases progressively with age, in a manner reminiscent of the findings at the ER gene locus. As above, the blots were reprobed with other CpG island probes to confirm complete enzymatic digestion.

***IGF2* Methylation Is Allele-Specific.** The finding of CpG island methylation in the promoter of an imprinted gene raises the possibility of allele-specific methylation on the parentally silenced allele (3). To study this for the *IGF2* P2–P4 promoters, we developed a PCR methylation assay that amplifies across a polymorphic *Bam*HI site 1 kb upstream of the CpG island (Fig. 3*A*). After PCR amplification and *Bam*HI digestion, DNA from individuals homozygous for allele A reveals only one band at 1.25 kb, whereas homozygotes for allele B have one band at 1.0 kb. Heterozygotes have both bands (Fig. 3*B Left*). Standardization of the PCR assay as outlined in *Materials and Methods* revealed that the PCR conditions allow both alleles to be amplified equally (data not shown). For allele-specific methylation analysis, genomic DNA from heterozygous individuals is first digested with the methylation-sensitive restriction enzyme *Tha*I, then PCR-amplified and digested with *Bam*HI. There are seven *Tha*I sites encompassed by the PCR primers, and all seven must be methylated for a PCR product to be produced. As shown in Fig. 3*B* (*Right*), after restriction with *Tha*I, only one allele is predominantly amplified in DNA from young individuals (<40 years), suggesting that the methylation is allele-specific. By contrast, an area 2 kb upstream of the CpG island (which is depleted in CpG sites) exhibited partial methylation of both alleles (data not shown), suggesting that the allele-specific methylation patterns are restricted to the P2–P4 promoter area.

As discussed above, in humans, *IGF2* P2–P4 derived transcripts are derived exclusively from the paternal allele. To study whether the methylated allele is indeed the silenced one, we determined allele-specific methylation in three heterozygous children from two families in which the mother is homozygous for allele A. In all three children, the predominant methylated allele was the one derived from the mother (Fig. 3*C*).

In the embryonal renal cancer Wilms tumor, the maternal allele of *IGF2* is frequently deleted, resulting in loss of heterozygosity at this locus. We studied eight such Wilms tumors with loss of heterozygosity, and all of them retained exclusively an unmethylated allele (see Fig. 5; data not shown).

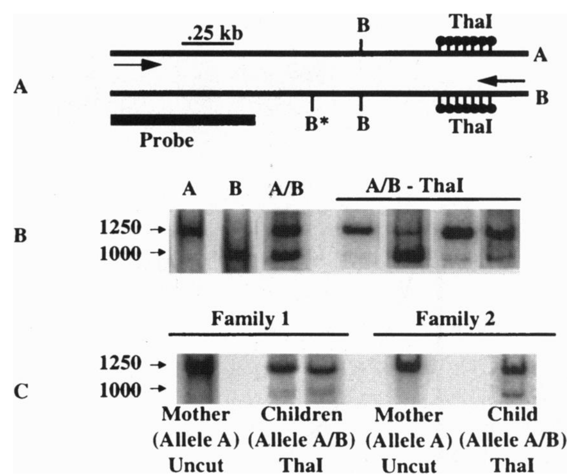

FIG. 3. Allele-specific methylation of *IGF2*. (*A*) Genomic map of a 2-kb fragment of *IGF2*, including 0.5 kb of CpG island sequence and 1.5 kb of 5′ flanking sequence. The location of PCR primers is indicated by arrows, and the location of the *IGF2* probe used is depicted by a filled box. B, *Bam*HI sites; B*, a polymorphic *Bam*HI site present in 40% of the U.S. population. Also depicted are seven sites for the methylation-sensitive restriction enzyme *Tha*I that are clustered in the *IGF2* CpG island. (*B Left*) Examples of Southern blots of *Bam*HI-digested PCR products. Lanes A and B are from individuals homozygous for alleles A or B, whereas lane A/B is from a heterozygous individual. The size of the bands (in bp) is shown to the left of the panel. (*Right*) Four examples of allele-specific methylation analysis. Normal colon genomic DNA from heterozygous individuals was PCR-amplified after digestion with *Tha*I. The PCR product was then digested with *Bam*HI, run on an agarose gel, and visualized by Southern transfer and probing with an *IGF2* probe. In all four cases, one band predominates, suggesting allele-specific methylation. (*C*) Maternal origin of the methylated *IGF2* allele. Peripheral blood DNA from two families was analyzed for *IGF2* promoter polymorphism and methylation as outlined above. In both families, the mother is homozygous for allele A, whereas the father and both children are heterozygous. After *Tha*I digestion, only allele A (the maternal allele) is predominantly amplified in all three children, indicating maternal allele-specific methylation.

