# Loss of imprinting of IGF2 correlates with hypomethylation of the H19 differentially methylated region in the tumor tissue of colorectal cancer patients

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**Abstract.** Expression of the imprinted genes insulin-like growth factor 2 (IGF2) and H19 depends on the methylation pattern of their differentially methylated region (DMR) located on chromosome 11p15. In the present study, we examined the imprinting status of the IGF2 gene in 120 human colorectal cancer (CRC) patients and 150 normal controls. In addition, we analyzed the DNA methylation of the sixth CTCF-binding site in the DMR of IGF2/H19 in 81 CRC patients using bisulfate sequencing. Of a total of 81 informative (heterozygous) samples, 51 samples showed bialleic IGF2 expression in tumor samples; however, only 15 of 69 informative samples showed loss of imprinting (LOI) of IGF2 in normal controls. Statistically significant differences in the methylation status between the retention of imprinting (ROI) and LOI groups (66.1±14.9 vs. 16.7±9.2, p=0.008) were observed. The results of the present study suggest that LOI of IGF2 is important in the carcinogenesis of CRC. Hypomethylation of the sixth CTCF-binding site in the DMR of IGF2/H19 is linked to LOI and the common IGF2-H19 enhancer competition model for IGF2 imprinting does not apply to human CRC.

## Introduction

By age 70, at least 50% of the Western population is likely to have developed a colorectal tumor and approximately 10% of these individuals progress to malignancies (1). Epidemiological studies suggest that 15% of colorectal cancers (CRC) occur according to a dominantly inherited pattern (2). Recent progress in molecular biology has revealed that several genes are involved in CRC.

Genomic imprinting is a form of gene regulation in which the two parental alleles of a gene are differentially expressed (3). Genomic imprinting is considered an epigenetic phenomenon, as the regulation of gene expression occurs without any change in the DNA sequence. The allele-specific expression of imprinted genes is thought to be based on allele-specific epigenetic modifications, such as cytosine methylation (4) and histone modifications (methylation, acetylation, phosphorylation and ubiquitination) (5,6). Alteration of the normal imprinting status is a common abnormality in embryonic and adult cancers, involving the loss of origin-specific gene expression, in a phenomenon known as loss of imprinting (LOI). LOI is found in various types of adult malignancies including ovarian (7,8), colon (9), lung (10) and bladder cancer (11), as well as chronic myelogenous leukemia (12).

Insulin-like growth factor 2 (IGF2) is a maternally imprinted gene and encodes a fetal peptide hormone regulating cell proliferation and differentiation (13). IGF2 has four promoter regions and promoter 3 is the most active promoter in the fetal liver (14). Pleomorphic adenoma gene 1 encodes a developmentally regulated transcription factor, which positively regulates IGF2 by binding to the promoter 3 region. Although IGF2 is downregulated in normal tissues after birth, except in liver tissues, it is overexpressed in a variety of childhood and adult cancers and serves as a tumor enhancer through autocrine and paracrine mechanisms (15). IGF2 has been studied extensively over the past decade as a key molecule involving CRC and Wilms' tumor (WT) pathogenesis. CTCF (CCCTC-binding factor) is a chromatin insulator with a conserved zinc finger phosphoprotein that shows methylation-sensitive binding to the seven target sites in the H19 differentially methylated region (DMR). The allelic expression of IGF2 is regulated by the methylation status of the sixth CTCF site in the H19 DMR that represents the parental origin of the IGF2 allele; whereas the paternal CTCF 6 allele is methylated, the maternal allele is unmethylated in normal tissues. The methylation status of binding sites of the CTCF in the H19 promoter has been

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suggested as being critical to the regulation of imprinting of the H19/IGF2 locus located on chromosome 11p15.

To obtain a better understanding of the relationship between DNA methylation and imprinting status in CRC, we determined the frequency of LOI between CRC tissues and peripheral blood and correlated these results to allele-specific DNA methylation changes of the sixth CTCF binding site in the H19 DMR.

## Materials and methods

*Materials*. The tumor tissues were obtained from 120 CRC patients in our hospital between January 1, 2009 and December 31, 2010. The study was conducted under the supervision of the Institutional Review Board of Nanjing First Hospital Affiliated to Nanjing Medical University and all the participants provided written informed consent. The patient age ranged from 33 to 83 years, with a median age of 64 years (63 males and 57 females). The tumor was confirmed by pathology and tumor markers such as carcinoembryonic antigen (CEA). For the normal controls, peripheral blood from 150 healthy individuals who underwent a physical examination in our hospital was obtained and all the results showed that they did not have any cancer or chronic inflammation. Of the 150 normal controls, 82 were male and 68 were female, and their age ranged from 34 to 85 years with a median age of 65 years.

