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Loss of imprinting and marked gene elevation are two forms of aberrant IGF2 expression in colorectal cancer

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Abstract

Loss of imprinting (LOI) of *IGF2* is a common event in many cancers and typically activates the maternally silenced allele. The resulting biallelic *IGF2* expression correlates strongly with the hypomethylation of a differentially methylated region (DMR) near its promoter. It has also been shown that *IGF2* undergoes overexpression in human malignancies; nevertheless, this phenomenon and its link to aberrant DMR methylation has not been reported in colorectal cancer (CRC). The aim of this study was to determine the relationship between *IGF2* LOI, overexpression and DMR hypomethylation in CRC. By analyzing *IGF2* and *H19* methylation in 97 primary CRC and 64 matched normal colorectal tissues, we have shown a significant correlation between *IGF2* LOI and DMR hypomethylation of *IGF2* and *H19*. Additionally, when analyzing Affymetrix expression data of 167 primary CRC tumor and 32 normal tissues, 15% of tumors showed marked *IGF2* elevation. We further investigated if substantially elevated *IGF2* levels were linked to *IGF2* or *H19* hypomethylation, but found no significant correlation. However, we demonstrated that noticeable *IGF2* overexpression, rather than LOI, negatively correlated with CRC microsatellite instability. These observations indicate that *IGF2* expression, particularly when transcribed at significantly high levels, is a result of mechanisms unrelated to LOI. Our results suggest that *IGF2* participates in CRC tumorigenesis through two different forms of aberrant gene expression.

Keywords

CpG methylation; LDR; colorectal cancer; *IGF2*; imprinting

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INTRODUCTION

Epigenetic alternations describe heritable changes in gene function that is not reflective of changes in the DNA sequence. Altered genomic imprinting and aberrant DNA methylation are two examples of epigenetic events that are hallmarks of human cancers. An imprinted gene is typically expressed in a monoallelic fashion from one of the parental alleles, whereas loss of imprinting (LOI) describes mechanisms that either activates the normally silenced allele or inactivates the normally active gene copy.

LOI was initially discovered at the insulin-like growth factor 2 (*IGF2*) locus of Wilms tumor (WT) and has subsequently been reported to be present in a wide range of human malignancies[1, 2]. *IGF2* is paternally expressed and is an important regulator of cell growth and apoptosis. LOI of *IGF2* is generally manifested by the activation of the normally silenced maternal allele with the subsequent expression of both gene copies[3]. The biallelic *IGF2* expression also correlates with aberrant *IGF2* / *H19* methylation. Studies have shown that *IGF2* LOI is linked to the hypomethylation of a differentially methylated region (DMR) close to the *IGF2* promoter, as well as linked to hypermethylation of a DMR upstream of the reciprocally imprinted *H19* gene[4–9]. The *H19* DMR contains six zinc finger CCCTC transcription factor (CTCF) binding sites (CBS) where their methylation levels reciprocally correlate with *IGF2* DMR in various cancers, including kidney, ovarian, bladder and lung[1, 2, 10–14]. In a normal imprinting state, CTCF binding on the hypomethylated *H19* DMR prevents a downstream enhancer from accessing the promoter of silenced maternal *IGF2* allele. However, LOI of *IGF2* is believed to occur through *H19* DMR hypermethylation and the alleviation of CTCF shielding results in the enhancer mediated biallelic expression [11, 15–17].

In CRC, LOI of *IGF2* occurs in 30% of tumors and 10% of non-cancerous tissues[18]. This phenomenon in CRC was originally thought to share the same enhancer competition model as described above[6]. However, Cui and colleagues have shown that *IGF2* and *H19* DMRs in CRC do not follow a reciprocal methylation pattern, suggesting LOI of *IGF2* in CRC associated with *H19* and *IGF2* DMR hypomethylation[4]. These observations suggest that the enhancer competition model of *IGF2* LOI may not apply to CRC, and that aberrant *IGF2* promoter methylation is more likely to play a critical role in LOI mediated *IGF2* expression.

The expression level of *IGF2* in which the tumor has undergone LOI at this locus is typically 2–3 times higher than in corresponding normal tissues[19]. However, *IGF2* overexpression at tumor-to-normal ratios of 10 times or higher is not unusual in human maligancies[5, 20]. Highly elevated *IGF2* expression is commonly seen in ovarian cancer and correlated with hypermethylation of CTCF binding site at *H19* DMR[5]. Using quantitative methods to study epigenetic and genetic abnormalities, we were interested in determining whether significant *IGF2* overexpression occurs in CRC and if it is related to LOI and other genetic abnormalities. We also investigated if aberrant *IGF2*/*H19* DMR methylation may explain these types of altered *IGF2* expression in CRC.

MATERIAL AND METHODS

Study population

A detailed description of the study population was published previously[21]. Briefly, it included patients who presented at Memorial Sloan-Kettering Cancer Center (MSKCC) with a colonic neoplasm between 1992 and 2004. Location of the primary colon tumor varied and included right, left, and rectal cancers. Tumor samples were spread among stages I–IV (AJCC criteria) in 17%, 24%, 24% and 35%, respectively. Ages ranged from 17 years old to 86 years old with a median age of 65. Biological specimens used in the study included

colorectal adenomas, primary colon adenocarcinomas and corresponding normal mucosa. All tissues were procured at the time of surgical resection and stored for future use under institutional IRB approved protocols.

Identification of *IGF2* polymorphism and LOI status

Genomic DNAs extracted from CRC specimen were genotyped for two SNPs (820 G/A, refSNP ID: rs680 and 266 C/T, refSNP ID: rs2230949) located in *IGF2* exon 9 using direct sequencing[22]. For all the tumor samples identified with heterozygous alleles, genomic DNAs from matched adjacent normal mucosas were also sequenced. Cases were scored as informative when the heterozygosity at ploymorphic sites presented in both tumor and matched normal tissues. All primer sequences and reaction conditions for Taqman assay were reported previously[22]. For the analysis of *IGF2* LOI status, total RNA from frozen tissues was extracted and RT-PCR was carried out to amplify the *IGF2* exon 9 region bearing SNP 820 G/A for LDR analysis. Complementary DNA (cDNA) was synthesized from 1μg total RNA using Multiscribe Reverse Transcriptase. A negative control reaction was prepared in parallel without reverse transcriptase to ensure the elimination of genomic DNA in cDNA synthesis. PCR was performed using the following protocol: 94 °C for 10 min, 35 cycles of 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 1 min, followed by a final extension step at 72 °C for 3 min. Inactivation of the polymerase was achieved by a thermocycled proteinase K reaction. The LDR was conducted in a final volume of 20 μl consisting of 20 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 100 mM KCl, 10 mM DTT, 1 mM NAD, 500 – 800 fmol each of the LDR primers, 40U of Taq DNA Ligase, and 2 μl of PCR amplicon. The reaction mixture was thermocycled with the following program: 94° C for 1.5 min followed by 20 cycles of 94° C for 1 min and 65° C for 4 min. The fluorescence intensity of LDR products was analyzed using ABI 3730 capillary DNA analyzer (Applied Biosystems, Foster City, CA). The sequences of PCR and LDR primers were shown in the Supplementary Table 1.

