Loss of Igf2 Imprinting in Monoclonal Mouse Hepatic Tumor Cells Is Not Associated with Abnormal Methylation Patterns for the H19, Igf2, and Kvlqt1 Differentially Methylated Regions*

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IGFII, the peptide encoded by the Igf2 gene, is a broad spectrum mitogen with important roles in prenatal growth as well as cancer progression. Igf2 is transcribed from the paternally inherited allele, whereas the linked H19 is transcribed from the maternal allele. Igf2 imprinting is thought to be maintained by differentially methylated regions (DMRs) located at multiple sites such as upstream of H19 and Igf2 and within Kvlqt1 loci. Biallelic expression (loss of imprinting (LOI)) of Igf2 is frequently observed in cancers, and a subset of Wilms’ and intestinal tumors have been shown to exhibit abnormal methylation at H19DMR associated with loss of maternal H19 expression, but it is not known whether such changes are common in other neoplasms. Because cancers consist of diverse cell populations with and without Igf2 LOI, we established four independent monoclonal cell lines with Igf2 LOI from mouse hepatic tumors. We here demonstrate retention of normal differential methylation at H19, Igf2, or Kvlqt1 DMR by all of the cell lines. Furthermore, H19 was found to be expressed exclusively from the maternal allele, and levels of CTCF, a multifunctional nuclear factor that has an important role in the Igf2 imprinting, were comparable with those in normal hepatic tissues with no mutational changes detected. These data indicate that Igf2 LOI in tumor cells is not necessarily linked to abnormal methylation at H19, Igf2, or Kvlqt1 loci.

Genomic imprinting is defined as an epigenetic change leading to differential expression of the two parental alleles in somatic cells (1–3). Igf2 is one of the known imprinted genes for which only the paternal allele is expressed, the maternal allele being silent. In cancers, the Igf2 imprinting is frequently relaxed so that the silent maternal allele becomes active, resulting in biallelic expression (4, 5). Such loss of imprinting (LOI) also occurs in normal tissues adjacent to some cancers with LOI (6, 7) and has been implicated in the Beckwith-Wiedemann syndrome (BWS), a congenital overgrowth disorder that predisposes to embryonal tumors (8, 9).

Imprinting of the Igf2 gene is thought to be controlled by sequences located at the H19, Igf2, and other loci (10, 11). Such cis elements usually contain the region that is differentially methylated on the parental alleles (DMR) (12–16), containing CpG-rich repeats, which are postulated to facilitate heterochromatinization and gene silencing at imprinted loci (17). In addition, antisense RNA has been shown to be transcribed from regions including the DMRs at Igf2 (18) and Kvlqt1 (KvDMR) (15, 16), which has been proposed to serve a regulatory role in silencing the sense orientation transcript (1, 2).

H19DMR is located upstream of the H19 promoter and is capable of binding CTCF, a highly conserved zinc finger DNA-binding protein with multiple roles in gene regulation. One of the important functions of CTCF is as a chromatin insulator acting in a methylation-dependent manner (19–22). H19 is expressed from the maternal allele, whereas the paternal allele is silent, indicating that the H19/Igf2 genes are reciprocally imprinted. Competition of these two genes for the use of sets of common enhancers located downstream of H19 is now a well documented model that explains this opposite allele specific expression (19–22). On the paternal allele, CTCF cannot bind to the hypermethylated H19DMR, and the common enhancer may be utilized by the Igf2 promoters. Such hypermethylation on the paternal H19DMR is established during male gametogenesis and is maintained throughout development (23). On the other hand, on the maternal allele where the H19DMR is unmethylated, CTCF binds to the H19DMR to insulate Igf2, and then the common enhancer may be utilized only by the H19 promoter.

