

Loss of imprinting in IGF2 in colorectal carcinoma assessed by microdissection

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Abstract. To ascertain the implications of loss of imprinting (LOI) of the insulin-like growth factor II gene (IGF2) for carcinogenesis, the precise frequency of LOI in colorectal carcinoma was examined using a laser capture microdissection method, and compared to the matched normal colorectal mucosa. LOI was examined by PCR-restriction fragment length polymorphism in combination with direct sequencing. The status was assigned as imprinting when PCR-RFLP showed only one band or sequence with a single peak, otherwise cases were classified as LOI. LOI was found in 13 of 24 informative cases of carcinoma (54%), which was higher than the ratios reported previously. LOI was also found in the normal colorectal mucosae in 14 cases (58%). The LOIs in carcinomas and in the normal mucosae were closely correlated: 10 of 13 LOI-positive carcinomas showed LOI in the matched normal mucosae. These results suggest that LOI of IGF2 in colorectal carcinoma and LOI in the background mucosa play important roles in carcinogenesis.

Introduction

The insulin-like growth factor II gene (IGF2) is imprinted genetically, and normally only the paternal allele is expressed. IGF2 is expressed at a high level in the fetus, and plays an important role in fetal growth and development, but its expression is down-regulated after birth (1-3). When it occurs in the germ cell, loss of imprinting (LOI) of the IGF2 causes overexpression of IGF2, leading to Beckwith-Wiedemann syndrome, in which macroglossia and splanchnomegaly appear (4). As LOI of IGF2 is advantageous for cell proliferation, it has been suggested that LOI in somatic cells is involved in

tumorigenesis (1-3,5). It has been reported that LOI of IGF2 occurs at high frequency in a wide variety of carcinomas, including colorectal carcinomas, gastric carcinomas, esophageal carcinomas, and hepatocellular carcinomas, although the reported incidence of LOI in these tumors shows considerable variation (6-13). LOI of IGF2 seems to play a unique role in tumorigenesis in colorectal carcinoma. LOI in the normal mucosa has been reported to be a potential marker of colorectal cancer risk, as the incidence of LOI in the normal mucosa with colorectal carcinoma is 3-fold higher than that in normal mucosa (14,15).

It is necessary to determine the precise incidence of LOI to ascertain its implications in carcinogenesis, but this is hampered by a number of problems. First, carcinoma tissues resected surgically contain normal cells, including lymphocytes, fibroblasts, and endothelial cells, which have the normal genome distinct from that of carcinoma cells, and it is extremely difficult to obtain the pure carcinoma genome from carcinoma tissues by surgical resection. Due to contamination, the reported LOI should be less frequent than predicted. The laser capture microdissection (LCM) method has been developed to allow pure carcinoma cells to be obtained more easily and precisely (16). Using LCM, loss of heterozygosity (LOH) of tumor suppressor genes in carcinoma cells has been investigated and the frequency determined using this method was higher than those obtained by analysis of whole tissue (17,18). Similarly, normal cells can be eliminated using LCM to analyze LOI precisely. However, the frequency of LOI using LCM has not been reported previously. Second, by PCR-restriction fragment length polymorphism (RFLP), a commonly used approach for quantifying imprinted alleles that distinguishes the *ApaI* polymorphic site, the presence of heteroduplexes in the PCR products cannot be recognized correctly by the endonuclease (19), and methods other than PCR-RFLP are necessary for correct assessment of LOI. DNA sequencing could be helpful for identifying LOI, as it has been used to detect a single sequence or heterogeneous sequences in a PCR product although it is poorly suited for quantification (19).

Here, we examine the precise incidence of LOI in colorectal carcinoma and matched normal colorectal mucosa; our results indicated higher frequencies of LOI in colorectal carcinoma and the matched normal mucosa than reported previously.

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Table I. Primer pairs and restriction endonuclease used for the semi-nested PCR-RFLP.