*DNA and RNA preparation*. DNA and RNA from CRC patients were isolated from tissues snap-frozen in liquid nitrogen. For DNA, tissues were incubated at 55˚C overnight in a lysis buffer (1% SDS, 150 mM NaCl, 50 mM EDTA, 500 mM Tris-Hcl and 500 $\mu$g/ml proteinase K), followed by extraction with phenol/chloroform and ethanol precipitation. RNA was prepared using the RNeasy Mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. As for the controls, the DNA and RNA of healthy individuals were isolated from the peripheral blood monocytes cells (PBMCs) according to the instructions of the Takara D9081 and R6814 kits, respectively.

*Genotyping of IGF2*. The restriction enzyme *Apa*I was used to genotype the exon 9 polymorphism (rs680) of IGF2 (Fig. 1). The primer sequence for genotyping was: IGF2-*Apa*I, forward: 5'-CTTGG ACTTT GAGT CAAAT TGG-3' and reverse: 5'-GGTCG TGCCA ATTAC ATTTCA-3'. The PCR conditions were as follows: denaturation at 98˚C for 5 min, followed by 94˚C for 1 min, 57˚C for 1 min and 72˚C for 1 min for 35 cycles, and elongation at 72˚C for 5 min. The PCR product was digested by *Apa*I at 37˚C for 24 h and then electrophoresed on a 2% agarose gel to detect the polymorphism of the IGF2 gene. Samples which had two bands (292 and 231 bp) between 200 and 300 bp were heterozygous for IGF2. Only the samples with heterozygous IGF2 were appropriate for our further study.

*Allelic expression analysis of IGF2*. To evaluate the allele-specific expression of IGF2, reverse transcription-PCR (RT-PCR) and restriction digestion by *Apa*I was performed for the informative samples. Total RNA was treated with DNase I (Takara D2270A) prior to the reverse transcription reaction to avoid genomic DNA contamination. The primer sequences were the same as for the genotyping analysis of IGF2. Following RT-PCR, the product was digested by *Apa*I at 37˚C for 24 h and electrophoresed on a 2% agarose gel. The samples that had two bands (292 and 231 bp) between 200 and 300 bp were confirmed as LOI of IGF2; however, retention of the imprinting (ROI) of IGF2 only had one band.

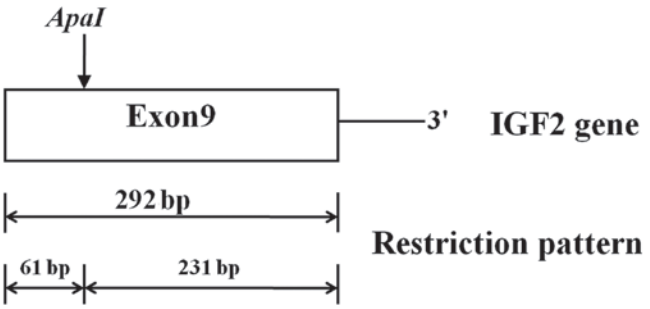


Figure 1. Map of the 3' end of the IGF2 gene and the restriction pattern of PCR product digested by *Apa*I. IGF2, insulin-like growth factor 2.

*Methylation analysis of the sixth CTCF binding site at H19 DMR*. Genomic DNA from 81 informative samples was treated with bisulfite (EpiTect Plus DNA Bisulfite kit 59124) to convert unmethylated cytosines to uracils, whereas methylated cytosines were unaffected. Bisulfite-treated DNA was subsequently amplified using the following primers: forward: 5'-TGGGTATTTTTGGAGGTTTTTTT-3' and reverse 5'-ATA AATATCCTATTCCCAAATAA-3' (16). These primers allowed the amplification of the methylated and the unmethylated alleles by spanning a region with 18 differentially methylated CpGs. The region corresponded to GenBank nucleotides 7881-8100 (accession no. AF125183). The PCR conditions were as follows: denaturation at 95˚C for 15 min, followed by 95˚C for 30 sec, annealing at 57˚C for 30 sec and 72˚C for 20 sec, respectively, for 50 cycles, and elongation at 72˚C for 5 min. Each PCR product (5 $\mu$l) was analyzed on a 2% agarose gel and the remaining 20 $\mu$l was purified using a QIAquick purification kit (Qiagen) to be sequenced using the forward primer. The sequencing results were analyzed using CpG viewer, a software tool for DNA methylation available by free download from http://dna.leeds.ac.uk/cpgviewer/download.php.