IGF2 and *H19* DMR methylation status

DNA methylation analysis using bisulfite/PCR/LDR/Universal Array has been described previously[23]. Briefly, genomic DNAs were bisulfite treated using EZ DNA methylation kit (Zymo Research, CA). This method was found to yield greater than 99% C to U conversion. Two segments of the *IGF2* DMR located upstream of exon 3, one segment each of *H19* CBS1 and CBS6 DMRs were subsequently multiplex PCR amplified. Three CpG sites were analyzed for each amplified fragment. LDR was also conducted in a multiplex fashion allowing all 36 LDR primers interrogating 12 CpG sites in one reaction. The final LDR products were resolved and displayed on a Universal Array. The raw methylation levels of the interrogated cytosines were calculated by the ratio of Cy3/(Cy3+Cy5). The sequences of PCR and LDR primers are shown in the Supplementary Table 1.

Determination of KRAS, BRAF mutation and MSI status

Methods to measure DNA mutations and MSI status were performed as previously reported[21, 24]. Briefly, *KRAS* and *BRAF* mutations were detected using PCR/LDR approaches. Fluorescently labeled LDR primers were designed to detect the 7 common *KRAS* (Val, Asp, Ala, Arg, Ser, and Cys at codon 12 and Asp at codon 13) and *BRAF* V600E mutations. For MSI study, fluorescently labeled oligonucleotide primers were designed to analyze microsatellite loci of BAT25, BAT26, D2S123, D5S346, and D17S250. MSI was scored as present when at least two of the five markers showed size instability.

Statistical analysis

The COPA analysis was performed using a R package[25]. Statistical comparisons were performed by either Student's t-test or Wilcoxon signed-rank test. The correlation studies were performed by computing the chi-square or Fisher's exact (two-sided) tests of the n × m contingency tables. P values of less than 0.05 were considered significant. Multiple testing correction was performed using the Benjamini-Hochberg method to find those tests with a false discovery rate (FDR) of at most 5%.

RESULTS

IGF2 and *H19* were hypomethylated in CRC

We measured DNA methylation status in the CBS1 and CBS6 regions of *H19* and the imprinting control region of *IGF2* using a quantitative bisulfite/PCR/LDR/Universal Array assay (Figure 1A and 1B)[23]. This approach provides unbiased amplification of a given genomic region with both methylated and unmethylated DNA sequences. Loci of *IGF2* and *H19* DMRs were amplified and the methylation levels of a total of 12 CpG sites were analyzed. As shown in Figure 1C and Supplementary Figure 1, the calibration curves for each CpG methylation level were established by mixing synthetic DNA templates with methylated and unmethylated sequences in the ratios of 0:5, 1:4, 2:3, 3:2, 4:1 and 5:0 followed by bisulfite conversion, PCR amplification and LDR/Universal microarray analysis. The linearity of these curves demonstrates the quantitative ability of our assay. The overall methylation status of a DMR was determined by averaging the methylation level of individual cytosines detected at that locus. By equally weighting the methylation levels of the queried cytosines, the overall DMR or promoter methylation status of a candidate gene may be represented by multiple CpG sites across a relatively larger region of DNA sequences. The cytosine methylation level of each interrogated CG dinucleotide was also confirmed using bisulfite sequencing in a subset of samples (data not shown).

The methylation status of *IGF2* and *H19* DMRs was profiled in 97 primary colorectal tumors and 64 matched normal colonic tissues (Figure 2). In all three DMRs, methylation levels were significantly lower in the tumors compared to the adjacent normal tissues ($p<0.0001$) (Figures 2 and 3). One of the CG dinucleotide (CpG 1) in *H19*CBS1 appeared to be hypomethylated in the majority of tumor and normal tissues. To validate the bislufite/PCR/LDR/Universal array results, we performed bisulfite sequencing in four tumor and four normal colorectal DNA samples (Supplementary Figure 2). In general, the sequencing data indicate that CG sites at *IGF2* and *H19* DMRs in tumors underwent hypomethylation, while sites in normal tissues remain hemimethylated. For CG sites measured at *H19* CBS1, relatively low level (around 20%) methylation was seen at site 1 in both normal and tumor samples. The majority of CG sites at *H19* CBS1 have medium level methylation (30–60%) in both tissues, nevertheless, higher methylation was found in normal tissues than in tumors with statistically significant differences. The bisulfite sequencing data are consistent with bisulfite/PCR/LDR/Universal array results and support our previous study suggesting that the overall promoter methylation status of a gene is optimally determined by averaging multiple cytosine methylation levels rather than basing methylation status on a single cytosine[21]. The *IGF2* DMR exhibited average methylation levels of 0.25±0.085 and 0.35±0.07 in the tumor and normal tissues, respectively. Hypomethylation was scored (<0.21) when the average methylation level of a given sample was two standard deviations (2SDs) below the mean of normal tissue methylation levels. Based on this criterion, 35% (n=34) tumors and 8% of normal tissues showed *IGF2* hypomethylation, which is consistent with a previous report indicating comparable percentages of *IGF2* LOI in tumor and normal colonic tissue[18]. Similarly, the average methylation levels were 0.31±0.065 and 0.34±0.048 at *H19*CBS1, and were 0.28±0.073 and 0.36±0.064 at *H19*CBS6 in the tumor and normal

tissues, respectively. Thus, hypomethylation at CBS1 and CBS6 was found in 14% (n=14) and 21% (n=20) of CRC when scored using the 2SDs cut-off. Over 75% of tumor samples with *H19*CBS1 or *H19*CBS6 hypomethylation were also hypomethylated at the *IGF2* DMR (p=0.0004 and 0.0001, respectively). This observation suggests that the methylation at *H19* and *IGF2* DMRs in CRC did not follow a reciprocal imprinting pattern.

