Loss of Imprinting of the IGF-II and H19 Genes in Epithelial Ovarian Cancer¹

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ABSTRACT

To establish a possible role of genomic imprinting in the carcinogenesis of epithelial ovarian cancer, we determined the imprinting status of both IGF-II and H19 genes in 43 ovarian cancers, 7 low malignant potential ovarian tumors, and their matched normal tissues. In ovarian cancer, loss of heterozygosity (LOH) of IGF-II, H19 RsαI, and H19 AluI was found in 4 of 24 (16.7%), 3 of 20 (15%), and 1 of 16 (6.3%) samples, respectively. All patients with tumor specimens exhibiting LOH are of advanced clinical stages. Loss of imprinting (LOI) was found in 5 of 20 (25%) for IGF-II and in 4 of 17 (23.5%) and 1 of 15 (6.7%) for the RsαI and AluI sites of H19 gene with no LOH. However, no LOH was found in low malignant potential tumors, and only one of them showed LOI in H19 AluI site. Overexpression of IGF-II was demonstrated in all five LOI samples with normal expression of H19. Three of the five tumor specimens exhibiting LOI were transcribed from P1 promoter, whereas the remaining two were from the P3 promoter. These results suggested that LOH of both IGF-II and H19 genes was associated with advanced ovarian cancer. LOI of IGF-II and H19 genes may be involved in the development of ovarian cancer. Transcription of IGF-II from the P1 promoter may account for the biallelic expression of the IGF-II gene.

INTRODUCTION

Epithelial ovarian cancer is a clonal disease that arises from a single cell in more than 90% of cases (1). Multiple genetic alterations must occur during malignant transformation of a single ovarian surface epithelial cell (2). A variety of oncogenes, tumor suppressor genes, and growth factors have been studied in normal and malignant ovarian epithelial cells to identify alterations occurring in cancers from this particular site (3–5). LOH⁴ at 11p15 has been demonstrated in ovarian cancer (6, 7). A few studies have proposed a correlation of 11p LOH with poorly differentiated and more advanced tumors (7). The human IGF-II gene, which is located in chromosome 11p15.5 and encodes a 67-amino acid autocrine growth factor, is highly expressed in various kinds of human malignant tumors, suggesting that IGF-II may act as a second signal in oncogene-induced tumorigenesis (8). In addition, IGF-II was found to be expressed only paternally; it has some effect on proliferation of ovarian epithelial cells when combined with epidermal growth factor and is involved in embryonic growth (2). The H19 gene, located closely downstream of IGF-II, for which no protein product has been detected, is believed to have a tumor suppressor activity in some tumor cell lines (9) and shows only maternal expression (10).

Genomic imprinting, the differential expression of parental alleles of a gene in somatic cells, is considered to play a role in human disease and cancer (11, 12). Thus far, a cluster of imprinting genes has been identified in chromosome 11p15.5, including IGF-II, H19, IPW, and p57Kip2. LOI of the IGF-II and H19 genes has been found in some embryonal and adult human cancers (11–18). Constitutional relaxation of IGF-II genomic imprinting has also been observed in normal kidney and peripheral blood leukocytes of a patient with Wilms’ tumor (13). LOI of IGF-II was found in 40–60% of Wilms’ tumors (11, 13). Also, LOI of both IGF-II and H19 has been identified in 50% (5 of 10) and 17% (2 of 12) of cervical cancers (18). LOI of IGF-II has been proposed to be one of the factors responsible for the abnormal overexpression of IGF-II in both human tumors and experimental animal models (13, 19).

It has been demonstrated that IGF-II gene imprinting is promoter-specific, in that expression from P1 promoter is biallelic, whereas that from the P2-P4 promoter is monoallelic, implying that both transcriptional repression (imprinting) and activation (LOI) can simultaneously occur within a single gene (20). Hypermethylation of promoter 3 in some tumor cell lines shows that activation of IGF-II promoter 1 is linked to LOI, which suggested that activation of the silenced alleles in cancer (LOI) may be due to the activation of promoter 1, which is expressed only in liver with biallelic expression (21).