*Correlation analysis of LOI and CpG island methylation of H19 DMR with clinical variables*. To determine whether LOI correlates with certain clinical variables, including age, pathology stage, tumor size and CEA value, we performed stratification to clarify the problem. The clinical variables were potential effect factors for CRC. A correlation analysis between the CpG island methylation of H19 DMR with the above clinical variables was also conducted. A CEA value of 5.0 and a 5.0 cm tumor size were set as the standard value.

*Statistical analysis*. Data were presented as the mean ± SE (standard error). $P \leq 0.05$ was considered statistically significant.

## Results

*Imprinting status of IGF2*. In total, 81 of the 120 CRC patients were heterozygous for IGF2 and were appropriate for our

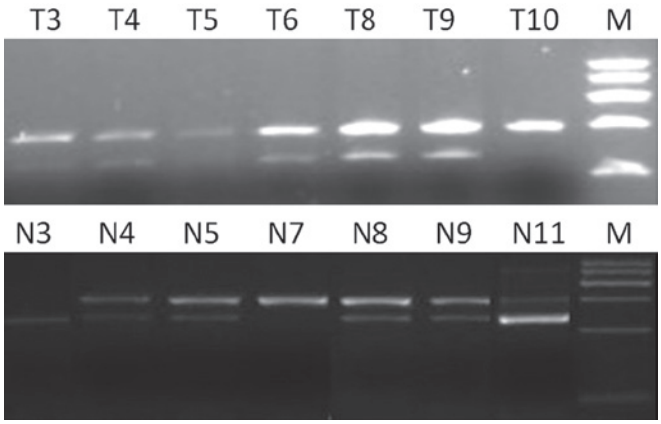


Figure 2. Genomic DNA was amplified to detect the *Apa*I restriction fragment length polymorphism. The PCR product was digested by *Apa*I to genotype the IGF2 exon 9 polymorphism. Samples with two bands were heterozygous. T, tumor tissues; N, normal peripheral blood; M, marker (MD101, Tiangen); IGF2, insulin-like growth factor 2.

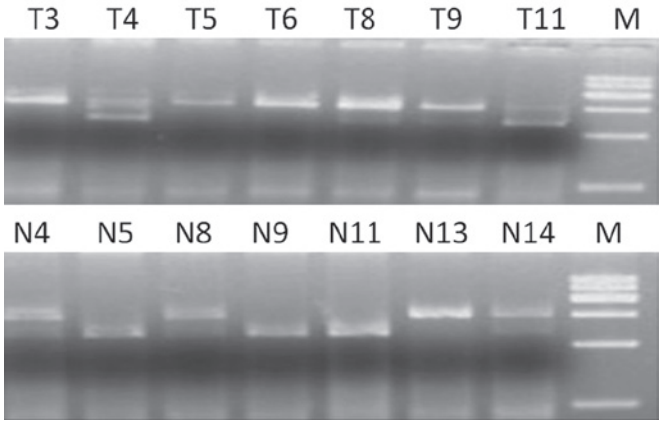


Figure 3. Complementary DNA was prepared to determine the allele-specific IGF2 expression. RT-PCR was carried out on samples that were confirmed to be heterozygous for IGF2. The products were digested by *Apa*I to determine IGF2 imprinting. The samples with two bands showed loss of imprinting. T, tumor tissues; N, normal peripheral blood; M, marker (MD101, Tiangen); IGF2, insulin-like growth factor 2.

