Frequent *IGF2/H19* Domain Epigenetic Alterations and Elevated *IGF2* Expression in Epithelial Ovarian Cancer

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Abstract

Overexpression of the imprinted *insulin-like growth factor-II* (*IGF2*) is a prominent characteristic of gynecologic malignancies. The purpose of this study was to determine whether *IGF2* loss of imprinting (LOI), aberrant *H19* expression, and/or epigenetic deregulation of the *IGF2/H19* imprinted domain contributes to elevated *IGF2* expression in serous epithelial ovarian tumors. *IGF2* LOI was observed in 5 of 23 informative serous epithelial ovarian cancers, but this did not correlate with elevated expression of *IGF2 H19* RNA expression levels were also found not to correlate with *IGF2* transcript levels. However, we identified positive correlations between elevated *IGF2* expression and hypermethylation of *CCCTC* transcription factor binding sites 1 and 6 at the *H19* proximal imprint center (*P* = 0.05 and 0.02, respectively). Hypermethylation of *CCCTC* transcription factor sites 1 and 6 was observed more frequently in cancer DNA compared with lymphocyte DNA obtained from women without malignancy (*P* < 0.0001 for both sites 1 and 6). Ovarian cancers were also more likely to exhibit maternal allele-specific hypomethylation upstream of the imprinted *IGF2* promoters when compared with normal lymphocyte DNA (*P* = 0.004). This is the same region shown previously to be hypomethylated in colon cancers with *IGF2* LOI, but this was not associated with LOI in ovarian cancers. Elevated *IGF2* expression is a frequent event in serous ovarian cancer and this occurs in the absence of *IGF2* LOI. These data indicate that the epigenetic changes observed in these cancers at the imprint center may contribute to *IGF2* overexpression in a novel mechanistic manner. (Mol Cancer Res 2006;4(4):283–92)

Introduction

Ovarian cancer is the leading cause of death from gynecologic malignancies because the majority of cases are not detected until the disease has metastasized. We previously did a microarray-based analysis on serous cancers from individuals with early-stage and advanced-stage disease (1). The gene expression profiles obtained from these cancers indicated that more than half exhibited a high level of expression of the *insulin-like growth factor-II* (*IGF2*) gene, which is located at chromosome 11p15.5. High *IGF2* expression was recently shown to be a predictor of poor prognosis in epithelial ovarian cancers and was associated with the most aggressive forms of this disease (2). In addition, serum levels of *IGF2* are one of four combinatorial diagnostic markers used in a newly developed test for early detection of ovarian cancer (3). Despite the strong association between *IGF2* expression and ovarian malignancies, little is known about the molecular underpinnings of *IGF2* deregulation in this disease.

*IGF2* encodes a potent mitogenic growth factor that is active in early development and plays an important role in embryonic and fetal growth (4). *IGF2* binds to the IGF-I receptor to initiate intracellular signaling cascades that lead to cell proliferation (5, 6). Increased expression of *IGF2* is a common feature of both pediatric and adult malignancies (5), and mounting evidence implicates *IGF2* as a major factor contributing to oncogenesis.