Hypomethylation of *IGF2* / *H19* correlated with *IGF2* LOI status in CRC

To study *IGF2* LOI, the heterozygous or informative samples were identified by analyzing a genomic polymorphism in *IGF2* exon 9[22]. A total of 38 informative cases were identified and the *IGF2* allelic expression levels were measured using LDR. Biallelic expression of *IGF2* was defined as the fluorescence intensity ratio of LDR products less than 3:1 between the more-abundant and less-abundant alleles. Of the 38 analyzed cases, 24 of them exhibited *IGF2* hypomethylation and biallelic gene expression (Table 1, p<0.0001). Correlation between *H19* hypomethylation and *IGF2* LOI was also seen at the CBS1 and CBS6 loci (Table 1, p<0.05). Only two of the hypomethylated *IGF2* samples showed allelically imbalanced expression. Our data is consistent with previous reports suggesting that *IGF2* and *H19* promoter hypomethylation correlated with *IGF2* LOI[4].

IGF2 overexpression correlated with microsatellite instability but not with its promoter hypomethylation

To determine if substantially elevated *IGF2* is a common event in CRC, the *IGF2* expression profiles of 167 primary colorectal tumors and 32 matched adjacent normal colonic tissues were obtained using Affymetrix HG-U133A2 array[26]. Tumor-specific *IGF2* expression was seen in the signals of probe sets 202410_x_at and 210881_s_at (p=0.029 and 0.0017, respectively, Figure 4). The microarray-based expression results were subsequently confirmed using real-time reverse transcription-PCR assay (Supplementary Figure 3). Histograms of these data revealed *IGF2* RNA levels skewed to the right, suggesting that the tumor-specific *IGF2* expression was heavily influenced by outlier samples with expression levels greater than 1.5 fold of the interquartil range (>6.4). By this criterion, 23 colorectal tumors (13.4%) were determined as having noticeable, outlier *IGF2* overexpression.

IGF2 overexpression has been shown to associate with uniparental disomy (UPD) at *IGF2* locus in Wilms tumors[27]. It is interesting to investigate if a similar correlation may exist in CRC. We have previously performed genome-wide copy number variation (CNV) study in a subset of colorectal tumors, which allows us to determine copy number neutral LOH or paternal UPD at 11p15 (*IGF2* locus)[21]. Among the 86 tumors in which the *IGF2* expression and methylation data were analyzed, CNV were measured in 37 samples using Affymetrix 50K SNP array (Figure 5 and Supplementary Figure 4). Among them, paternal UPD at 11p15 was revealed in only three tumor samples. Although, all three tumors possessed *IGF2* DMR hypomethylation and duplication of the hypomethylated allele, none of them associated with marked *IGF2* overexpression.

We further investigated the association between significantly elevated *IGF2* expression, promoter hypomethylation and some common genetic alterations in CRC. As shown in figure 5, among the 86 tumors where the gene expression and methylation data were both available, only five samples exhibited marked *IGF2* elevation and promoter hypomethylation (p=0.20). *IGF2* overexpression did not correlate with either *H19* DMRs hypomethylation (p=0.64 for CBS1 and 0.27 for CBS6), KRAS (p=0.43) or BRAF mutations (p=0.32). Nevertheless, a significant negative correlation was found between samples of markedly elevated *IGF2* expression and microsatellite instability (MSI) phenotype (p=0.031, Figure 5). These results indicate that marked *IGF2* elevation did not

arise through *IGF2* and *H19* promoter hypomethylation, but instead may involve other oncogenic event such as chromosomal instability.

DISCUSSIONS

Three probe sets were fabricated on Affymetrix HG-U133A2 array to study *IGF2* transcription levels. Our data indicated that CRC-specific *IGF2* expression was detectable by two probe sets (202410_x_at and 210881_s_at) located in the *IGF2* coding region and capable of capturing multiple *IGF2* isoform transcripts. However, the third probe set (202409_at) that hybridizes to loci near the end of *IGF2* 3' UTR did not reveal signals of tumor-specific expression (Supplementary Figure 5) and was not employed in the data analysis. It is worth noting that *IGF2* transcription may be controlled by several promoters, including a biallelically expressed P1 promoter and the imprinted P2-P4 promoters[28]. To assure our study primarily focused on measuring *IGF2* imprinting rather than P1 derived transcription, the determination of *IGF2* methylation status was performed at exon 3 and the analysis of allelic expression ratio was assayed according to a SNP at exon 9[4, 22].

Using quantitative assays, we have shown that the LOI of *IGF2* significantly correlated with *IGF2* and *H19* DMR hypomethylation in CRC. This mechanism is unique to CRC and differs from the *H19* DMR hypermethylation observed in other cancer types[4]. In fact, hypermethylation of *H19* CBS1 and CBS6 was not observed in our study and the biallelic *IGF2* expression correlated only with *H19* CBS hypomethylation. These results suggest that the model of *IGF2*/*H19* competing for a common downstream enhancer may not be entirely applicable to CRC. Moreover, although *IGF2* promoter hypomethylation resulted in LOI, neither *IGF2* nor *H19* aberrant methylation correlated with the substantially elevated *IGF2* expression. The observation that noticeable *IGF2* overexpression was unlikely to occur in MSI or tumors with a near-diploid genome, supporting the notion that aneuploidy or chromosomal instability may cause significantly elevated *IGF2* expression.