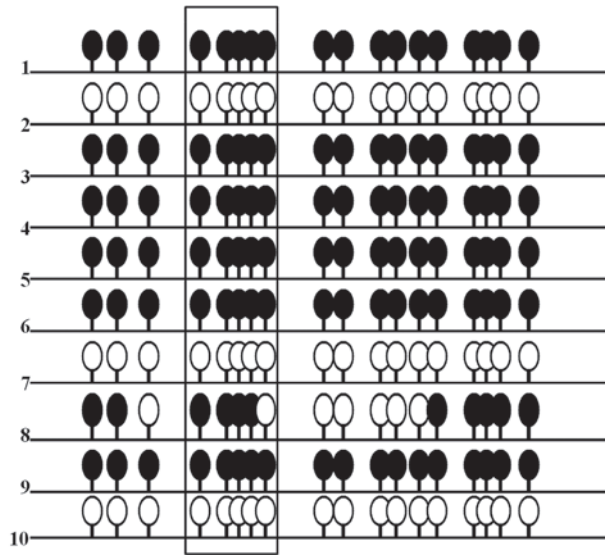


Figure 4. The methylation of CpG islands in ROI samples is shown. CpG position located in H19 differentially methylated regions corresponding to GenBank nucleotides 7881-8100 (accession no. AF125183). Each line is a separate ROI sample. Black circles show the methylated CpG sites; white circles show the unmethylated CpG sites. Boxed area is the imprinting control region, the core CTCF-binding site domain. ROI, retention of imprinting.

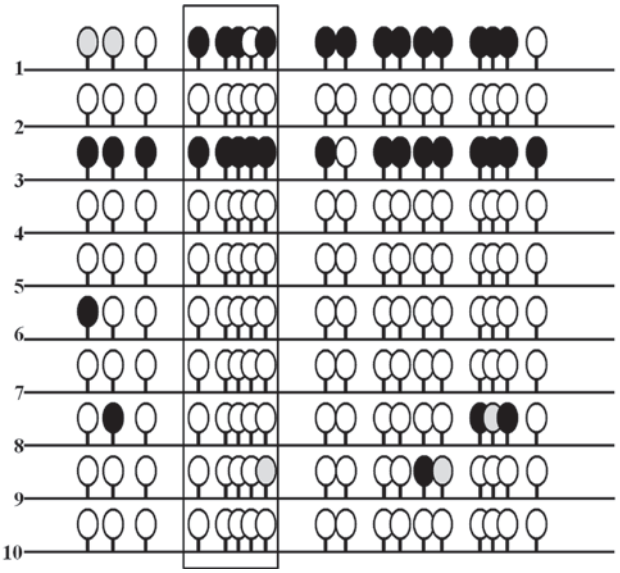


Figure 5. The methylation of CpG islands in LOI samples is shown. CpG position located in H19 differentially methylated regions corresponding to GenBank nucleotides 7881-8100 (accession no. AF125183). Each line shows a separate LOI sample. Black circles show the methylated CpG sites; white circles show the unmethylated CpG sites; grey circles show the non-CpG position where there is a CpG in the genomic sequence. Boxed area is the imprinting control region, the core CTCF-binding site domain. LOI, loss of imprinting.

study, and 69 of 150 IGF2 genes from the PBMCs of normal controls were appropriate (Fig. 2). A total of 51 of 81 informative samples (63%) were found to have LOI in tumors, while 15 of 69 (21.7%) were found to have LOI in the normal control group (Fig. 3), indicating that there was a significant difference ($p<0.01$) in the frequency of LOI.

*Methylation status of the sixth CTCF binding site at H19 DMR.* ROI (n=30) and LOI samples (n=51) were analyzed for methylation of the DMR region containing 18 CpG islands. ROI samples exhibited normal CpG island methylation (Fig. 4). Seventeen of 30 samples exhibited hypermethylation, where all CpGs were methylated. In contrast, LOI samples exhibited hypomethylation of CpG islands (Fig. 5) and none of the 51 LOI samples were completely methylated. A significant difference was observed between ROI and LOI samples (66.1±14.9 vs. 16.7±9.2, $p=0.008$). Moreover, we observed differences in the methylation status between ROI and LOI samples in the imprinting control region (ICR), which contained a core binding domain for the methylation-specific enhancer CTCF. The percentage of methylated CpGs in the ICR of the ROI group was 4-fold higher than that of the LOI group.

Table I. The correlation analysis of LOI with clinical variables.

| Variables | LOI/samples | p-value |
|---|---|---|
| Age (years) | | |
| >70 | 12/25 | 0.062 |
| ≤70 | 39/56 | |
| Stage | | |
| T1-T2 | 17/31 | 0.233 |
| T3-T4 | 34/50 | |
| CEA | | |
| >5.0 | 11/16 | 0.593 |
| ≤5.0 | 40/65 | |
| Tumor size (cm) | | |
| >5.0 | 15/23 | 0.791 |
| ≤5.0 | 36/58 | |

LOI, loss of imprinting; CEA, carcinoembryonic antigen.