To further understand the possible role of genomic imprinting in epithelial ovarian cancer, we studied the allelic gene expression of both IGF-II and H19 in ovarian cancer and LMP ovarian tumor. LOI of both IGF-II and H19 was involved in ovarian cancer with overexpression of IGF-II but not of H19.

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³The abbreviations used are: LOH, loss of heterozygosity; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IGF-II, insulin-like growth factor II; LMP, low malignant potential; LOI, loss of imprinting; RT, reverse transcription.
PATIENTS AND METHODS

Characteristics of Patients. Forty-three ovarian cancers and 7 LMP ovarian tumor tissues were obtained at operation in the Department of Obstetrics and Gynecology, Queen Mary Hospital, University of Hong Kong. The tissue was quickly frozen in liquid nitrogen and stored at −80°C until analysis. Adjacent normal tissues, including normal ovary, cervix, endometrium, or lymphocytes, were also obtained from these patients. Histological diagnosis and staging were performed according to the Fédération Internationale des Gynaecologistes et Obstétristes criteria. Among the 43 epithelial ovarian cancer specimens, 11 were of stage I (29.3%), 9 of stage II (27.6%), 15 of stage III (31%), and 6 of stage IV (12.1%). There were 20 serous cystadenocarcinomas, 9 mucinous cystadenocarcinomas, 7 endometrioid carcinomas, and 7 clear cell carcinomas. The histology of the borderline ovarian tumors included five mucinous tumors and two serous tumors. Three benign ovarian cysts and two normal ovaries were also included.

DNA and RNA Extraction and Purification. DNA from cancer samples and normal tissues or lymphocytes were extracted using proteinase K/phenol chloroform protocol. RNA was extracted by TriPure isolation reagent (Roche Molecular Biochemicals) according to the manufacturer’s protocol. One μg of RNA was treated with RNase free-DNase I (Life Technologies, Inc.) for 15 min at room temperature to eliminate contamination by genomic DNA. Ten μg of RNA was treated with RNase-free DNase I and 280°C until analysis.

Identification of Genomic Polymorphism of IGF-II and H19. IGF-II exon 9 Apal polymorphism site and the last two exons of H19 RsaI and AluI polymorphism sites were studied using PCR with 1 μg of total reaction volume. Ten μl of PCR products were digested with Apal, RsaI, or AluI at 37°C for 3 h (10 units of enzyme with 10 μl of PCR product and 2 μl of buffer at a final volume of 20 μl); run through a 3% agarose gel; and visualized with ethidium bromide. The primers used were as follows: for IGF-II Apal, P1F, 5'-CTTGGACCTTTGAGTCCAAATTTG-3’, and P1R, 5'-GGTCTGCGAAATTAATTATCACA-3’; for H19 RsaI, P2F, 5'-TGCTGACCTTTACAACTCCTTGGG-3’, and P2R, 5'-GGTCGGAGCTTCCAGACTAG-3’. The PCR conditions for IGF-II were as follows: DNA was denatured at 94°C for 4 min, amplified by 35 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 1 min) and extension at 72°C for 4 min. Conditions for H19 both RsaI and AluI sites were as follows: 95°C for 2 min, 30 s and 35 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min; extension at 72°C for 7 min.