Gene fusion due to chromosomal translocation has been reported in several type of human cancers[29]. A well-known example is the *BCR-ABL* fusion protein in chronic myelogenous leukemia where the *ABL* proto-oncogene was activated by the 5' activation region of *BCR*[30, 31]. Recently, gene fusion has also been identified in epithelial tumors[32]. In prostate cancer, fusion between *TMPRSS2* and oncogenomic *ETS* family transcription factors (*ERG* or *ETV1*) has been found in many instances (>50%), accompanied with high *ETS* gene expression. This phenomenon has been reasoned that oncogenes driving tumorigenesis are drastically activated by the fusion of a strong promoter element translocated from other genes upstream genomic region. Applicable methods of cancer outlier profile analysis (COPA) were developed to identify chromosomal rearrangement events and their corresponding overexpressed candidates[25, 32]. We analyzed the gene expression profiles of 167 primary CRC and 32 matched normal tissues using the open source COPA package[25]. This method was developed on the assumption that gene translocation may involve the fusion of an activating domain to multiple downstream candidates, nevertheless only one fusion event or the highest activated candidate is likely to be found in a given tumor sample. Namely, the fusion induced gene activation is likely to be seen in a mutually exclusive fashion in a sample if there were multiple fusion products from a same activating domain. Unexpectedly, our result indicates that *IGF2* was ranked the first in the outlier analysis (Supplementary Figure 6). This finding suggests that the substantially elevated *IGF2* expression may involve a previously uncharacterized molecular event. This preliminary data may potentially explain why the highly expressed *IGF2* does not correlate with its DMR hypomethylation.

Our study of CRC did not show a correlation between *IGF2* UPD and marked overexpression like those observed in Wilms tumors. The discrepancy may be explained in several ways. First, the correlation of *IGF2* UPD and overexpression may exist in a tumor type specific manner. *WT1* is an *IGF2* repressor and its mutation facilitates *IGF2* up-regulation. Since paternal UPD duplicates the expressed *IGF2* allele and is commonly found in Wilms tumors with a *WT1* mutation, it has been suggested that *IGF2* overexpression results from a *WT1* abnormality and *IGF2* paternal UPD. However, despite a *WT1* mutation that occurs in 15–25% of Wilms tumors, it has not been reported in CRC. Additionally, UPD at 11p15 is neither a frequent event in colorectal tumors (relative to marked *IGF2* overexpression). A similar example of tumor-specific abnormality is the presence of reciprocal methylation patterns between *IGF2* and *H19* DMRs in Wilms tumors, but not in CRC. Thus, one may not expect to see the correlation between *IGF2* UPD and overexpression in CRC. Second, change of *IGF2* expression in CRC may involve mechanisms other than LOI. An example is the discovery of a long-range interchromosomal association between one allele of *IGF2* imprinting control region and *Wsb1/f1* gene, mediated by a transcription factor CTCF[33]. The participation of cis-acting elements and trans-acting factors may explain the alteration of transcription levels at these loci. It is likely that the colocalization of looped chromosomes bring regulatory elements such as enhancers to allow mutual influence of transcription from separated genes[34–36]. Alternatively, multiple gene expression may be activated by shared transcription factors in which the confined chromosomal geometry permits a transcription machinery interacting with several juxtaposed promoter regions[37, 38]. These examples are consistent with our preliminary result suggesting that chromosomal instability, including gene fusion, might be a mechanism for marked *IGF2* overexpression. Third, UPD at *IGF2* locus may not be adequately identified using 50k SNP array. The interrogated SNPs in Affymetrix 50k array has a mean spacing of 47kb, which does not support a detailed mapping of chromosomal aberrations at *IGF2* locus spanning 20.5kb region. To avoid the underestimation of *IGF2* UPD in tumors, platforms with higher resolution (e.g. a mean spacing at 5kb) will be employed in future studies to accurately identify short genomic sequence alterations at the *IGF2* locus.

A recent study demonstrated that LOI of *IGF2* enhances *IGF2* signaling by increasing the proliferation-related gene expression (*Akt/PKB* signaling)[39]. Interestingly, the signaling enhancement does not correlate with the increase of overall *IGF2* levels. Markedly sustained *Akt* activation was seen in LOI cells treated with a low dose of *IGF2*, compared to those treated with four-fold higher *IGF2* dosage. The detection of up-regulated *IGF2* signaling components, *IGF1* and insulin receptors (*igf1r* and *insr*) in LOI cells provided further evidence indicating that hypersensitivity of the signaling pathway, rather than *IGF2* levels, accounts for the proliferation-related gene activation of *Akt/PKB* signaling. These observations suggest a plausible mechanism of *IGF2* LOI involvement in early tumor development. The strong biological impact of LOI in responding to low *IGF2* has been reasoned to facilitate the proliferation of tumorigenic cells at early cancer stages in which the density of *IGF2* producing cells was low. While the above study suggested that increasing *IGF2* level has little impact on *IGF2* signaling, it is also likely that the marked *IGF2* overexpression (typically expressed at 10-fold or greater, than the average CRC *IGF2* level), especially in non-LOI tumor cells, may affect tumorigenesis through mechanisms other than LOI enhanced signal sensitivity, including the interaction with *H19* gene, transcription factor mediated interchromosomal association, and other signaling pathways[33, 40].

Clinically and pathologically it has been suggested that the *IGF2* LOI is linked to MSI tumors[41, 42]. Since adult MSI patients have a favorable prognosis, the correlation between LOI and MSI may represent a valuable clinical application of *IGF2* as a biomarker. However, in a similar study performed by Sasaki et al. using 95 informative *IGF2* LOI, no

significant correlation was found between LOI and MSI[43]. The authors argued that those earlier reports were conducted in a relatively small sample size or patients were recruited with a bias that resulted in a high prevalence of MSI cases (30–40%). In contrast, the number of MSI cases in our study cohort is comparable with a typical MSI prevalence (15%) in sporadic CRC. Our data showed that genetic abnormalities (e.g. MSI and *KRAS/BRAF* mutations) in CRC did not correlate with either *IGF2* LOI or DMR hypomethylation. The results are consistent with those reported by Sasaki et al. indicating no significant correlation between MSI and biallelic *IGF2* expression. The fact that *IGF2* LOI was not linked to MSI may represent a unique opportunity to subclassify colorectal carcinogenesis. We conclude that the aberrant *IGF2* expression in CRC consists of at least LOI and other mechanisms resulting in elevated *IGF2* expression.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

CRC	colorectal cancer
DMR	differentially methylated region
IGF2	insulin-like growth factor 2
LDR	ligase detection reaction
LOI	Loss of imprinting
MSI	microsatellite instability