*IGF2* transcription is subject to genomic imprinting, an epigenetic form of gene regulation that leads to mRNA production from only one allele in a manner depending on the sex of the parent from whom the allele was inherited. *IGF2* expression is from the paternal allele, whereas the maternal allele is normally inactive. *IGF2* imprinting is relaxed in many different types of tumors, including osteosarcoma (7), lung adenocarcinomas (8), head and neck squamous cell adenocarcinomas (9, 10), Wilms’ tumor (11), prostate cancer (12), and colorectal carcinomas (13–17). This loss of imprinting (LOI) occurs when there is abnormal activation of the maternal copy of *IGF2* and the resultant overexpression contributes to tumor growth. *IGF2* expression is coordinately regulated with the maternally expressed *H19* gene that produces a noncoding RNA. Studies in mice and humans have shown that the reciprocal imprinting of these two genes is at least partially dependent on the presence of binding sites for the *CCCTC* transcription factor (*CTCF*; ref. 18), which are located upstream of the *H19* promoter and comprise part of the germ-line imprinting mark (19).
CTCF is a zinc finger transcription factor that functions to preclude potential associations between promoters and enhancers by inducing formation of chromatin structure that physically separates the relevant sequences (20, 21). CTCF is blocked from binding the H19 proximal binding sites by methylation on the paternal chromosome. CTCF binds the unmethylated maternal chromosome and prevents enhancers located downstream of H19 from accessing the maternal IGF2 promoter. Acquisition of methylation on the maternal chromosome in this region or loss of methylation from the paternal chromosome occurs in a highly tumor-specific manner. For example, in bladder cancer, paternal hypomethylation leads to biallelic H19 expression (22), whereas in Wilms’ tumor, maternal hypermethylation and biallelic IGF2 expression are common (11, 23-25).

H19 RNA itself has also been ascribed a role in the regulation of IGF2. Using a hepatoblastoma cell line disomic for chromosome 11, Wilkin et al. showed that transgenic expression of H19 antisense transcripts resulted in increased IGF2 mRNA and protein expression, whereas expression of H19 sense transgenes resulted in IGF2 transcript and protein levels equivalent to control cells (26). The level of H19 RNAs in Wilms’ tumor is also found to inversely correlate with levels of IGF2 mRNA (27). In this study, H19 RNAs were found in polysomes, indicative of H19 translation and/or potential transregulation of IGF2 translation.

Another epigenetic element recently implicated in the control of IGF2 imprinting is the differentially methylated region (DMR) located upstream of the imprinted IGF2 promoters. This DMR normally carries a maternal methylation mark in both mouse and human, but this methylation is specifically reduced or lost in Wilms’ tumors (28) and colorectal cancers (14-16) and this correlates with IGF2 LOI (14, 15).

We sought to determine whether IGF2 LOI or deregulated H19 expression might explain the elevated levels of IGF2 transcripts frequently observed in serous epithelial ovarian cancers. We also examined the epigenetic profiles of the IGF2/H19 domain in these tumors to determine if deregulated methylation status correlated with IGF2 expression and potential LOI.

Results

Our recent microarray analysis of serous epithelial ovarian cancers indicated that IGF2 was expressed at high levels in more than half of the cases analyzed compared with normal ovarian surface epithelium. We independently validated the microarray data using quantitative real-time reverse transcription-PCR (1) and these specimens and others were analyzed in the present study. There are several DMRs in the IGF2/H19 imprinted domain that contribute to regulation of IGF2 expression and may therefore be involved in the overexpression observed in the ovarian tumors. One of these is located upstream of the imprinted IGF2 promoters (Fig. 1) and is normally maternally methylated (IGF2 DMR; ref. 28), whereas another is located upstream of H19 and is paternally methylated (29). The latter region harbors sequences known to bind to the zinc finger protein CTCF in a methylation-sensitive manner (18, 30). Altered methylation of each of these regions bears relevance to various types of cancer.

IGF2 DMR

We first examined the methylation profile of the IGF2 DMR by PCR and nucleotide sequencing followed by determining the percent methylation for each of the CpG dinucleotides using phosphorimaging analysis. Lymphocytes from individuals without evident malignancy (n = 43) exhibited an average