Allele-specific Gene Expression. One μg of RNA was reverse-transcribed by 200 μl of Moloney murine leukemia virus reverse transcriptase with 200 ng of random primers, 0.5 μM dNTP, 40 units of RNase inhibitor (Promega), 50 μg of Tris-HCl, 75 mM KCl, 3 mM MgCl₂, 10 μM DTT in a total volume of 20 μl for 1 h at 37°C. RT was inactivated by heating for 10 min at 70°C. cDNA (50 ng) was amplified by PCR using primers for IGF-II and H19 RsaI and H19 AluI sites, respectively, under the same conditions used for genomic DNA, except that the number of cycles was increased to 40. The amplified cDNA was also digested with Apal for IGF-II (yielding a 292-bp fragment or 231- and 61-bp fragments) and with RsaI or AluI for H19. Because the amplified area of IGF-II gene did not include an intron, RT-PCR was performed both with and without reverse transcriptase. No PCR products were observed when reverse transcriptase was not added. We used P2F and P2R for the H19 RsaI site and P2F and P3R for the H19 AluI site, both of which included an 80-bp intron. Contamination by genomic DNA can be identified by the size of the DNA bands as well as by carrying out the experiment without reverse transcriptase. For H19 RsaI site, the size of the DNA band is 635 bp for allele a or 497 bp and 138 bp for allele b. However, RNA is 555 bp for the allele A, or 417 bp and 138 bp for the allele B. For the H19 AluI site, the size of the DNA band is 239 bp for allele a and 138 and 101 bp for allele b. RNA is 159 bp for the allele A and 101 bp and 58 bp for the allele B.

Semi-quantitative RT-PCR in LOI Samples and Matched Normal Samples. To determine whether imprinting status affects IGF-II or H19 mRNA levels, semi-quantitative RT-PCR was performed. One μg of total RNA was reverse transcribed, and the amount of cDNA was compared with GAPDH. Primers of GAPDH were as follows: P4F, 5'-CACCACTCTTCCAGGAAGCCGAG-3’; P4R, 5'-TCACGCCCACGTCCCGGA-3’. The cycles were determined by the standard curve amplified from 16, 20, 24, 28, 32, 36, or 40 cycles. Conditions were chosen to give a linear relationship between the amount of amplified product and the input RNA (data not shown). For IGF-II and GAPDH, only 25 cycles were selected, and 30 cycles were performed for H19. Ten μl of PCR product were electrophoresed on a 2% agarose gel and analyzed by UVP Gel Work 1D for Windows. All data were compared with GAPDH.

P1 and P3 Promoter Expression of IGF-II in LOI Samples. Activation of the P1 promoter concurrent with silencing of the P3 promoter through hypermethylation has been observed in several epithelial cancer cell lines, suggesting the promoter-specific regulation of the IGF-II gene (21). To study the role of promoter-specific regulation in the LOI mechanism, we determined promoter-specific expression in the five LOI samples and their matched normal tissues. For IGF-II LOI samples, cDNA were amplified by PCR using standard buffer conditions. For IGF-II P1 promoter-specific PCR, the primers used were as follows: P5F, 5'-GGCCAAGAGCCGCCATCTGCTAGGAG-3’ (located in IGF-II exon 2, which is exclusively transcribed from P1 promoter); P5R, 5'-TGGCCAGTTTCCAGAAGAG-3’ (located in IGF-II exon 9). For IGF-II P3 promoter, the primers used were P6F (5'-TCCTCCCTGGAATTACAGCATGAA-3’; located in IGF-II 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 P4F and P4R for GAPDH as an internal control. All samples were performed twice with or without reverse transcriptase (21).
LOI of IGF-II and H19 in Ovarian Cancer

Table 1  Frequency of LOH and LOI for IGF2 and H19 in 43 ovarian cancers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Frequency, no. (%)</th>
<th>Heterozygous</th>
<th>LOH</th>
<th>LOI</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF2 Apal site</td>
<td>24 (55.8)</td>
<td>4 (16.7)</td>
<td>5 (25)</td>
<td></td>
</tr>
<tr>
<td>H19 Rsal site</td>
<td>20 (46.5)</td>
<td>3 (15)</td>
<td>4 (23.5)</td>
<td></td>
</tr>
<tr>
<td>H19 A1al site</td>
<td>16 (37.2)</td>
<td>1 (6.3)</td>
<td>1 (6.7)</td>
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</table>

*Percentage of LOI in cancer with no LOH.