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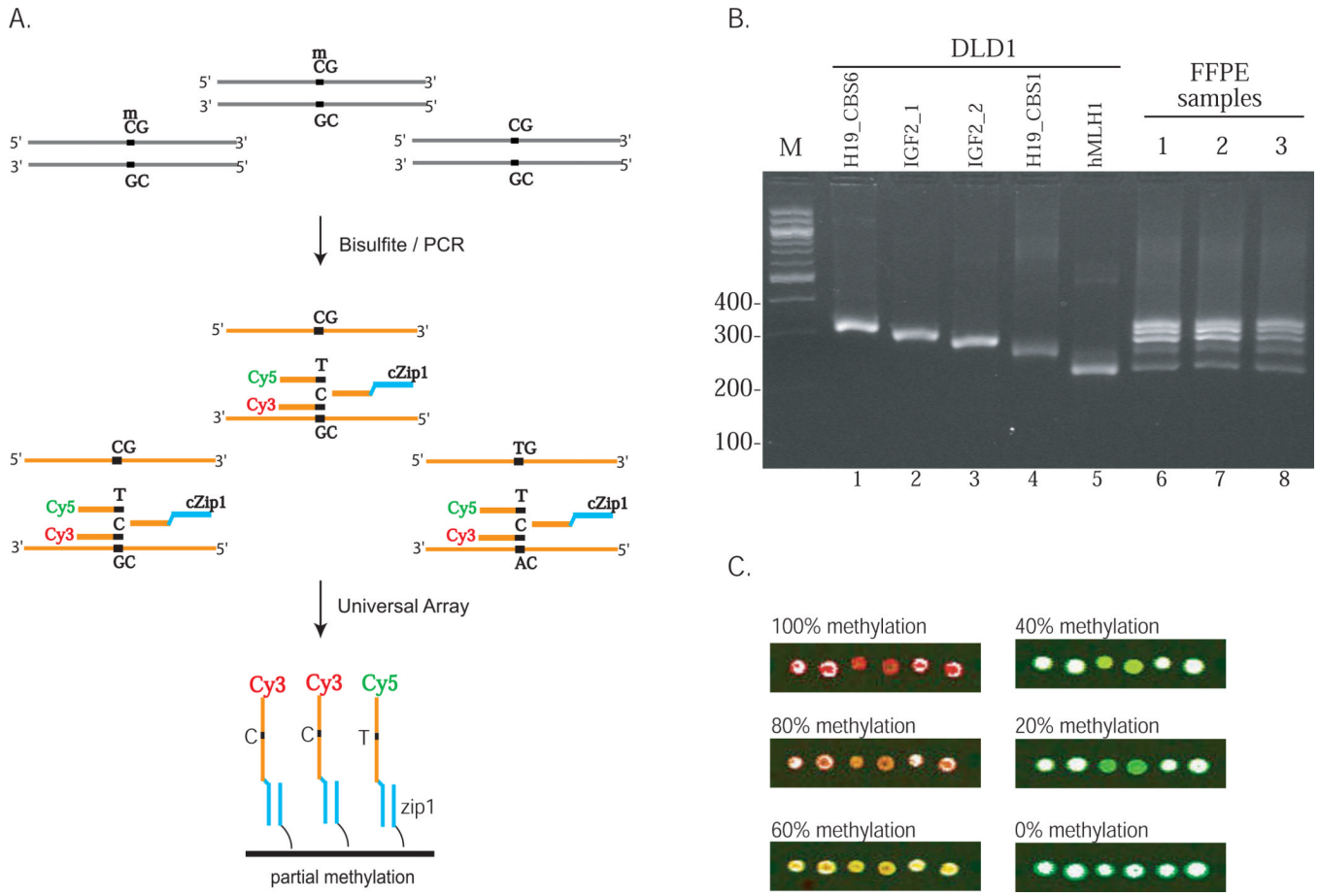

Figure 1.

Quantitative analysis of aberrant DNA methylation. (A) Schematic diagram of the bisulfite/PCR/LDR/Universal Array assay. The diagram depicts a hypothetical scenario in which the methylation levels of three copies of the same genomic locus were analyzed. After bisulfite treatment, only top-strand DNAs were PCR amplified and shown. Three LDR primers were designed to analyze the methylation status of each cytosine. Two upstream primers were fluorescently labeled and the downstream primer was tagged with a zipcode complementary sequence. LDR products with the same complementary zipcode tags were captured onto a specific array address. LDR/Universal Array is able to identify the 2:1 ratio of the methylated vs. unmethylated DNA fragments. (B) Multiplex amplification of the interrogated genomic loci. Genomic DNAs extracted from CRC cell line DLD1 (lanes 1–5) and formalin-fixed, paraffin-embedded (FFPE) tissues (lanes 6–8) were PCR amplified after bisulfite treatment and resolved on an agarose gel. The primer and assay designs were suitable for multiplex analysis of genomic DNAs extracted from FFPE tissues. The *hMLH1* was shown for the demonstration of multiplex PCR and was not further investigated in this study. (C) Examples of the quantitative determination of *IGF2* DMR methylation. Different amount of synthetic methylated and unmethylated DNAs were mixed and subjected to bisulfite/PCR/LDR/Universal Array analysis. The red-green color scale represents the percentage of methylation levels at each CpG dinucleotide from high to low determined by the fluorescence intensity ratio of LDR products[23]. The zipcode oligos corresponding to each interrogated CpG sites were double spotted to increase the detection accuracy.