![FIGURE 1. Schematic representation of the imprinted IGF2/H19 domain at 11p15.5 (not to scale). IGF2 and H19 are paternally and maternally expressed, respectively, and are separated by ~144 kb of intervening sequence. Vertical bars, exons; black, expressed allele, gray, silenced allele; arrowheads, direction of transcription for IGF2 and H19; white, imprinted expression (P2, P3, and P4 promoters of IGF2 and H19 promoter); gray, nonimprinted expression (IGF2 P1 promoter). There are three known DMRs in the IGF2/H19 domain; they are indicated by the allele-specific methylation (gray circles). The intergenic sequence contains a differentially methylated imprint control region that harbors seven binding sites (vertical oval) for the zinc finger protein, CTCF (represented as one horizontal oval). Methylation on the paternal chromosome (PAT) blocks CTCF binding, silences the H19 promoter, and allows activation of paternal IGF2 transcription by downstream enhancers (white oval). Methylation on the maternal chromosome (MAT) upstream of the imprinted IGF2 promoters (IGF2 DMR) is thought to contribute to maternal IGF2 silencing. Abnormal maternal activation of IGF2 can occur through hypomethylation of the IGF2 DMR and/or hypermethylation of the CTCF-binding sites. CTCF-binding sites 1 and 6 were examined in the present study. Note the relative position of the SNP used to analyze potential IGF2 LOI in ovarian cancer.](Image 94x136 to 491x305)
methylation level of 38.3 ± 9.6% for the IGF2 DMR (Fig. 2). The methylation status was confirmed for selected samples by sequencing individual cloned alleles following PCR amplification of the bisulfite-treated DNA (data not shown).

Of 72 tumors, 20 exhibited hypomethylation of this DMR (defined as an average methylation level <20% or 2 SDs below the average level observed in normal lymphocytes), but this did not correlate with the expression of IGF2 based on the microarray data (P = 0.15), with survival (P = 1.0), or with age at diagnosis (P = 0.21; see Table 1; data with matching microarray values are summarized in Fig. 3). In contrast to the frequent hypomethylation observed in ovarian cancer, only 2 of 43 lymphocyte samples from individuals without malignancy exhibited hypomethylation of this region (P = 0.004).

H19 Expression

H19 has been implicated previously in the negative regulation of IGF2 and has been proposed to function as a tumor suppressor (26, 27). We therefore analyzed the expression level of H19 and compared it with that of IGF2 to determine if there was any correlation between transcript levels of these genes in serous epithelial ovarian malignancies. We did quantitative real-time reverse transcription-PCR (Taqman) using assays specific for IGF2 and H19 with the expression values normalized to those obtained in parallel for β2-microglobulin (B2M) in a subset of the cancer specimens. Linear regression showed lack of correlation between expression of IGF2 and H19 in these tumors (r² = 0.016; P = 0.54; data not shown).

CTCF-Binding Sites at IGF2/H19 DMR

We next examined the methylation profile of the imprint control region and specifically focused on two of the seven CTCF-binding sites located within this region that have been shown previously to exhibit methylation abnormalities in other cancers (23). We examined the methylation profile of these two CTCF-binding sites (5’-CCCGCGCGGC-3’; ref. 15) using bisulfite sequencing (see Fig. 4A and B for representative data).