RESULTS

**LOH of IGF-II and H19 Genes.** Of the 43 cases of ovarian cancer, 24 cases were found to be heterozygous for the Apal polymorphic site of IGF-II gene. LOH was shown in 4 of 24 (16.7%) of the heterozygous tumor specimens. As for the Rsal and A1al polymorphic sites of the H19 gene, LOH was demonstrated in 3 of 20 (15%) and 1 of 16 (6.3%) of the heterozygous cases, respectively. All patients with tumor specimens exhibiting LOH are of advanced clinical stages, i.e., stage III or IV and LOH of all three polymorphic sites of these two genes was found in one stage IIIc serous adenocarcinoma. Of the 24 heterozygous IGF-II, 12 cases were advanced ovarian cancer (stages III and IV). The incidence of LOH in advanced ovarian cancer is 33.3% (4 of 12). No LOH was found in LMP ovarian tumors. LOH of IGF-II might correlate with advanced ovarian cancer (P < 0.05). The results are summarized in Tables 1 and 2.

**LOI of IGF-II and H19 Genes.** Of the 20 informative cases of IGF-II with no LOH in ovarian cancer, LOI was shown in 5 specimens, 4 of which were in early clinical stages. LOI was also found in 4 of 17 (23.5%) and 1 of 15 (6.7%) of the Rsal and A1al sites of the H19 gene, respectively. LOI of both IGF-II and H19 was found in two specimens. One stage Ia1 sample showed LOI in both tumor and matched normal ovarian in the H19 Rsal site. LOI was also identified in the normal cervix of the same patient, and the biallelic expression of H19 (RsaI) in normal tissue was unlikely to be due to contamination from the ovarian carcinoma. There were a total of nine LOI samples from tumor and one in normal ovary. Among them, six were in early stage with different histological diagnosis, and one was a borderline ovarian tumor exhibiting H19 LOI in the A1al site. Data are shown in Tables 1–3 and Figs. 1 and 2. No LOI was found in all tumors with LOH.

**Overexpression of the IGF-II Gene in LOI Samples.** The results of the expression level of both IGF-II and H19 in five LOI samples of IGF-II and six LOI samples of H19 are shown in Fig. 2. The normal and tumor samples showed approximately the same amounts of the RT-PCR products for GAPDH. All five tumors with LOI of IGF-II demonstrated significantly elevated levels of IGF-II mRNA when compared with the normal matched specimens, whereas all five of these tumors showed uniformly low levels of H19 mRNA expression regardless of the imprinting status. The other six tumors with LOI of H19 showed same expression of H19 and IGF-II except the two specimens with both LOI of IGF-II. The results are shown in Fig. 3.

**Activation of P1 Promoter of the IGF-II Gene in LOI Samples.** Three of the five tumors expressed from P1 promoter and lack of expression from P3 promoter (Fig. 4). In contrast, other samples all showed transcription from P3 promoter and lack of expression from P1 promoter. Thus, expression of IGF-II from the nonimprinted P1 promoter may account for some of the biallelic expression the gene observed in ovarian cancer (Fig. 4).

DISCUSSION

Ovarian carcinomas have the highest mortality rate of all gynecological carcinomas (22). It is widely accepted that human neoplasm is the result of a multistep process, in which accumulation of several genetic alterations plays a definite role. In epithelial ovarian neoplasm, these genetic changes are poorly understood. Frequent LOH of 11p15.5 has been demonstrated in borderline ovarian tumors (5, 23). A correlation of 11p LOH with poorly differentiated and more advanced ovarian tumors has been reported (23). Our present study revealed that the frequency of LOH of IGF-II and H19 in ovarian cancer was about 16.7 and 21.3%, respectively; LOH was demonstrated only in advanced diseases, stage III or IV ovarian cancer. The rate of LOH of IGF-II in advanced ovarian cancer was 33.3%. One patient with stage IIIc disease showed LOH in all of the three sites of IGF-II and H19. The remaining LOH tumors included two adenocarcinomas, two endometrioid carcinomas, one mucinous carcinoma, and one clear cell carcinoma. LOH was not detected in any LMP ovarian tumors, benign cysts, or normal ovaries in this study. Our results suggest that LOH of both IGF-II and H19 may be associated with late stage ovarian cancer. The frequency of LOH in cervical cancer reported by Douc-Rasy et al. (18) is similar to our result, about 23 and 14% for IGF-II and H19, respectively.