The Igf2 gene contains three DMRs, two of which (Igf2DMR0 and DMRI) are located far upstream of the Igf2 coding region, and the other (Igf2DMR2) existing within the gene body (12–14, 18). Igf2DMR0 is methylated on the inactive maternal allele (18), whereas Igf2DMR1 and DMRI are methylated on the active paternal allele (12–14). Deletion of the region including maternal Igf2DMR1 has been reported to result in Igf2 LOI (24), and it has been proposed that a putative repressor may bind to the maternal unmethylated Igf2DMR1 to silence the maternal allele (12–14, 24). Furthermore, there seems to be a functional link between the H19DMR and Igf2DMRs, because deletion of the maternal H19 gene results in decreased methylation at the maternal Igf2DMR0 (18) and paternal Igf2DMR1 and DMRI (25). Moreover, deletion of a large segment of Igf2 upstream, either from the maternal or paternal alleles, is reported to lead to LOI not only of Igf2 but also H19 (26).

KvDMR is located within an intron of the Kvlqt1 gene, where the maternal allele is hypermethylated, whereas the paternal allele is unmethylated (15, 16). Although the Kvlqt1 gene is transcribed from the paternal allele, antisense transcripts,
referred to as LIT1, are expressed from the unmethylated paternal allele in both humans and mice (15, 16). Demethylation of the maternal KvDMR associated with biallelic expression of LIT1 has been detected in the majority of BWS cases (15, 16), Smilinich et al. (15) reporting IGFR2 LOI to occur independently of changes in methylation or expression of H19. Thus, KvDMR appears to be an additional control center for the Igfr2 imprinting, independent of Igfr2/H19 loci. However, this remains controversial, because Lee et al. (16) reported similar results but found that KvDMR demethylation was not correlated with the IGFR2 LOI, although it was consistently associated with LIT1 LOI.

The molecular events involved in the Igfr2 LOI in tumors have not been determined. In a subset of Wilms’ tumors (27, 28) and intestinal tumors (7), it has been shown to be accompanied by methylation at the maternal H19DMR together with silencing of the maternal H19 allele (27, 28). Although this pattern of Igfr2/H19 expression is consistent with the Igfr2/H19 chromatin insulation model, there is no reciprocal pattern of Igfr2 expression as well as no strict relationship between the Igfr2 LOI and methylation at the H19DMR in various tumors (29–34). Furthermore, some cases of Wilms’ tumors with the Igfr2 LOI show hypomethylation at the region corresponding to mouse Igfr2DMR0 (34), suggesting the possibility that altered methylation at Igfr2DMRs may be also involved.

In the present study, we therefore investigated whether Igfr2 LOI is associated with specific alterations of methylation at H19DMR, Igfr2DMR1, and KvDMR. In an earlier report, we documented that cells of hepatic tumors (HTs) chemically induced in C3H/HeJ × MSM (Mus musculus molossinus Mishima, Japanese wild mice) frequently show Igfr2 LOI and express Igfr2 at high levels (35). Furthermore, consistent polymorphisms found between the parental C3H/HeJ and MSM strains allow investigation of allele-specific methylation correlating with allele-specific expression of Igfr2/H19. We here report that HT cells with Igfr2 LOI are composed of diverse cell populations, with and without Igfr2 LOI, but even the monoclonal cells with the Igfr2 LOI retain the maternal H19 expression with normal methylation patterns at H19DMR, Igfr2DMR1, and KvDMR.

**EXPERIMENTAL PROCEDURES**

**Mouse HT Cell Lines**—Male F1 hybrid mice derived from breeding female C3H/HeJ and male MSM mice were in utero-transplanted in a sterile manner with diethylnitrosamine (5 µg/g, body weight) at the age of 2 weeks. Cells were isolated by the collagenase perfusion method from HTs that developed 12–14 months later. The HT cells were cultured at a low density as described previously (36), and colonies that were present 4–5 days after the start of cultivation were individually isolated using a cloning ring and expanded to establish cell lines. These were then examined for Igfr2 LOI, and when LOI (+) clones were found, they were further cloned to determine how many subclones showed the Igfr2 LOI.