Table 1. Epigenetic Analysis of IGF2 in Serous Epithelial Ovarian Tumors

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n</th>
<th>Age (Avg)</th>
<th>CTCF-1&lt;sup&gt;c&lt;/sup&gt;</th>
<th>CTCF-6&lt;sup&gt;b&lt;/sup&gt;,&lt;sup&gt;1&lt;/sup&gt;</th>
<th>IGF2 DMR&lt;sup&gt;c&lt;/sup&gt;</th>
<th>LOI&lt;sup&gt;x&lt;/sup&gt;</th>
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<tr>
<td>Borderline</td>
<td>5</td>
<td>41.4</td>
<td>2/2</td>
<td>0/5</td>
<td>2/3</td>
<td>0/2</td>
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<tr>
<td>Early stage (I/II)</td>
<td>9</td>
<td>55.2</td>
<td>6/2</td>
<td>1/8</td>
<td>4/5</td>
<td>0/2</td>
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<tr>
<td>Advanced stage (III/IV)</td>
<td>64</td>
<td>60.9</td>
<td>37/16</td>
<td>28/29</td>
<td>14/44</td>
<td>5/16</td>
</tr>
<tr>
<td>Normal lymphocytes&lt;sup&gt;k&lt;/sup&gt;</td>
<td>43</td>
<td>N/A</td>
<td>3/30</td>
<td>2/37</td>
<td>2/41</td>
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<tr>
<td>≤50</td>
<td>17</td>
<td>44.9</td>
<td>10/5</td>
<td>5/11</td>
<td>7/10</td>
<td>1/3</td>
</tr>
<tr>
<td>&gt;50</td>
<td>56</td>
<td>64.8</td>
<td>33/13</td>
<td>24/25</td>
<td>11/39</td>
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<td>Fisher’s exact P</td>
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<th>Survival (advanced stage)</th>
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<td>≤3 y postdiagnosis</td>
<td>30</td>
<td>59.7</td>
<td>16/6</td>
<td>12/13</td>
<td>7/20</td>
<td>3/7</td>
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<td>≥7 y postdiagnosis</td>
<td>24</td>
<td>60.1</td>
<td>15/6</td>
<td>11/31</td>
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<tr>
<td>Fisher’s exact P</td>
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<th>IGF2 expression</th>
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<tr>
<td>Microarray ≤90</td>
<td>26</td>
<td>57.6</td>
<td>11/9</td>
<td>5/17</td>
<td>10/14</td>
<td>4/3</td>
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<tr>
<td>Microarray &gt;90</td>
<td>35</td>
<td>60.7</td>
<td>25/5</td>
<td>18/14</td>
<td>7/24</td>
<td>1/12</td>
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<td>Fisher’s exact P</td>
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<td>Borderline vs malignant</td>
<td></td>
<td></td>
<td>0.58&lt;sup&gt;£&lt;/sup&gt;</td>
<td>0.07&lt;sup&gt;£&lt;/sup&gt;</td>
<td>0.61&lt;sup&gt;£&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;£&lt;/sup&gt;</td>
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<td>Stage I/II vs III/IV</td>
<td></td>
<td></td>
<td>1.00&lt;sup&gt;£&lt;/sup&gt;</td>
<td>0.07&lt;sup&gt;£&lt;/sup&gt;</td>
<td>0.24&lt;sup&gt;£&lt;/sup&gt;</td>
<td>1.00&lt;sup&gt;£&lt;/sup&gt;</td>
</tr>
<tr>
<td>Malignant vs lymphocytes</td>
<td></td>
<td></td>
<td>&lt;0.0001&lt;sup&gt;£&lt;/sup&gt;</td>
<td>&lt;0.0001&lt;sup&gt;£&lt;/sup&gt;</td>
<td>0.004&lt;sup&gt;£&lt;/sup&gt;</td>
<td>N/A</td>
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Abbreviation: N/A, not applicable.
<sup>a</sup>Number exhibiting hypermethylation/number exhibiting differential methylation.
<sup>b</sup>Number exhibiting hypomethylation/number exhibiting differential methylation.
<sup>c</sup>One sample was not tabulated here because it was hypermethylated at CTCF-6.
<sup>1</sup>Number with loss of IGF2 imprinting/number with maintenance of imprinting.
<sup>k</sup>Normal lymphocytes were from individuals without evident malignancy.
<sup>£</sup>Fisher’s exact P.
For a subset of the cancer cases (n = 8; Fig. 4C) and normal lymphocytes (n = 17; data not shown), the methylation status was also examined using phosphorimaging analysis. We found that CTCF-binding site hypermethylation, defined by an average methylation level of ≥70% for the CpGs contained within the CTCF sites, was much more frequent in advanced-stage disease for both sites combined (≈59% of the total sites examined) than borderline (22%) or early-stage (41%) disease (Table 1). In normal lymphocytes, 7% of these sites exhibited hypermethylation. When analyzed independently, CTCF site 1 was more frequently hypermethylated than site 6. Although 39% of the serous tumors were hypermethylated at both CTCF sites, 3% were hypermethylated at site 6 only, whereas 31% were hypermethylated only at site 1. One cancer specimen exhibited hypermethylation of CTCF site 1, whereas CTCF site 6 was hypomethylated (see Figs. 3 and 4C).