Primer pairs	Sequence	Restriction endonuclease	Fragment size (bp) total (digest)
1st run			
sense	5'-CTTGGACTTTGAGTCAAATTGG-3'	<i>ApaI</i>	292 (231+61)
antisense	5'-GGTCGTGCCAATTACATTTCA-3'		
2nd run			
sense	5'-CTTGGACTTTGAGTCAAATTGG-3'	<i>ApaI</i>	257 (196+61)
antisense	5'-GGTTTTTCATGCTCTGTCCTC-3'		

Materials and methods

Materials. Tissue samples were obtained from 53 patients with colorectal carcinoma. Portions of carcinoma tissues and non-tumor mucosa were cut out and frozen immediately after surgery at Kanazawa University Cancer Research Hospital. The tissues were stored at -80°C until analysis. All patients gave informed consent before sample collection according to our institutional guidelines.

PCR-RFLP. Genomic DNA from normal tissues was extracted using a DNA isolator PS kit (Wako, Osaka, Japan) in accordance with the manufacturer's recommendations. Total RNA was extracted from the normal or carcinoma tissues by the acid guanidinium phenol chloroform method (20), and contaminating DNA was digested by DNase I (Takara, Tokyo, Japan). Aliquots of 1 µg of extracted total RNA in a volume of 20 µl were converted to cDNA using AMV reverse transcriptase (Promega, Madison, WI). Reverse transcription reaction was performed for 30 min at 42°C and then heated for 5 min at 99°C to inactivate the enzyme. Genomic DNA and cDNA were amplified by the PCR method with the primer pairs for exon 9 of human IGF2 (10) listed in Table I. The PCR product was re-amplified by semi-nested PCR with the same sense primer and the other antisense primer for microdissected specimens (Table I). As the amplified area was restricted to the exon, fragments of the same length were amplified from genomic DNA and cDNA. Digestion of contaminating genomic DNA by DNase I was confirmed by PCR to amplify the exon-intron spanning region of the integrin β5 gene (21). Analysis of *ApaI* polymorphism in the IGF2 gene was then performed. The PCR products were digested with *ApaI* (Toyobo, Osaka, Japan) for 3 h at 37°C and run on 2% agarose gels containing ethidium bromide. The electrophoresis patterns appeared as three bands for heterozygotes, where the restriction enzyme recognition sequence was present in one allele and absent in the other. Digital images of the bands scanned into a computer were analyzed using NIH image.

Laser capture microdissection. Colorectal tissues were frozen and embedded in OCT compound (Tissue-tech, Torrance, USA). Sections 10-µm thick were cut and fixed with 70% ethanol on silanized slides (Dako Japan, Kyoto, Japan) for 10 min. A laser capture microdissection system (LCM, LM100, Olympus, Tokyo, Japan) was used to obtain individual

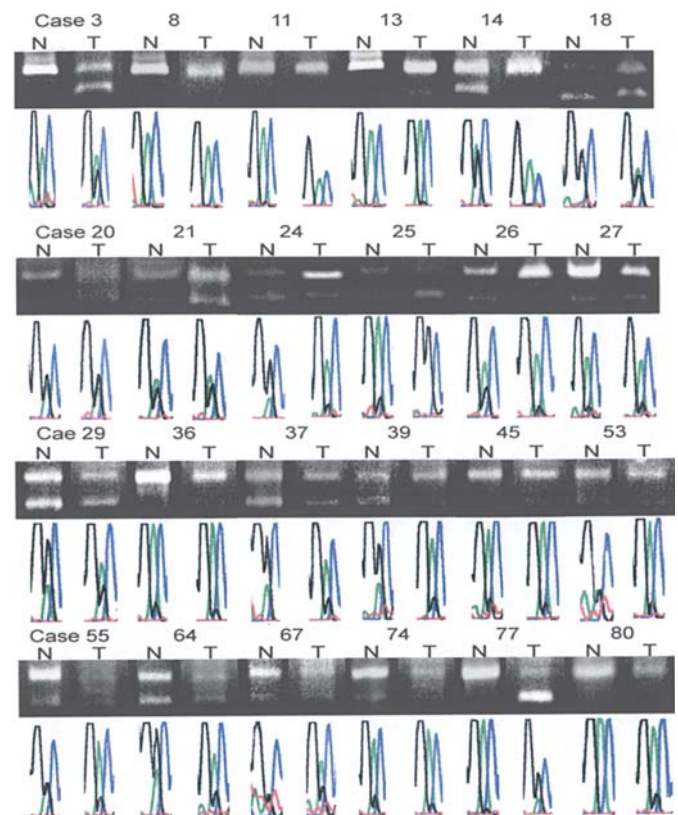


Figure 1. PCR-RFLP analysis using *ApaI* polymorphism and sequencing for whole tissues of normal mucosa and microdissected colorectal carcinomas. PCR products were digested with *ApaI* and run on 2% agarose gels. In the electropherogram, green shows adenine, black shows guanine, and red shows thymine. N, normal mucosa; T, tumor.