In the present study, LOI of IGF-II gene was found in 25% (5 of 20) of ovarian cancer with no LOH. The frequency is lower than the report of Kim et al. (24), which showed 54% (6 of 11) LOI. In that study, LOI was also involved in three benign ovarian cysts, suggesting that IGF-II LOI is one of the early event of ovarian carcinogenesis. In our study, LOI occurred in four early stage and one late stage ovarian cancer, including two mucinous, one serous, one endometrioid, and one clear cell carcinoma. This study was in contrary to the report of Yun et al. (16) that only monoallelic expression was found in 11 informative ovarian cancer cases. The frequency of LOI of IGF-II in ovarian cancer is lower than that of other malignant gynecological tumors, such as cervical cancer (Ref. 18; 50%, 5 of 10), and it is similar to that of endome-
trium carcinoma (Ref. 25; 20%, 1 of 5). In our study, LOI of the H19 gene was 30.2% on the RsaI and AluI sites. Two ovarian cancer samples showed LOI in both IGF-II and H19 sites. LOI was identified in one IA1 mucinous cystadenocarcinoma in tumor and both normal ovary and normal cervix of the same patient at the H19 RsaI site. LOI also occurred in one LMP ovarian tumor in the H19 AluI site. Similar findings have been reported in lung cancer (15), brain gliomas (26), and renal cell carcinomas (27). The incidence of H19 LOI in our study is lower than that (62%) in the report of Kim et al. (24). The authors of that study also indicated that LOI was more frequent in malignant epithelial ovarian carcinomas and was associated with advanced stages. The discrepancy between their study and ours is probably due to the differences in tumor cell types and total number in the study. This is the first report that LOI occurred in the LMP ovarian tumor and normal ovary in the H19 gene. From our results, LOI was detected not only in ovarian cancer but also in LMP ovarian tumor and normal ovary. LOI of IGF-II and H19 is probably an early event in ovarian carcinogenesis.

We observed that the expression of IGF-II in LOI samples was higher than that of the matched normal tissues, whereas there was no difference in the H19 expression in both the IGF-II LOI and H19 LOI samples. The findings of the present study are unlike that in Wilms’ tumor, in which LOI of IGF-II is associated with down-regulation of H19 (28, 29). The mechanism by which the genomic imprint is first established and then maintained is not well understood. However, in the case of IGF-II, the expression of a neighboring gene, H19, has been suggested to influence its transcription.

Table 3: Stage, histology, and sites of LOI

<table>
<thead>
<tr>
<th>Stage</th>
<th>Histology</th>
<th>LOI</th>
<th>T48</th>
<th>T32</th>
<th>T21</th>
<th>T45</th>
<th>T61</th>
<th>T37</th>
<th>T58</th>
<th>T17</th>
<th>T38</th>
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<tr>
<td>IGF2</td>
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<td>+</td>
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<td>H19</td>
<td>RsaI</td>
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<td>AluI</td>
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* Mucin, mucinous adenocarcinoma; Endo, endometrioid carcinoma; Serou, serous adenocarcinoma; BorM, borderline and mucinous tumor; NorOvCx, normal ovary and cervix.

Fig. 1 IGF2 LOI in ovarian cancer. Left lane, 50-bp DNA ladder; T, tumor; N, normal; −RT, tumor without RT; Ctr, control. The RT-PCR product was digested with ApaI. The 292- and 231-bp bands are a and b alleles, respectively. All cases demonstrated LOI through biallelic expression in cancer and monoallelic expression in normal tissues.