To further verify our data for the methylation status of CTCF sites 1 and 6, we cloned PCR amplicons generated from the bisulfite-modified DNA into plasmid vectors where each clone contains a single allele generated from the PCR. We then sequenced multiple individual cloned alleles for each of four tumor specimens (Fig. 4D). Tumor 1 in Fig. 4D exhibits elevated IGF2 expression, hypermethylation of both CTCF sites 1 and 6 as determined by bisulfite sequencing of the PCR amplicon, and IGF2 LOI. In agreement with our prior assessment, the cloned alleles from both CTCF sites in this tumor are predominantly methylated. This individual is heterozygous for a CpG-altering C/T single nucleotide polymorphism (SNP) in CTCF site 6 (position 7966 of accession no. AF125183) and another nearby A/T SNP (position 8008 of accession no. AF125183). As such, the parental alleles can be distinguished from the sequence. In this case, there was one clone (7966T and 8008A) that was completely unmethylated, suggesting that it may have been derived from normal cells (fibroblasts, stroma, lymphocytes, etc.) present in the tumor tissue used to purify DNA for this sample. Because the maternal allele is normally unmethylated, these results suggest that the 7966T/8008A allele is maternally derived as are the other 7966T/8008A clones that are unmethylated at CpG site 7. The remaining clones are methylated at site 7, are 7966C/8008T, and are therefore likely paternally derived. Similarly, tumor 2 (corresponding to tumor 7 in Fig. 4C) also exhibits very high IGF2 expression and 89.2%

FIGURE 3. Summary of IGF2 expression and corresponding epigenetic profile. Y axis, relative expression of IGF2 from 56 serous ovarian tumors detected by hybridization to Affymetrix U133A GeneChip Arrays (Santa Clara, CA; ref. 1). Dominos, methylation status for each tumor sample at the IGF2 DMR and CTCF sites 1 and 6; filled squares, presence of methylation; half-filled dominos, differential methylation (top and bottom domino halves, paternal and maternal alleles, respectively). The absence of a domino indicates that the sample was not analyzed. Diamonds, heterozygosity at the DMR and CTCF sites 1 and 6; filled squares, presence of methylation; half-filled dominos, differential methylation (detected by hybridization to AffymetrixU133A GeneChip Arrays (Santa Clara, CA; ref. 1). Dominos, methylation status for each tumor sample at the CTCF sites 1 and 6 was hypomethylated (see Figs. 3 and 4C).
methylation at CTCF site 1 and 95.5% methylation at CTCF site 6 (excluding CpG 7 because it is polymorphic in this individual) by phosphorimaging analysis. The results obtained from sequencing cloned alleles from this tumor again agree with the phosphorimaging results, with both CTCF sites exhibiting near complete methylation. This individual is also heterozygous for both SNPs within the sequenced region, but parental identity cannot be determined because all alleles are heavily methylated.

For tumor 3 in Fig. 4D (corresponding to tumor 1 in Fig. 4C) with low IGF2 expression, the status of the cloned alleles also corroborates our phosphorimaging analysis, where we observed 77.0% and 9.7% methylation of CTCF sites 1 and 6, respectively. Intriguingly, the hypermethylation present within the genomic region encompassing CTCF site 1 seems to specifically target the core CpGs within this binding site. Finally, tumor 4 exhibits elevated IGF2 expression and was assigned a hypermethylated status for CTCF site 1 and normal differentially methylated status for CTCF site 6 by bisulfite sequencing. Analysis of the cloned alleles completely corroborates the bisulfite sequencing results. Together, these data indicate that there is good correlation between methylation results obtained by bisulfite sequencing, phosphorimaging, and sequencing of cloned alleles for these sites.