**Reverse Transcription–PCR (RT-PCR)**—RNA was extracted from HT cells and tissues from normal liver tissues of male C3H/HeJ, MSM, and C3H/HeJ × MSM F1 mice at various ages. Total RNAs were treated with RNase-free DNase (GenHunter, Nashville, TN) to destroy contaminating genomic DNA, and first strand cDNAs were generated using Superscript II (Invitrogen). The specific primers and conditions for each RT-PCR are listed in Table I.

**Igfr2 LOI**—The Igfr2 exon 6 containing the polymorphic CA repeat (35) was amplified by genomic or RT-PCR using the 5′-6-carboxyfluorescein-labeled forward and unlabeled reverse primers, and the products were analyzed by an Applied Biosystem automatic sequencer (ABI Prism 377; Foster City, CA) with the GeneScan software. RT negative controls were run in parallel and demonstrated to be consistently negative.

**Fig. 1. Allelic Igfr2 expression assay by the gene scanning.** A, the sequence including the polymorphic CA repeats in the Igfr2 exon 6 was amplified from the genomic DNA from C3H/HeJ, MSM, and C3H × MSM F1 mice and analyzed by gene scanning. The three major peaks are observed for C3H/HeJ and MSM mice, but those of MSM mice are 4 bases longer than those of C3H/HeJ mice. The peaks of C3H × MSM F1 mice show the overlapping pattern of the C3H/HeJ and MSM peaks. B, RT-PCR analysis of subclones with (top) and without Igfr2 LOI (bottom). The pattern of the LOI (+) cells is similar to that of C3H × MSM F1 mice, whereas that of the LOI (−) cells is similar to that of the paternal MSM mice.

**Table I**

| Genes | Region | PCR type | Forward* | Reverse* | Acc. no.* | annot. Tm.* |
|-------|--------|----------|----------|----------|-----------|------------|
| Igfr2 | Exon 6 | RT        | 25,313–25,336 | 25,473–25,494 | MMU71085 | 59 |
|       | DMR1   | MS        | 12,756–12,770 | 13,146–13,170 | MMU71085 | 48 |
| H19   | Exon2–3 RT | 7011–7031 | 7191–7210 | 3584–3611 | AF049091 | 48 |
|       | DMR    | MS        | 3308–3334 | 1408–1427 | MMU51037 | 54 |
| CTCF  | Exon2–3 RT | 1057–1076 | 1380–1399 | 1739–1760 | MMU51037 | 54 |
|       | Exon3–6 RT | 1670–1689 | 2005–2024 | MMU51037 | 54 |
|       | Exon6–7 RT | 2901–2931 | 2495–2573 | MMU51037 | 48 |
| Kiolq1| DMR    | MS        | 3611 AF049091 | 3611 AF049091 | 48 |

* Nucleotide numbers.
* Acc. no., GenBank accession numbers.
* annot. Tm., annealing temperature for PCR.
* RT, RT-PCR.
* MS, methylation-specific PCR.
Igf2 Imprinting Relaxation and Methylation

**RESULTS**

Establishment of Monoclonal HT Cell Lines with Igf2 LOI—

Since the RT-PCR fragments from Igf2 exon 6 including the polymorphic CA repeat were four bases longer in the paternal MSM allele than the maternal C3H/HeJ allele (35), it was possible to distinguish the parental alleles from which Igf2 was expressed (Fig. 1). Although Igf2 was expressed in all 10 HT tissues and 54 of 62 cell lines, only 4 of 54 HT cell lines were demonstrated to show the Igf2 LOI (Table II). Then the four cell lines with the Igf2 LOI were subcloned, and 11–33 sublines were produced from each. Analysis of each subclone revealed mosaicism in terms of monoallelic/biallelic as well as positive/negative Igf2 expression (Table II). Therefore, three Igf2 LOI (+) clones from the first cell lines were further subcloned, and 9–12 sublines were produced from each. Analysis of these revealed 75–100% of sublines to show the Igf2 LOI (Table II).