These results further indicate that it is the transcriptionally silent maternal allele that is normally methylated. Interestingly, one out of two samples of Wilms tumor without loss of heterozygosity displayed distinct increases in methylation at the *IGF2* CpG island (see Fig. 5; data not shown).

Age-Related Biallelic *IGF2* Promoter Methylation. The age-related increase in methylation described in Fig. 2 could be interpreted as either extension of methylation on one allele, or de-novo methylation involving the opposite allele. To distinguish between these two possibilities, we analyzed allele-specific methylation patterns of 25 individuals heterozygous for the 5′ *Bam*HI polymorphism ranging in age from 8 to 85 years. As shown in Fig. 4*A*, after digestion with the methylation-sensitive enzyme *Tha*I, only one allele is predominantly amplified in young individuals, whereas both alleles begin to show methylation around age 50. By age 82, both alleles are equally amplified, indicating equivalent methylation. We quantitated the relative intensities of the amplified bands and established an allelic ratio of the intensity of the predominantly amplified allele divided by the second allele. As seen in Fig. 4*B*, this allelic ratio decreases logarithmically with age and approaches 1 after age 70. These data demonstrate that part of the increased methylation with age shown in Fig. 2 is due to *de novo* methylation of the originally unmethylated allele.

Hypermethylation of *IGF2* in Human Tumors. Having established age-related increased methylation at the *IGF2* P2–P4 promoters, we sought to determine the fate of this methylation in a variety of human tumors (Fig. 5). As compared with normal tissues, we observed more extensive meth-

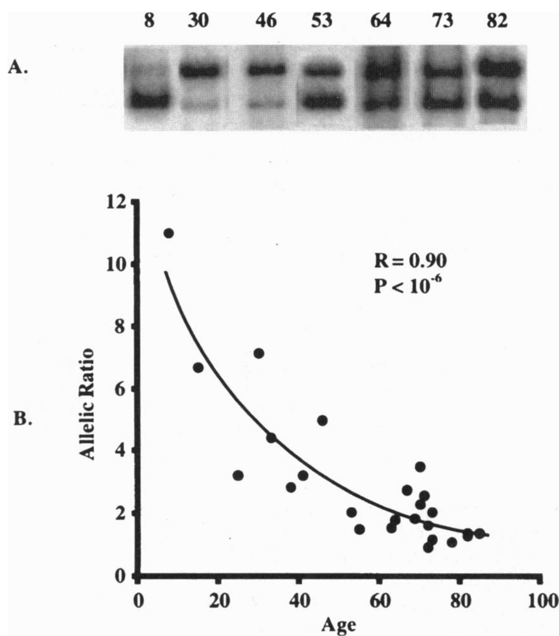

FIG. 4. Age-related biallelic *IGF2* promoter methylation. (*A*) Normal colon genomic DNA from individuals heterozygous for the *Bam*HI polymorphism 5′ of *IGF2* was analyzed for allele-specific methylation as in *B*. The age of each person is indicated on top of the lanes (shown are representative examples). (*B*) The relative intensity of each of the two bands seen in the C lanes in *A* was measured in a PhosphorImager, and an "allelic ratio" was determined by dividing the density of the predominant band by the density of the other band. A ratio of 1 means equal amplification of both alleles. This allelic ratio was then plotted against age for 25 individuals. The data best fit a logarithmic curve ($R = 0.9$) and are statistically significant by regression analysis ($P < 10^{-6}$).

ylation at the P2–P4 promoter area in most of 31 cultured cell lines derived from human tumors, including colon (4/4), breast (6/6), leukemia (8/8), prostate (4/5), fibrosarcoma (1/1), Wilms tumor (1/1), and nonsmall cell lung cancer (4/5) (examples in Fig. 5). The only cell line that showed relative hypomethylation at this CpG island is Hep-G2, a hepatoma cell line that expresses high levels of *IGF2* from the P3 promoter. In addition, all 30 primary colonic tumors examined also exhibited hypermethylation when compared with adjacent normal tissue (examples in Fig. 5). These included premalignant adenomas, indicating that hypermethylation of *IGF2* is a very early event in colorectal neoplasia. Other primary tumor types also demonstrated similar hypermethylation, including renal cell carcinoma (1/2), endometrial carcinoma (2/2), leukemias (3/4), nonsmall cell lung cancers (3/6), gastric carcinomas (2/2), esophageal carcinomas (2/2), and Wilms tumors without 11p loss of heterozygosity (1/2) (see examples in Fig. 5). In all evaluable cases, including six primary colorectal cancers and four cell lines, *IGF2* hypermethylation was present without loss of heterozygosity at the *IGF2* locus and involved both alleles, suggesting *de novo* methylation of the paternal allele early in the process of carcinogenesis.