We did not find that there were differences in the methylation profile of these sites that correlated with age at diagnosis, survival, or stage of disease. However, cancers with IGF2 expression levels greater than the average observed for normal ovarian surface epithelium (92.8; n = 3) by microarray analysis were significantly more likely to be hypermethylated at CTCF site 1 (P = 0.05) or CTCF site 6 (P = 0.02) than cancers with low IGF2 expression (Table 1).

IGF2 Imprinting

To determine if increased IGF2 expression was associated with IGF2 LOI, we identified 24 tumors that were heterozygous for a SNP in exon 9 of IGF2 and compared the nucleotide sequence of their genomic DNA with tumor cDNA (Fig. 5). Of the 24 heterozygotes, 5 exhibited biallelic IGF2 expression. Surprisingly, 4 of the 5 expressed IGF2 without malignancy, and this observation is consistent with the proportion of the general population reported to have this characteristic (14).

A role for noncoding RNAs is implicated in the regulation of imprinted genes due to the preponderance of these transcribed sequences located in imprinted domains and empirical data suggesting their importance (26, 27, 31-42). For example, the genes Delta, Drosophila homologue-like 1 (DLK1) and maternally expressed gene 3 (MEG3) in the imprinted domain on human chromosome 14 bear remarkable similarity to the IGF2/H19 domain in terms of the genomic organization and putative regulatory features (43-45). A regulatory role has been postulated for MEG3 RNA in modulating the expression of the paternally expressed DLK1. This is based on analysis of these two genes in sheep (Ovis aries) that exhibit a muscular hypertrophy phenotype characterized by enhanced DLK1 expression and maintenance of normal imprinting (31). In this case, the ratio of expression of the protein coding to the RNA encoding gene seems to be an important factor in the development of the hypertrophic phenotype. It is plausible that a similar regulatory scenario for the IGF2/H19 domain occurs in ovarian cancer as has been postulated (26, 27). However, we determined the ratio of expression of H19 mRNA in 26 of these cancers to the level of IGF2 expression using quantitative real-time reverse transcription-PCR and found no

Discussion

This study is the largest to date that has analyzed the quantitative expression of IGF2 combined with potential LOI in gynecologic malignancies. It is also the first to begin to examine the epigenetic characteristics of the IGF2/H19 imprinted domain in ovarian cancers in the context of elevated IGF2 expression. We have shown that there are substantial changes in the normal methylation profile of specific regions within the IGF2/H19 imprinted domain, including those associated previously with other types of cancer. We have also determined that ~22% of informative serous epithelial ovarian cancers exhibit IGF2 LOI but, surprisingly, LOI in these cancers is not correlated with elevated IGF2 expression levels.

The DMR upstream of the IGF2 imprinted promoters exhibits substantial hypomethylation in serous epithelial ovarian cancers, but this change is not associated with IGF2 LOI. This is an unexpected result based on the prior reports of association between hypomethylation of this DMR, LOI, and incidence of colon cancer (14-16). This could be explained by loss of heterozygosity specific to the maternal chromosome, although it is unclear what advantage loss of the normally silent maternal allele would confer to tumor growth unless the locus driving such loss is linked to IGF2. Our results from serous ovarian cancers suggest that there is considerable tissue-specific variability in the ability of the IGF2 DMR to affect imprinting of IGF2. We did find hypomethylation of this region in a small fraction of the lymphocyte DNAs we analyzed from women without malignancy, and this observation is consistent with the proportion of the general population reported to have this characteristic (14).
statistical correlation between expression levels of these genes. These results indicate that the level of H19 RNA produced in serous epithelial ovarian cancers is unlikely to be critical for regulation of IGF2 expression.

In contrast to the 22% LOI we observed in serous ovarian cancers, LOI in Wilms’ tumors can approach 90% for specific subtypes (11), whereas the incidence in colorectal carcinoma is ~44% (46). Previous studies agree with our findings that elevated IGF2 levels, but relatively infrequent LOI of IGF2, are characteristic of serous ovarian adenocarcinomas. Yun et al. reported that although IGF2 is overexpressed in ovarian cancers, LOI was not an apparent contributing factor in seven informative serous cases (47). Kim et al. also showed that IGF2 LOI was not a prominent mechanism for IGF2 overexpression in serous epithelial tumors, but IGF2 LOI was actually more frequently observed in benign tumors (48).