Fig. 2 H19 LOI in ovarian cancer. Left lane, 100-bp DNA ladder; TD, DNA PCR product in tumor; T, cDNA PCR product in tumor; N, cDNA PCR product in normal tissue; −RT, tumor without RT. PCR and RT-PCR products were digested with RsaI. The DNA bands at 635 and 497 bp are heterozygous a and b alleles, respectively. However, cDNA bands were seen at 555 bp for the A allele and at 417 and 138 bp for the B allele. Samples 32, 37, and 58 demonstrated LOI in tumor sample and imprinting in normal tissues. Sample 48 showed biallelic expression (LOI) in both tumor and normal ovary. Sample 10, used as a control (Ctr) for complete digestion, showed imprinting in tumor and normal tissues.

Fig. 3 Expression of IGF2 and H19 by semiquantitative RT-PCR. Top panel, expression of IGF2 (229 bp) in tumor (T), normal (N), and tumor without RT (−RT). Ctr, control. Middle panel, expression of GAPDH (400 bp). Bottom panel, expression of H19 (555 bp). Overexpression of the IGF2 was found in all LOI samples with normal expression of the H19 gene. The H19 LOI sample T58 did not show overexpression of IGF2.

Fig. 4 Activation of the P1 promoter in the LOI samples. Top panel, P3 promoter expression (500 bp), expression in tumor (T) normal tissue (N), and normal human liver (HL) as a positive control (Ctr). Middle panel, P1 promoter expression (600 bp). Bottom panel, GAPDH expression (400 bp). T32, T45, and T48 showed transcription from the P1 promoter. The remaining tumor and normal tissues are all transcribed from P3 promoter.
by competition for a common enhancer, thereby generating a mutually exclusive and allele-specific pattern of gene expression. Associated changes in CpG methylation in discrete areas of both genes have been implicated in maintenance of the imprint (30). Joyce et al. (30) also reported in sporadic Beckwith-Wiedemann syndrome that IGF-II showed biallelic expression although H19 expression and methylation status were normal. This indicates that there must be an alternative H19-independent pathway by which allele-specific IGF-II expression is established or maintained. Thus, it is possible that the mechanisms of involvement in ovarian cancer may be different from Wilms’ tumor.

It has been demonstrated that IGF-II gene imprinting is promotor-specific, in that expression from P1 promotor is biallelic, whereas that from the P2-P4 promotor is monoallelic, which implies that both transcriptional repression (imprinting) and activation (LOI) can simultaneously occur within a single gene. In the present study, the expression of the IGF-II P1 promotor not P3 was identified in three of the five LOI tumors, and others were all transcribed from P3 promotor. This suggests that activation of the P1 promotor may attribute to the biallelic expression of IGF-II. Issa et al. (21) demonstrated that hypermethylation of the IGF-II gene was associated with low or absent activity of P3 promotor but maintained expression from P1, suggesting that it may contribute to the biallelic expression in cancer cells showing LOI (21). However, not all LOI samples behave in the same way, suggesting that a different mechanism of LOI of IGF-II may exist. Yun et al. (16) suggest that IGF-II gene expression from the maternal P1 promotor can occur without expression from the maternal P3 promotor within the same IGF-II gene. They demonstrated independent allele activation of the promotor-specific transcription from a single cell. Biallelic P1 transcription can occur with monoallelic P2-P4 transcription.

Our present studies revealed that: (a) LOH of both IGF-II and H19 genes, although not frequent, tend to be found in advanced clinical stages of ovarian cancer; (b) LOI of IGF-II and H19 genes may be involved in ovarian carcinogenesis and were found in both LMP and invasive ovarian cancer; and (c) LOI of IGF-II associated with transcription from the P1 promotor may account for the biallelic expression of the IGF-II gene. However, reciprocal regulation of the IGF-II gene and the H19 gene was not found.

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