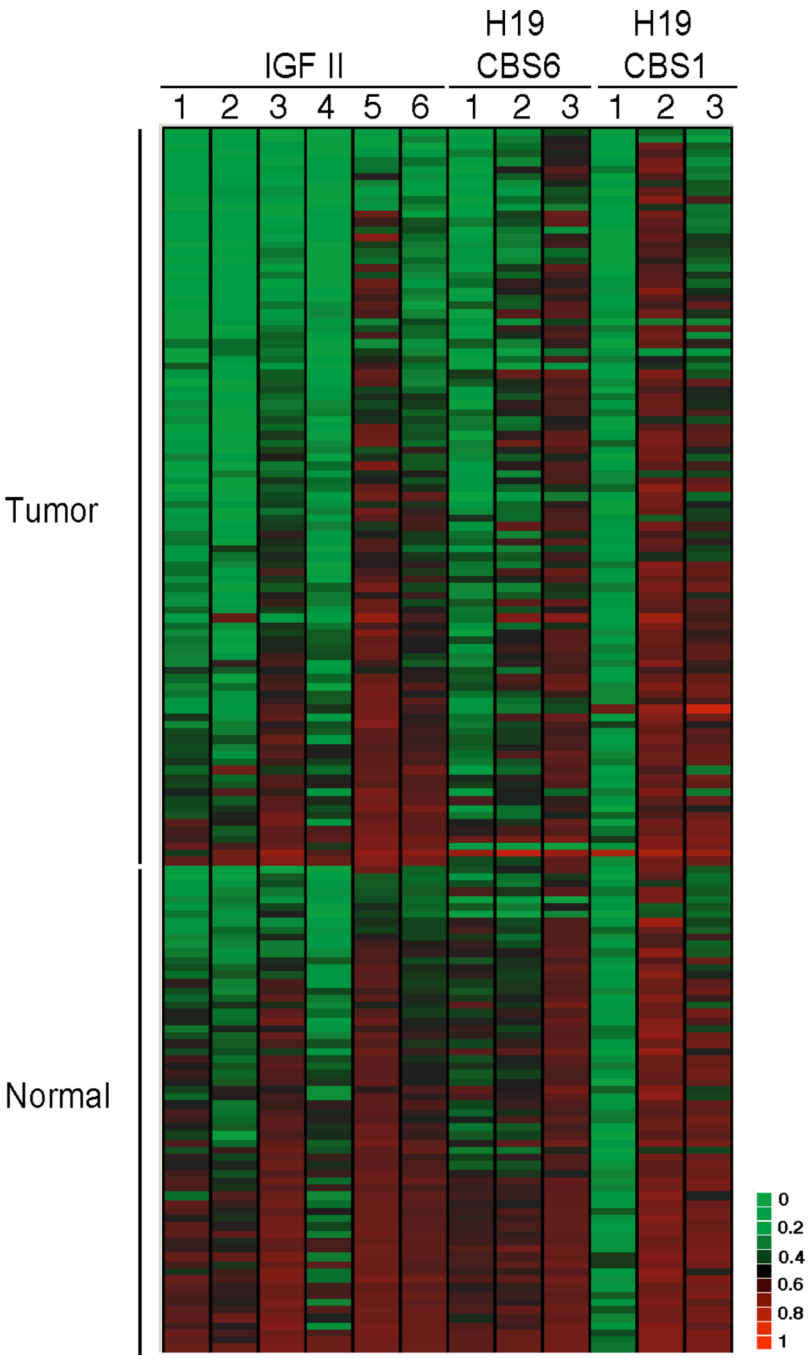

Figure 2.

The cytosine methylation levels of 97 primary CRC and 64 matched normal mucosas were profiled using bisulfite/PCR/LDR/Universal Array assay. Six, three and three CpG sites at the *IGF2* DMR, *H19*CBS1 and *H19*CBS6 were analyzed, respectively. A heat map diagram depictes the percentage of methylation at each CpG dinucleotide site. The methylation levels of these sites were calibrated using the standard curve shown in supplementary figure 1. Matched normal tissues were used as controls to calculate the statistical significance of tumor-specific aberrant methylation at each locus.

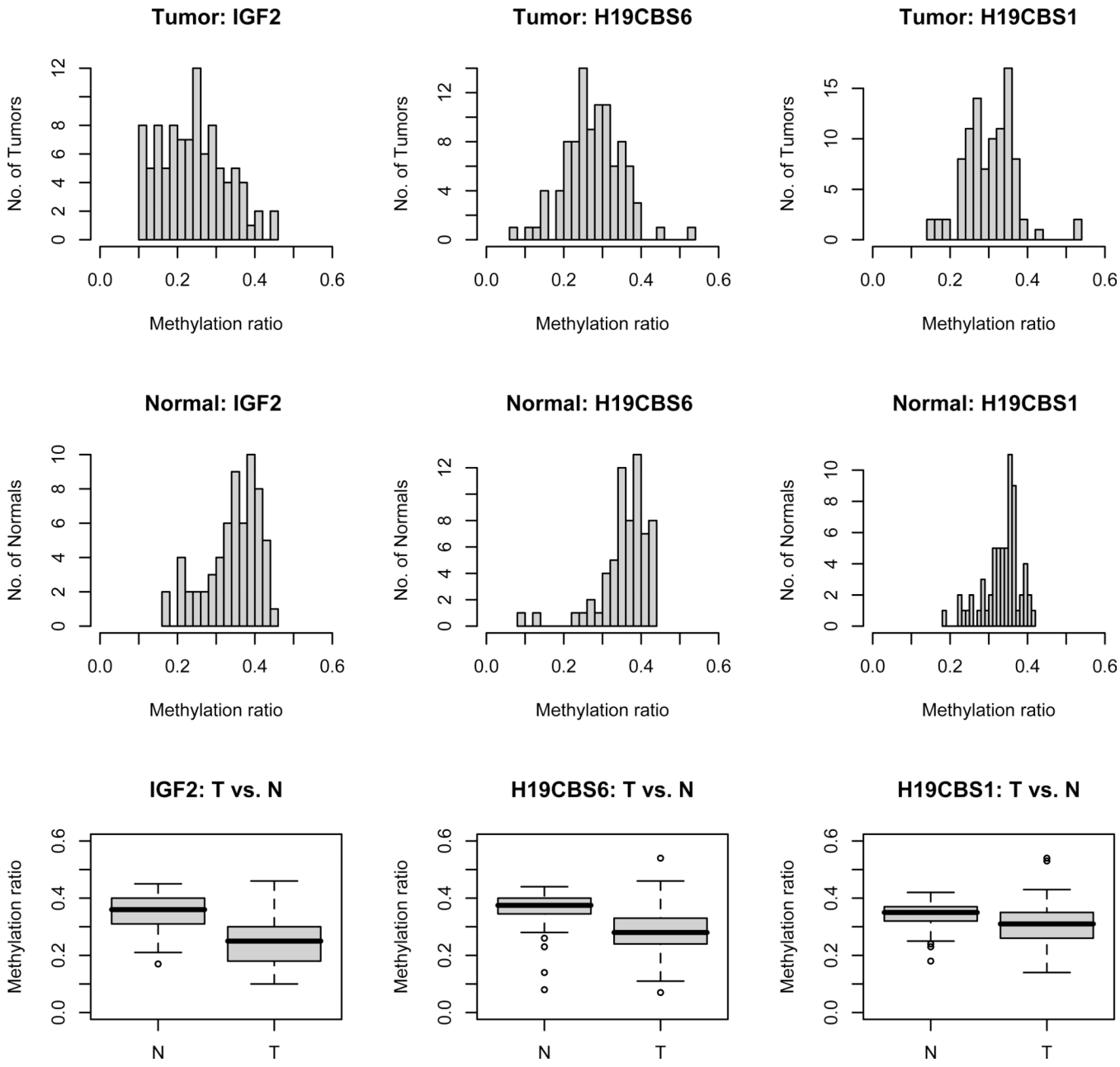

Figure 3.