Although these results indicate that LOI is not a frequent event in ovarian cancers and that LOI does not consistently lead to elevated expression of IGF2 in serous epithelial tumors, they do not account for the elevated expression of IGF2 observed in about half of the cases we analyzed. Overexpression of IGF2 is clinically relevant and it is therefore important to understand how this occurs in serous epithelial ovarian cancer in the absence of LOI. Our results now implicate an imprinting-independent role for hypermethylation at the IGF2 imprint control region in the overexpression of IGF2. Current dogma holds that hypermethylation of the CTCF sites within the imprint control region blocks CTCF binding, leading to IGF2 LOI (Fig. 1). This is the first report to our knowledge that links aberrant hypermethylation of this imprint control region to elevated IGF2 expression with maintenance of normal imprinting and again underscores the complexity associated with the transcriptional regulation of this domain.

Our data suggest that whereas the maternal IGF2 allele remains silent the paternal allele in many tumors has become more transcriptionally proficient. It is unclear how biallelic CTCF site methylation would contribute to enhanced IGF2 expression from only the paternal allele. This could be due to changes in histone acetylation or histone methylation status (e.g., see refs. 49, 50) that promote increased IGF2 expression.
from the paternal allele, more efficient transcriptional activation via transcription factor binding to the paternal allele, or other epigenetic changes that regulate IGF2 expression. In this regard, analysis of the methylation status of the intragenic DMR of IGF2 (see Fig. 1) would help determine whether this region, which was shown previously to be evolutionarily conserved among imprinted mammals and to contain putative CTCF-binding sites (51), is epigenetically modulating IGF2 expression in ovarian cancer.

The high frequency of epigenetic alterations observed in serous epithelial ovarian cancers at the IGF2/H19 domain suggests that these changes may serve as a useful disease biomarker. Of the three epigenetic markers we analyzed, only 13% of the specimens exhibited a normal methylation profile. Of particular relevance, each cancer in this study that was diagnosed at an early stage (II; n = 9) exhibited IGF2 DMR hypomethylation and/or CTCF site 1 hypermethylation. In contrast, hypermethylation of CTCF site 6 was found mostly in advanced cases. Unlike CTCF site 1 hypermethylation, IGF2 DMR hypomethylation was not associated with high expression of IGF2. Nevertheless, both epigenetic changes may be an early indicator of disease. One of the major limitations to the successful treatment of women with epithelial ovarian cancer is the difficulty in detecting early-stage disease. Additional studies are required to evaluate the potential to use epigenetic characteristics, like those reported here for the IGF2/H19 domain, to improve the ability to detect early-stage disease by analyzing tumor DNA present in serum, plasma, or peritoneal fluid. Further evaluation of the mechanistic basis for epigenetic-mediated up-regulation of IGF2 could also lead to development of novel therapeutic targets.

Materials and Methods

Samples

Borderline (low malignant potential), early-stage (I or II), and advanced-stage (III or IV) serous ovarian carcinomas (see Table 1), normal ovarian tissues, and peripheral blood lymphocytes (from individuals without evident malignancy) were obtained and used with approval from the Institutional Review Board of Duke University. Samples were immediately processed and stored at −80°C before nucleic acid extraction. DNA was prepared using standard phenol/chloroform extraction followed by ethanol precipitation. RNA was prepared using the Qiagen RNeasy Mini kit (Valencia, CA).