Expression of the *IGF2* P1 Promoter in Human Tumors. Biallelic expression of *IGF2* has been observed in a variety of human tumors (18–22). Our methylation data would suggest that, in adult tumors, such expression is unlikely to be derived from the P2–P4 promoters because they are extensively methylated. P1, the upstream promoter of *IGF2* is not embedded in a CpG island and has been reported to be normally expressed biallelically (16). To study this issue in cancers, we determined promoter-specific *IGF2* expression by reverse transcription–PCR in a panel of 13 human epithelial tumor cell lines that showed *IGF2* hypermethylation, as well as in Hep-G2 and normal liver. As shown in Fig. 6, none of the cell lines expressed *IGF2* from the P3 promoter, except Caco-2, which had trace expression, and the hypomethylated cell line Hep-G2, which expressed a relatively high amount of mRNA from the P3 promoter. In contrast, 12 out of the 14 cell lines expressed *IGF2* from the P1 promoter. Furthermore, in a well-described model of simian virus 40 immortalization of normal human fibroblasts, during which *IGF2* hypermethylation can be observed (8), we found lack of expression of P1 in the precrisis stage but reactivation of P1 by Northern blotting using a probe-specific for P1 initiated transcripts and reverse transcription–PCR in the immortalized cells (data not shown). Thus, expression of *IGF2* from the nonimprinted P1 promoter may account for the biallelic expression of the gene observed in some adult human tumors.

DISCUSSION

Our results indicate that (*i*) the *IGF2* P2–P4 promoter-associated CpG island is methylated on the silenced maternal allele in young individuals; (*ii*) with age, this methylation also appears on the paternal allele, resulting in biallelic methylation; (*iii*) most adult tumors extend methylation of all retained alleles at this CpG island; and (*iv*) higher methylation in tumors is associated with low or absent *IGF2* expression from P3 but preserved *IGF2* expression from P1.

We cannot determine from the present data whether this newly defined *IGF2* methylation on the maternal allele precedes or follows imprinting at this locus. If this allele-specific methylation can be detected at the gamete stage, then it might be one of the signals for imprinting at *IGF2*, in a manner similar to H19 and other imprinted genes (23, 24). On the other hand, methylation may simply follow transcriptional inactivation of this locus initiated by other mechanisms, such as the enhancer competition model involving the reciprocally im-

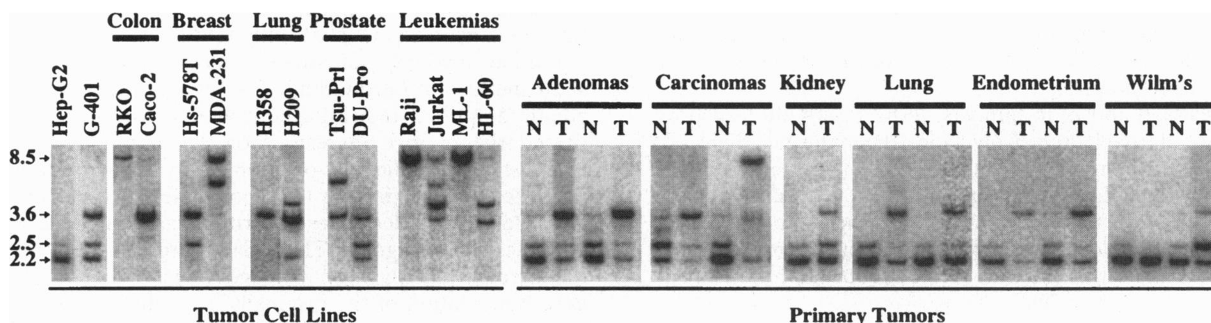

FIG. 5. Hypermethylation of *IGF2* in human tumors. Shown are representative Southern blots of genomic DNA isolated from human malignant cell lines or primary tumors. DNA was digested and probed as in Fig. 2*C*. The source of DNA is indicated on top of each lane. (*Left*) Several tumor cell lines. (*Right*) Primary tumors. In the primary tumors, N is normal tissue adjacent to the tumor and T is the tumor itself. Adenomas refer to premalignant colonic adenomas, whereas carcinomas are malignant primary colonic tumors.