The distributions of *IGF2* and *H19* DMR methylation status in 97 primary CRC and 64 matched normal colorectal tissues. The overall promoter methylation status of *IGF2*, *H19*CBS1 and *H19*CBS6 in each sample was determined by averaging the methylation levels of six, three and three interrogated cytosines in that particular locus, respectively. The Student's t-test was conducted to determine the significance of differential methylation between tumor and normal tissues ($p<0.0001$ at all three loci). T: tumor and N: normal tissues.

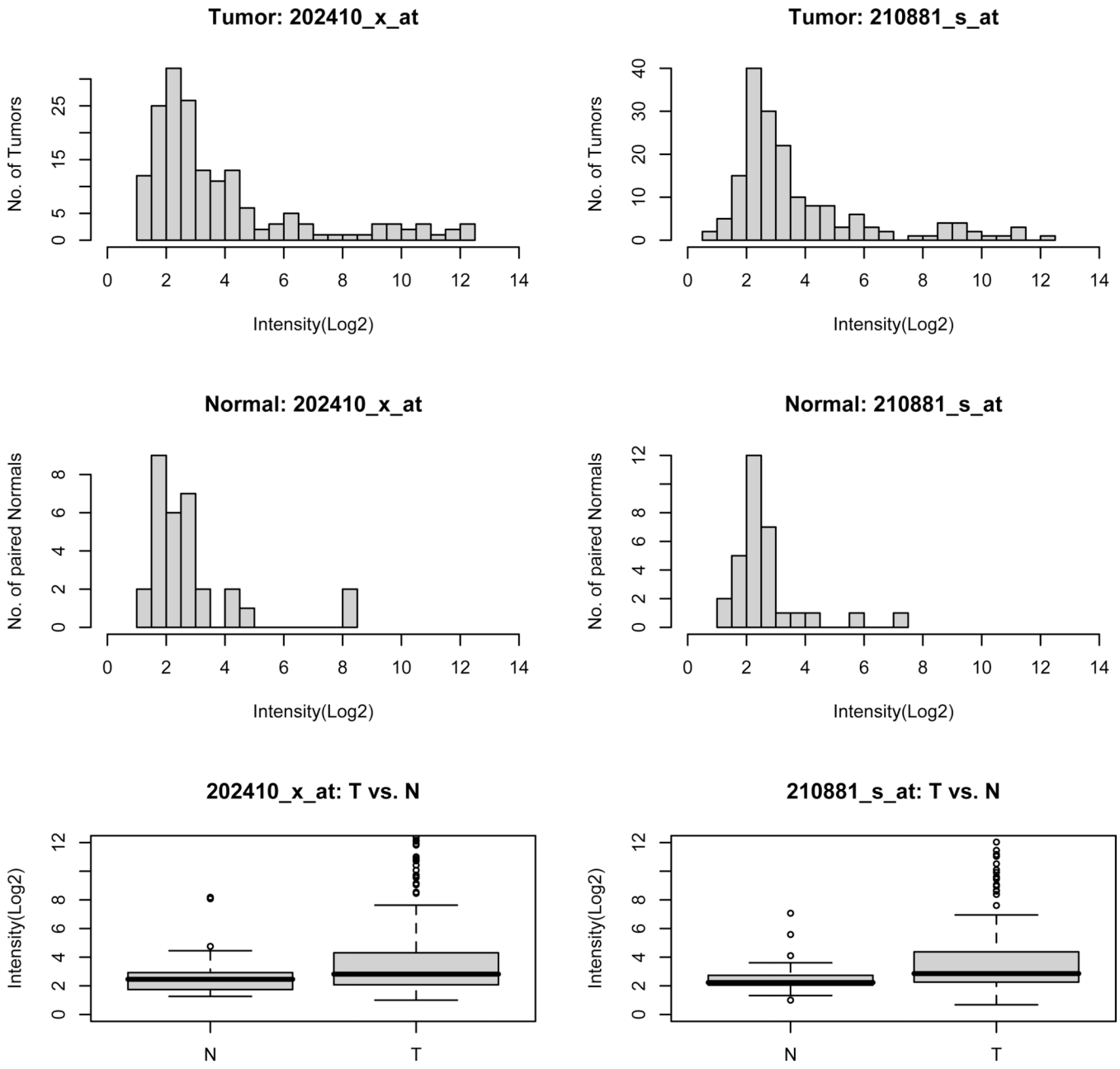

Figure 4.

The distribution of *IGF2* gene expression in 167 primary CRC tumors and 32 matched normal colorectal tissues. The *IGF2* transcription level of each sample was measured by two corresponding probe sets on Affymetrix HG-U133A2 array. The Wilcoxon signed-rank test was conducted to determine the significance of differential *IGF2* transcription between tumor and normal tissues (p=0.029 for 202410_x_at and 0.0017 for 210881_s_at). T: tumor and N: normal tissues.