Methylation Analyses

Genomic DNA (1 μg) was treated with sodium bisulfite as described previously (52, 53). Sodium bisulfite converts unmethylated cytosines to uracils, whereas methylated cytosines are unaffected. Bisulfite-treated DNA (20–50 ng) was subsequently used as template in PCR. Primers were designed to amplify the bisulfite-converted DNA by annealing to regions devoid of CpG dinucleotides to avoid bias in amplification. Primer sequences (5’-3’) include IGF2 DMR TAATTATTT-TAGGTTGTGT (forward) and TCCAAACACCC-CACCCTT (reverse; ref. 15), CTCF site 1 GTATAGGTA-TTTTGGAGGT (forward) and CCTAAAATATATAACACATAAACC (reverse; ref. 23), and CTCF site 6 GTATATGGGTATTTTTGGAGGT (forward) and CTAAATCCCAACCAAAAAACTA (reverse; ref. 15). PCR amplicons were resolved on agarose gels and purified using Sigma GenElute spin columns (St. Louis, MO) or directly using the Qiagen MinElute PCR Purification kit. The amplicons were manually sequenced with the Thermosequenase Dideoxy Terminator Cycle Sequencing kit (U.S. Biologicals, Swampscott, MA) using the following primers: IGF2 DMR (forward primer listed above), CTCF site 1 GAGGTGGTTTTAGTTGG (reverse; ref. 15), and CTCF site 6 (TATCCTATCCCCATAACC). Following resolution of the sequencing reactions on denaturing polyacrylamide gels, the gel was exposed to radiographic film (Kodak X-OMAT MR, New Haven, CT) and/or a phosphor-imager screen followed by percent methylation determination using the Storm PhosphoImager System and ImageQuant Software (GE Healthcare Life Sciences, Piscataway, NJ). Specimens with average methylation of <20% for the three CpGs in the IGF2 DMR were considered to exhibit hypomethylation, whereas those exhibiting methylation levels ≥70% for the CpGs within CTCF sites 1 and 6 were considered hypermethylated.

For analysis of the methylation status of individual alleles, PCR amplicons were generated as described above and resolved on 2% agarose gels. Following purification with the Qiagen MinElute PCR Cleanup kit, the amplicons were ligated into pGEMT-Easy vectors (Promega, Madison, WI) and plasmids were transformed into competent JM109 Escherichia coli (Promega) followed by plating to LB agar with 100 μg/mL ampicillin (Teknova, Hollister, CA). Whole-cell PCR was used to amplify plasmids from individual colonies using SP6 and T7 primers (94°C for 5 minutes followed by 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds and a final 5-minute extension at 72°C). The amplicons were resolved on agarose gels, purified using Sigma GenElute spin columns, and sequenced.

Quantitative Real-time Reverse Transcription-PCR

Total RNA (1 μg) was reverse transcribed in a 20 μL reaction volume with random hexamer primers using the AMV First-Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Subsequent real-time PCRs (Taqman Assays-On-Demand; Applied Biosystems, Foster City, CA) were done using 11.25 μL of a 1:15 dilution of the cDNA according to the manufacturer’s recommendations on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems) with the exception that a 25 μL reaction volume was used with 50 total cycles. The relative expression levels of IGF2 and H19 were obtained for each sample by normalization to the expression level of B2M (Taqman Assays-on-Demand, Hs99999907_m1). All assays were done in parallel.

Imprint Status Determination

DNA from tumors was genotyped for a SNP located in exon 9 of IGF2 using primers CTGGGACTTGTGCAAATT (forward) and GTGCTGCCATACATTCA (reverse). cDNAs generated from DNase I–treated RNAs of heterozygotes were analyzed for allelic expression by manual


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nucleotide sequencing with the forward primer. Laser capture microdissection using an Arcturus PxnCell II LCM System (Mountain View, CA) was done on selected tumors to isolate homogeneous populations of tumor cells to confirm imprint status. Samples with evident biallelic cDNA expression were considered to exhibit LOI.

Statistical Analysis

Fisher’s exact tests were used to calculate two-sided Ps using InStat 3.0 for Mac (GraphPad Software, San Diego, CA) to determine if there were nonrandom associations between categorical variables. Ps ≤ 0.05 were considered significant.

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References


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