carcinoma cells in the tissue sections stained with Kernechtrot (Merck, Darmstadt, Germany). Between 100 and 1000 cells were collected on each plastic cap (CapSure™ LCM Caps, Arcturus, Mountain View, CA). To extract total RNA from microdissected carcinoma cells, we used a PicoPure™ RNA isolation kit (Arcturus) in accordance with the manufacturer's instructions. All specimens were assessed histologically by a pathologist (E. Kawahara).

Statistical analysis. Statistical analyses were performed using the χ^2 test.

Table II. The status of imprinting or loss of imprinting (LOI) in informative cases using whole normal tissues or laser capture microdissected carcinoma cells evaluated by PCR-RFLP in combination with direct sequencing.^a

Case	Normal tissue			Carcinoma cells		
	PCR (%A)	Sequence (%A)	Status	PCR (%A)	Sequence (%A)	Status
3	100	100	Imprinting	55	65	LOI
8	100	100	Imprinting	100	100	Imprinting
11	100	100	Imprinting	100	100	Imprinting
13	100	100	Imprinting	100	100	Imprinting
14	60	53	LOI	100	100	Imprinting
18	25	0	Imprinting	42	52	LOI
20	94	50	LOI	71	44	LOI
21	93	57	LOI	73	56	LOI
24	46	24	LOI	66	81	LOI
25	100	79	Imprinting	0	0	Imprinting
26	70	64	LOI	100	91	Imprinting
27	91	89	LOI	78	82	LOI
29	40	37	LOI	49	60	LOI
36	100	92	Imprinting	100	92	Imprinting
37	40	40	LOI	64	77	LOI
39	43	33	LOI	79	22	LOI
45	100	92	Imprinting	100	93	Imprinting
53	76	50	LOI	100	90	Imprinting
55	78	50	LOI	86	75	LOI
64	57	64	LOI	62	71	LOI
67	81	40	LOI	81	69	LOI
74	93	77	LOI	100	90	Imprinting
77	100	95	Imprinting	15	27	LOI
80	100	100	Imprinting	100	85	Imprinting

^aThe ratios of the densities of the top and bottom bands (top/top+bottom) by PCR-RFLP and the ratios of the height of A and G peaks (A/A+G) on sequencing are listed. When there was only one band by PCR-RFLP (ratio of 0 or 100%) or the sequence pattern showed a single peak (ratio of 0 or 100%), the status was considered to be imprinting, otherwise the status was considered to be LOI.

Results

Genomic DNA of whole non-tumor mucosa was used for screening whether the samples were informative or not, using PCR-based *ApaI* polymorphism. Of the 53 colorectal carcinoma cases (56.6%) 30 were informative. All of the informative cases for which tissue sections were available for LCM (24 of 30 cases) were examined for the imprinting status in both normal mucosa and in microdissected carcinoma cells. PCR products from cDNA in the normal mucosa and carcinoma cells showed two bands or a single band on *ApaI* analysis, and two A and G peaks or a single A or G peak by sequence analysis (Fig. 1). Prior to determining whether the status is imprinting or LOI, the density ratio between the top and bottom bands by PCR-RFLP and the ratio of heights of A and G peaks by sequence analysis were measured and calculated (Table I). Samples were judged as showing LOI when two bands were seen even if the bottom bands were faint. If the top bands were faint (cases 18 and 77), we had

to consider the possibility of incomplete *ApaI* digestion, and examined the results of sequencing analysis carefully. Cases with a single G peak were considered to be imprinting (case 18). Cases with a low A peak in addition to the G peak were considered to be LOI (case 77). As sequencing data indicated whether the ratio by PCR-RFLP was <10 or >90%, *ApaI* analysis in almost all cases showed only one band, and 10% was considered the background level. However, the normal mucosae of cases 25 and 80, in which peaks were within 10 to 90%, were assumed to show imprinting as PCR-RFLP analysis showed only one band. Thus, all cases were assumed to be assigned correctly to imprinting or LOI, and eventually we identified simple criteria for the imprinting status: when there is only one band on PCR-RFLP (ratio of 0 or 100%) or the sequence pattern shows a single peak (ratio of 0 or 100%), the status is considered to be imprinting; otherwise, the status is considered LOI.