**TABLE II**

| HCC tissues and cell lines | No. Subclones examined | Igf2 expressiona | Igf2 L0Ib |
|---------------------------|------------------------|------------------|-----------|
| HCC tissues               | 10                     | 10 (100)         | 10 (100)  |
| HCC cell lines            |                        |                  |           |
| First cell lines          | 62                     | 54 (87)          | 50 (93)   |
| Second cell lines         | 4                      | 33 (100)         | 23 (70)   |
| 2–1                       | 33                     | 19 (90)          | 11 (58)   |
| 2–2                       | 21                     | 8 (67)           | 2 (25)    |
| 2–3                       | 12                     | 11 (100)         | 3 (27)    |
| 2–4                       | 11                     | 10 (100)         |           |
| Third cell lines          | 3                      | 9 (100)          |           |
| 3–1                       | 9                      | 12 (100)         | 3 (25)    |
| 3–2                       | 12                     | 10 (100)         | 1 (10)    |
| 3–3                       | 10                     |                  |           |

a Numbers in parenthesis represent percentages of Igf2-expressing tissues or cell lines among the total samples examined.

b Numbers in parenthesis represent percentages of LOI (−) and (+) cells among the Igf2 expressing cells.

c Subclones of the second Igf2 LOI (+) cell lines (2–1, 2–2, and 2–3) were further subcloned, and each subclone was examined for Igf2 LOI.

d Subclones of the first Igf2 LOI (+) cell lines (3–1, 3–2, and 3–3) were further subcloned, and each subclone was examined for Igf2 LOI.

Finally, bound antibodies were visualized using the ECL system (Amersham Biosciences, Uppsala, Sweden).

Statistics—The data were statistically evaluated with Statview software (SAS Institute, Cary, NC). Differences were analyzed using the χ² or Fisher’s test, and significance was concluded at p < 0.05.
are retained (Table III and Fig. 2B).

**Methylation at the H19DMR**—Allele-specific methylation at the H19DMR, including 13 CpGs and one of the four CTCF sites (the fourth CTCF site at the 3' end in Refs. 19 and 20), was then analyzed (Fig. 3A). This region includes a single polymorphic base at nt 3534 (GenBankTM accession number AF049091), with G for C3H/HeJ and C for MSM, allowing distinction of the parental alleles. This region was found to be generally heavily methylated on the paternal allele and hypomethylated on the maternal allele in the normal liver in two mice as previously described (23), but one sample showed less methylation on the paternal allele (Fig. 3, A-I). Nonmethylated CpGs on the paternal allele were more frequent on the 5' side, including the CTCF binding site. When comparing cells with and without the Igf2 LOI (Fig. 3, B and C), aberrant methylation in the region including the CTCF site was found on the maternal allele at low frequency. This change was detected in all of the four Igf2 LOI (+) cells (Fig. 3C) but not in the Igf2 LOI (−) cells (Fig. 3B) (*p < 0.05). Although the same region was less methylated on the paternal allele in the Igf2 LOI (−) cells (Fig. 3B) than the Igf2 LOI (+) cells (Fig. 3C), this could be due to physiological variation as observed in normal hepatic tissues, because the Igf2 LOI (−) cell lines were derived from different tumors that were present in different hepatic lobes in a single mouse.

**Status of CTCF**—RT-PCR revealed CTCF to be expressed in all of the cell lines (Fig. 4A), and direct sequencing of the CTCF cDNA detected no mutations (data not shown). Western blotting analysis revealed that the expression levels of CTCF protein were not different between the cell lines with and without Igf2 LOI (Fig. 4B), indicating that the CTCF may not be altered in any of them.

**Methylation at the Igf2DMR1**—Five CpGs in the Igf2DMR1 were examined for allele-specific methylation using the polymorphic base at nt 12826 (GenBankTM accession number MMU71085), A for C3H/HeJ and G for MSM as a marker. Although degrees of methylation were variable in each normal hepatic tissue and cell line, some characteristic methylation patterns were noted (Fig. 5). First, the CpGs at the first and fifth positions were more methylated than the others, although this was not constant either in normal livers or cell lines. Second, the CpG at the fifth position tended to be differentially methylated on the paternal allele, but such a tendency was not apparent for other CpGs, for which the results were variable in each sample. However, when cells with and without the Igf2 LOI were compared, there was no clear cut difference in the methylation patterns.