Table II. The correlation analysis of CpG island methylation with clinical variables.

| Variables | Number of cases | Mean ± SE (18 CpGs) | p-value |
|---|---|---|---|
| Age (years) | | | |
| >70 | 25 | 14.21±10.88 | 0.023 |
| ≤70 | 56 | 33.77±13.97 | |
| Stage | | | |
| T1-T2 | 31 | 30.96±13.06 | 0.606 |
| T3-T4 | 50 | 28.49±15.73 | |
| CEA | | | |
| >5.0 | 16 | 22.8±12.97 | 0.393 |
| ≤5.0 | 65 | 28.58±12.25 | |
| Tumor size (cm) | | | |
| >5.0 | 23 | 23.21±10.28 | 0.920 |
| ≤5.0 | 58 | 25.32±14.23 | |

CEA, carcinoembryonic antigen; SE, standard error.

*Correlation of LOI and CpG island methylation of H19 DMR with clinical variables*. There were no correlations between IGF2 LOI and clinical variables in CRC patients ($p<0.05$) (Table I). IGF2 LOI had no correlation with age ($p=0.062$), pathology stage ($p=0.233$), CEA value ($p=0.593$) or tumor size ($p=0.791$), respectively. We found a correlation between CpG island methylation and age ($p=0.023$) (Table II).

## Discussion

Genomic imprinting is the silencing of one copy of an autosomal gene while the other copy is expressed (17). This allele-specific expression is regulated by epigenetic modifications, such as DNA methylation. Imprinting occurs primarily by allelic-specific methylation of cytosines in areas of DNA that are rich in CpG islands (18). IGF2 and H19 are two oppositely expressed genes, located adjacent to each other at 11p15.5, that share the same transcription regulatory epigenetic mechanisms (19). In the majority of human tissues the imprinting of IGF2 depends on a DMR that is located upstream of H19 promoters (20). Aberrant DNA methylation that is capable of modifying imprinted gene expression may provide an attractive mechanism linking other causes to CRC.

Firstly, we examined the imprinting status of the IGF2 gene. In total, 51 of 81 informative samples (63%) were found to have LOI in tumors. Certain studies (21-23) reported a 44, 33 and 38% frequency of LOI in CRC patients, respectively. Our result was higher than findings from the abovementioned studies, possibly due to the large sample size. A significant difference ($p<0.01$) was observed in the frequency of LOI between the tumor tissue of CRC patients and normal controls, indicating that LOI of IGF2 is important in the carcinogenesis of CRC. Thus, LOI of IGF2 may be a manifestation or biomarker of CRC (24).

Secondly, we conducted DNA methylation analysis of the sixth CTCF-binding site. This result may show that in primary human CRC, LOI is asssociated with hypomethylation of the sixth CTCF-binding site at H19 DMR. Our result is consistent with other reports (9,11), whose results showed that LOI of IGF2 is linked to hypomethylation of H19 DMR. However, our results are in contrast to the findings of other studies (25-27), which reported that hypermethylation of the H19 DMR correlated with LOI of IGF2. This result suggests a mechanism for the regulation of IGF2 imprinting independent of enhancer competition. The enhancer competition model demonstrates that IGF2 and H19 promoters compete on the same chromosome for a shared enhancer, and access of the maternal IGF2 allele to this enhancer is blocked by the H19 DMR when unmethylated, likely due to the insulator activity of CTCF binding to the unmethylated H19 DMR (28-32). Thus, our result showed that the common IGF2-H19 enhancer competition model for IGF2 imprinting does not apply to human CRC.

Thirdly, we carried out a correlation analysis of LOI and CpG island methylation with clinical variables, respectively. No correlations were found for IGF2 LOI with any of the clinical variables, indicating that the incidence of LOI is an early event in cancer progression. We found a correlation between CpG island methylation and age, showing that the methylation decreased with increasing age. This finding again confirmed that aberrant DNA methylation is an important factor in the carcinogenesis of CRC.

Future studies may address the correlation between the methylation status and its regulation mechanism. Understanding DNA methylation/demethylation and histone modifications in the CTCF-binding domain of imprinted genes in more detail is likely to shed light on carcinogenesis and may aid in improving the prognosis and therapy of CRC.

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