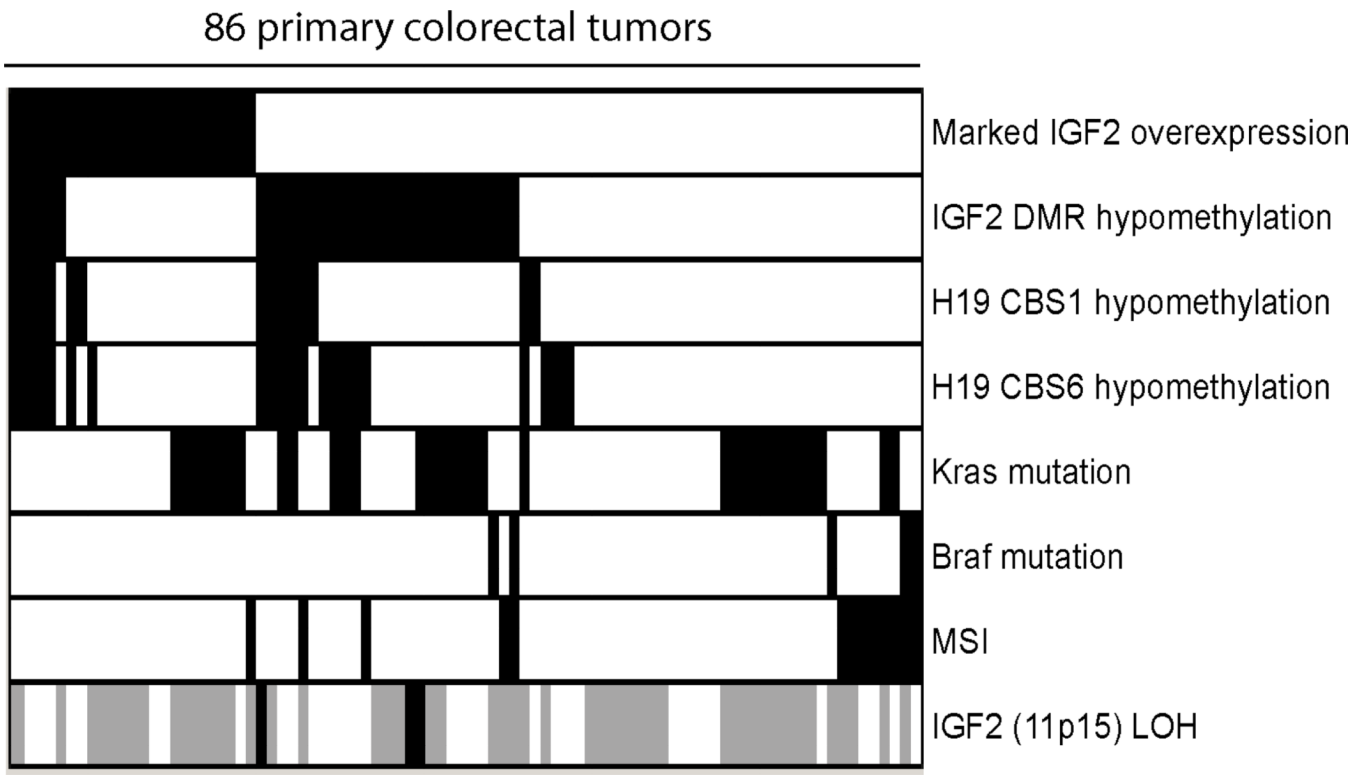

Figure 5.

A dichotomous heat map of *IGF2*/*H19* methylation, *IGF2* expression, selected mutation and genomic instability status in 86 primary CRC. The *IGF2* overexpression status was determined by averaging signals from two probe sets (202410_x_at and 210881_s_at) and scored by the criteria described in the result section. For each DMR, the scoring of hypomethylation was determined by the two standard deviation criteria described in the result section. The MSI, *BRAF* and *KRAS* mutation statuses, and copy number neutral LOH at *IGF2* locus were also determined for each tumor sample. The alignment of each tumor was maintained across. The presence of noticeable *IGF2* overexpression, DMR hypomethylation, MSI, *BRAF* and *KRAS* mutations, and copy number neutral *IGF2* LOH were indicated in black. The presence of normal *IGF2* expression, DMR hemimethylation, MSS, *BRAF* and *KRAS* wild-type alleles, and *IGF2* locus not undergone copy number neutral LOH were indicated in white. Tumor samples lack of SNP array experimental results were indicated in gray.

Table 1

The relationship between *IGF2* expression and the DMR methylation status.

Sample ID	*IGF2* LOI	Methylation status			marked *IGF2* elevation	MSI
		IGF2	*H19*CBS1	*H19*CBS6		
1	Yes	Half	Half	Half	No	No
2	Yes	Half	Half	Half	No	No
3	Yes	Hypo	Half	Hypo	No	No
4	Yes	Hypo	Half	Hypo	No	No
5	Yes	Hypo	Half	Hypo	No	No
6	Yes	Hypo	Half	Half	No	Yes
7	Yes	Hypo	Half	Half	No	Yes
8	Yes	Hypo	Half	Half	No	Yes
9	Yes	Hypo	Half	Hypo	No	No
10	Yes	Hypo	Hypo	Hypo	No	No
11	Yes	Hypo	Half	Hypo	No	No
12	Yes	Hypo	Half	Half	No	No
13	Yes	Hypo	Half	Hypo	No	No
14	Yes	Hypo	Hypo	Hypo	No	No
15	Yes	Hypo	Hypo	Hypo	No	No
16	Yes	Hypo	Hypo	Hypo	No	No
17	Yes	Hypo	Hypo	Hypo	No	No
18	Yes	Hypo	Hypo	Hypo	No	No
19	Yes	Hypo	Hypo	Hypo	No	No
20	Yes	Hypo	Hypo	Hypo	No	No
21	Yes	Hypo	Hypo	Hypo	No	No
22	Yes	Hypo	Hypo	Hypo	No	No
23	Yes	Hypo	Hypo	Hypo	No	No
24	Yes	Hypo	Hypo	Half	Yes	No
25	Yes	Hypo	Half	Half	Yes	No
26	Yes	Hypo	Half	Half	Yes	No

Sample ID	*IGF2* LOI	Methylation status			marked *IGF2* elevation	MSI
		IGF2	*H19*CBS1	*H19*CBS6		
27	No	Half	Hypo	Half	No	No
28	No	Half	Half	Half	No	Yes
29	No	Half	Half	Half	No	Yes
30	No	Half	Half	Half	No	No
31	No	Half	Half	Half	No	No
32	No	Half	Half	Half	No	No
33	No	Half	Half	Half	No	No
34	No	Half	Half	Half	No	No
35	No	Hypo	Half	Hypo	No	No
36	No	Hypo	Half	Hypo	No	No
37	No	Half	Half	Hypo	Yes	No
38	No	Half	Half	Half	Yes	No

Summary		*IGF2* DMR methylation		*H19*CBS1 DMR methylation		*H19*CBS6 DMR methylation		marked *IGF2* elevation		MSI	
		hypo	hemi	hypo	hemi	hypo	hemi	yes	no	yes	no
*IGF2*LOI	yes	24	2	12	14	17	9	3	23	3	23
	no	2	10	1	11	3	9	2	10	2	10
		p=0.000008		**p=0.03**		**p=0.035**		p=0.643		p=0.643	