**Methylation at the KvDMR**—Twelve CpGs in the KvDMR, including a polymorphic base at nt 2494 (GenBankTM accession number AF119985), with G for C3H/HeJ and C for MSM, were generally hypermethylated on the paternal allele but hypomethylated on the paternal allele in the normal livers (Fig. 6A), as previously described (15). However, some PCR fragments derived from the paternal allele showed hypermethylation, and a few maternal fragments showed hypomethylation, indicating that the KvDMR creates a mosaic in terms of methylation at the individual DNA strand level in normal hepatic tissues. On the other hand, such a mosaic pattern was less prominent in the HT cell lines, presumably due to extensive selection of the clones. However, there was no significant difference in methylation patterns between the cells with and without Igf2 LOI.
DISCUSSION

Although the examined mouse HT cell lines showed Igf2 LOI at low frequency, all 10 HT tissues were negative in this study. This suggests that the Igf2 LOI (+) cells may be derived from rare cells within the HT tissues or generated de novo during establishment of the lines. Because Igf2 LOI has been reported in cultured mouse and rat fibroblasts (37, 38) and human T lymphocytes stimulated to proliferate by phytohemagglutinin in vitro (39), culture conditions may be responsible. Furthermore, Ungaro et al. (38) reported that the Igf2 LOI that occurred in rat fibroblasts held in the confluent state persisted over cell generations, when the cell confluence was released by trypsinization and dilution, suggesting that the Igf2 LOI may be irreversible. On the other hand, we demonstrated that even the established HT cell lines were composed of heterogeneous cell populations with/without the Igf2 LOI, as well as with/without Igf2 expression. Heterogeneity of cell populations in solid tumors was reported according to Igf2 LOI in Wilms’ tumors (40) and to E-cadherin gene methylation and expression in breast cancers (41).

Previous studies have demonstrated Igf2 LOI to be associated with loss of H19 expression, together with aberrant methylation on the maternal H19 gene, in a subset of Wilms’ and other tumors (7, 27, 28), which lends support to the Igf2/H19 chromatin insulation model (19–22). However, in the present study, all the Igf2 LOI (+) cells retained monoallelic H19 expression from the maternal allele. Such a pattern of Igf2/H19 expression may be due to mixed populations of cells, with and without Igf2 LOI (i.e. if one population had biallelic Igf2 expression without the H19 expression and another maintained the normal Igf2/H19 imprinting, the outcome would be biallelic Igf2 expression with maternally monoallelic H19 expression). We therefore cloned the Igf2 LOI (+) cells and isolated four subclones of cells with biallelic Igf2 expression. Allele-specific expression analysis, however, revealed the biallelic Igf2 expression with maternally monoallelic H19 expression to still be evident in all cases.
Although methylation-specific sequencing detected aberrant methylation at the maternal H19DMR at low frequency in the Igf2 LOI (+) cells, the normal differential methylation pattern was retained in individual DNA strands in both Igf2 LOI (+) and (−) cells. This is in contrast to the reported cases of Wilms’ and other tumors (7, 27, 28), cells of individuals affected by Beckwith-Wiedemann syndrome (8, 9), and human T lymphocytes stimulated to proliferate in vitro (39), in which Igf2 LOI occurs in association with aberrant methylation at the maternal H19DMR. Thus, Igf2 LOI definitely occurs independently of altered methylation at H19DMR, and aberrant methylation of the maternal H19DMR may not, to a major extent, contribute to the Igf2 LOI in our cell lines.

It was noted that some samples showed less methylation at the paternal H19DMR, especially at one of the four CTCF sites (19, 20) and its flanking regions (Fig. 3, A–I and B–I–4), indicating variation in the degree of methylation at the paternal H19DMR in individual mice. Such demethylation might allow the binding of CTCF to paternal H19DMR, resulting in the insulation of the paternal Igf2. However, the fact that the paternal Igf2 and maternal H19 expressions were maintained in such cells indicates that such partial demethylation on the paternal H19DMR does not affect the Igf2/H19 imprinting.