FIG. 6. *IGF2* expression in human tumors. RNA extracted from human epithelial tumor cell lines and normal human liver was reverse transcribed using random primers and amplified using primers specific for the *IGF2* P1 promoter, the *IGF2* P3 promoter, and actin as a control for RNA integrity. One-third of the PCR products were run on an agarose gel and visualized by ethidium bromide staining. The sizes of the PCR products were 600 bp for P1, 500 bp for P3, and 400 bp for actin. The source of RNA is indicated on top of each lane.

printed gene H19 proposed by Leighton *et al.* (25, 26). Nevertheless, even if promoter methylation followed transcriptional silencing, additional factors must account for the establishment of age-related biallelic methylation at this locus because most human genes, including unexpressed genes, do not become methylated with age (J.-P.J.I., unpublished observations).

Although it is clear that *IGF2* promoter methylation starts on the inactive maternal allele, the mechanism of age-related methylation on the opposite allele remains to be determined. It is possible that methylation on the paternal *IGF2* allele is independent of methylation on the maternal allele and simply arises as a result of *de novo* methylation, as observed for the ER gene during aging and carcinogenesis (9). On the other hand, the relative rarity of age-related *de novo* methylation (J.-P.J.I., unpublished observations), even for nonexpressed genes, suggests a possible interaction between methylation of the maternal and paternal alleles that would increase the likelihood of age-related biallelic methylation. One model that would account for such an interaction involves the "spreading" of methylation from one allele to the other through physical interaction between the two alleles. A well-studied analogous situation is the promoter methylation and silencing of the endogenous gene sometimes associated with transgene methylation in plants, a phenomenon termed cosuppression (27). This silencing has been proposed to be due to the formation of DNA–DNA duplexes between the methylated transgene and the endogenous gene. This complex would be recognized by the DNA-methyltransferase enzyme as hemimethylated DNA, an excellent substrate for this enzyme, which would then place methyl groups on the previously unmethylated allele. Imprinted areas would be especially sensitive to this process because methylated and unmethylated alleles coexist within the same cell. Indeed, such pairing between two oppositely imprinted alleles has recently been observed (28). Similarly, X chromosome genes might also be candidates for such methylation spreading in trans, and it would be interesting to study whether such spreading occurs during aging. Finally, it has been previously shown that some pseudogenes and Alu repeats are also hypermethylated (29), and it is plausible that this might direct age-related *de novo* methylation at previously nonimprinted areas.

The age-related increase in methylation at the *IGF2* locus has profound implications for the fate of imprinted areas during development and aging. If promoter methylation can spread from a silenced to a transcriptionally active allele, it would inevitably result in complete loss of expression of the imprinted gene. The resulting changes in expression may underlie some of the physiologic changes observed during aging. In this respect, it is important to note that although *IGF2* expression is generally low in most adult tissues, it has been reported that adult rat brain expresses *IGF2*, and that this expression declines with age (30), as might be predicted by our findings.

Most human cancers are predominantly diseases of aging cell populations. Given the dramatic increase in *IGF2* methylation during aging, it is not surprising that the majority of human tumors examined displayed hypermethylation of the *IGF2* promoter. Indeed, with regard to *IGF2* methylation, cancer cells could simply be seen as extending a process begun during aging. Such hypermethylation would be expected to silence the *IGF2* P2–P4 promoter, as observed in cell lines. This hypermethylation would not, however, affect the nonimprinted P1 promoter that lies more than 20 kb upstream of P2–P4. In fact, human liver that expresses high levels of P1 biallelically also displays more methylation at the *IGF2* P2–P4 CpG island than other normal tissues, raising the possibility that P2–P4 hypermethylation could induce P1 expression, perhaps through the release of a P1 transcriptional repressor. In this respect, it is interesting to note that the tumor-suppressor gene WT-1 binds to the CpG-rich area in the P3 promoter and serves as a repressor of *IGF2* expression (31). Most of the cell lines we examined that hypermethylate *IGF2* also transcribed the upstream P1 promoter. By analogy therefore, it is possible that age-related hypermethylation of *IGF2* P2–P4 may result in a higher expression from the nonimprinted P1 promoter, leading to deregulated autocrine growth stimulation and ultimately to neoplasia. In fact, many cancers overexpress *IGF2* (32), *IGF2* activation occurs early in a mouse tumorigenesis model (33), and *IGF2*-overexpressing mice have a high incidence of neoplasia (34, 35).

Biallelic expression of *IGF2* has been observed in many human tumors (18–22). In a few cases of childhood tumors such as Wilms tumor and rhabdomyosarcoma, this was directly attributable to biallelic expression from P2–P4 through loss of imprinting (36, 37). However, Vu *et al.* (16) have proposed that *IGF2* expression through the nonimprinted P1 promoter could also explain the biallelic expression patterns in some neoplasms. Although we have not directly examined allele-specific expression patterns of *IGF2*, our findings of heavy methylation of the *IGF2* P2–P4 promoters in adult human tumors, along with absent expression from P3 but maintained expression from P1, are consistent with this hypothesis.

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