LOI of IGF2 was detected in 14 of the 24 informative cases (58%) in matched normal mucosa (Table II). Of the 24

informative cases 13 (54%) showed LOI using LCM (Table II). There was a significant difference between LOI-positive cases in the normal mucosa and LOI-positive cases in carcinoma cells ($P < 0.05$): 10 of 14 LOI-positive cases in the normal mucosa also showed LOI in the carcinoma cells. In two cases, the imprinting status in the normal mucosa changed to LOI in the carcinoma cells. Furthermore, there were various patterns of change; in four cases, LOI in the normal mucosa converted to imprinting in carcinoma cells (cases 14, 26, 53, and 77), and imprinting in the G allele converted to imprinting in the A allele in one case (case 25).

Discussion

Contamination by normal cells is a major problem in analysis of gene expression by carcinoma cells using tissue samples. Microdissection is advantageous to obtain pure carcinoma cells. In the present study, we found LOI in 54% of microdissected colorectal carcinoma cells, which was higher than the rates of 33-44% reported previously (6-9,14). This high incidence was probably due to the use of microdissection, which also enabled us to improve the evaluation of LOI. Previous studies had to take into account not only true contamination by normal cells but also the possibility of contamination. For example, cases in which one of two bands obtained by PCR-RFLP was faint were not considered to be LOI. Application of the microdissection method allows the possibility of contamination to be excluded. Using microdissection we found that cases in which one band derived from the G or A allele was faint represented LOI. The evaluation method seems reasonable, as partial biallelic expression would occur (22) and the imprinting status could vary within the tumor (23). Indeed, case 77 may show partial imprinting in which one band on PCR-RFLP was faint and the peak of sequence was low, but use of a precise method for quantification, such as hot-stop PCR using radioisotopes (19), would be necessary to confirm this. Yun *et al* (24) further suggested that variation in the incidences of LOI reported to date could have been due to partial digestion with the restriction enzyme and contamination by genomic DNA. In the present study, we used DNase for RNA extraction and confirmed removal of genomic DNA. Direct DNA sequencing method could be applied if there was a problem of partial digestion by *Apa*I. Thus, the high frequency of LOI (>50%) found here suggests that overexpression of IGF2 by LOI plays a critical role in tumorigenesis in colorectal carcinomas. However, in a few cases LOI in the normal mucosa showed the converse alteration to imprinting status in the tumor, whereas it is disadvantageous for cell proliferation. Furthermore, imprinting of the A allele in the normal mucosa was converted to imprinting of the G allele in one case, which seemed to be neither advantageous nor disadvantageous for cancer. These phenomena may reveal a basic feature of the diversity of these tumors.

Cui *et al* (14,25) found LOI of IGF2 in the tumors and matched normal colonic mucosa in 30% of colorectal carcinoma patients, as compared with 10% of individuals without colorectal carcinoma, suggesting that LOI in the normal tissue may be a marker of colorectal cancer risk (15). This suggestion is similar to the concept that DNA mutation

targeting oncogenesis appears in normal colon tissue and progresses toward adenoma and then carcinoma (26). Similarly, we also clarified the high incidence of LOI in the normal mucosa (58%), and 77% of LOI-positive carcinomas showed LOI in the normal mucosa, supporting the hypothesis proposed by Cui. Other studies also showed a high incidence of LOI in matched normal mucosa of cases with LOI-positive carcinoma, ranging from 60 to 100% (13,14,22). LOI causes only a modest increase in IGF2 expression, theoretically double, but tumorigenesis may occur through alterations in intestinal maturation (27).

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