The observed low frequency of aberrant methylation of the maternal H19DMR raises the possibility that the Igf2 LOI may be caused by alterations of other components in the Igf2/H19 imprinting machinery. We therefore investigated alterations in CTCF, because chromosomal loss at human Ch16q, where CTCF is localized, has been frequently detected, in many types of tumor including HTs (42, 43). Mutational changes in the CTCF gene have been found in some human tumors (44), and overexpression of CTCF in tumor cells leads to growth arrest and apoptosis (45). Furthermore, CTCF can act as a silencer for c-myc (44), which is frequently overexpressed in various tumors including HTs. However, because no CTCF mutations were detected, and also because its protein levels were not different between Igf2 LOI (+) and (−) cells, the CTCF function may be intact in these cells, although further experiments are required to confirm this.

The Igf2DMR1 is suggested to be able to bind to a putative silencer in a tissue-specific manner (12, 13, 24). Deletion of the 5-kb region corresponding to the mouse Igf2DMR1 results in activation of the silent maternal Igf2 in mesenchymal tissues, while not affecting the maternal H19 expression (24). Such tissue specificity is speculated to be due to the possibility that, although the access of the endodermal-specific common H19/Igf2 enhancer to the Igf2 promoter is efficiently blocked by the CTCF insulator on the maternal chromosome, the mesodermal-specific common enhancer may further need the Igf2 silencer to suppress maternal Igf2 expression. Methylation analysis revealed that the patterns of differential methylation at the Igf2DMR1 were not apparent as compared with the H19DMR and KvDMR in the normal hepatic tissue. Especially, although differential methylation was observed at the fifth position of the five CpGs analyzed in the some samples, it was not apparent for other CpGs. The fact that there was no significant difference in the methylation pattern between the Igf2 LOI (+) and (−) cells suggests that this region may not be responsible for the Igf2 LOI in these cell lines.

In addition to the H19DMR and Igf2DMRs, the elements so far substantiated according to the Igf2 LOI are the KvDMR (15) and some newly found enhancers upstream and downstream of H19 (46–48). Aberrant demethylation at the maternal KvDMR, associated with biallelic LIT1 expression, is thought to be a major cause of BWS (8, 9, 15, 16). Because such BWS cases do not show any abnormality at the H19DMR and loss of H19 expression, the KvDMR is thought to be the locus for BWS independent of H19DMR. LIT1 expression from the paternal Igf2 is indicated to be related to regulation of various maternally expressed neighboring genes at human 11p15.5 and mouse distal Chr7, with a special importance for p57Kip2 expression (2). However, because such demethylation at the maternal KvDMR has not been detected in Wilms’ tumors (49, 50) and also because the frequency of embryonic tumors in BWS cases with the maternal KvDMR demethylation and LIT1 LOI is much lower than in the cases with maternal H19DMR methylation and loss of H19 expression (51, 52), KvDMR may be less important than H19DMR, or not relevant to pathogenesis of Wilms’ tumors. The present observation of no abnormality at KvDMR in our cell lines may support the notion that the KvDMR may not have a major role in maintenance of Igf2 imprinting (16).

Further potential candidates are newly found tissue-specific enhancers located upstream and downstream of H19, which are conserved between mouse and humans (46–48). These novel sequences may not only interact with H19DMR and Igf2DMR1 but also affect the methylation status of H19DMR, and deletion of these sequences may lead to the Igf2 LOI. Furthermore, in brain, where Igf2 expression is biallelic, the relevant enhancers are located upstream of the H19DMR (53). Therefore, the possibility that alteration(s) of these elements may result in Igf2 LOI in tumors remains to be investigated.

In conclusion, the present study demonstrated that Igf2 LOI can occur independently of changes in H19DMR, Igf2DMR1, and KvDMR in mouse HT cell lines.

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