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Loss of imprinting of insulin-like growth factor II is associated with increased risk of proximal colon cancer

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ABSTRACT

The aim of this study was to determine the clinicopathological features of sporadic colon cancers with loss of imprinting (LOI) of insulin-like growth factor II (*IGF-II*) in Chinese patients. DNA from peripheral blood leucocytes and RNA from tumours were amplified and then digested with *Apa*I to determine the LOI status. Of the 316 patients enrolled for analysis, 149 were informative for *IGF-II* LOI. The positive rate of *IGF-II* LOI of colon cancer tissue was 47% (70/149) in Chinese patients. Proximal colon (64%) cancers were more likely to have LOI of *IGF-II* in tumour than distal colon (40.9%) cancers (odds ratio (OR) = 2.60, 95% confidence intervals (CI) = 1.21–5.56, $p = 0.014$). LOI of *IGF-II* in tumours was also associated with more advanced diseases (OR = 2.90, 95% CI = 1.05–8.04, $p = 0.04$). *IGF-II* LOI is present in high frequency in Chinese colon cancer patients, especially those with proximal cancer.

1. Introduction

Insulin-like growth factor II (*IGF-II*) has been reported as playing a crucial role in the growth and invasion of cancer cells.[1–3] Overexpression of *IGF-II* mRNA or protein was detected in 30 to 40% of advanced colorectal cancer tissues.[4,5] It has been reported that the *IGF-II* expression level in the carcinoma lesion was >40 times higher than that in the adenoma lesion.[6] Excessive expression of *IGF-II* can result in a 10-fold increase in the number and the diameter of colon adenoma and increased susceptibility to colon carcinoma in mice with *Apc*-Min/+, a murine model of human familial adenomatous polyposis.[7] Increased systemic availability of *IGF-II* may result from either biallelic expression, increased delivery from a transgene, or disruption of the *IGF-II* receptor. Loss of imprinting (LOI) of *IGF-II* gene is an important cause of biallelic expression of *IGF-II*.

Genomic imprinting is an epigenetic modification in the gamete or zygote of a specific parental allele of a gene that leads to parental origin-specific differential expression of the two alleles of a gene in somatic cells of the offspring.[8] LOI can result in either activation or silencing of the normally

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silent or expressed allele of a growth promoting gene or a growth inhibitory gene, respectively.[9] Normally, the *IGF-II* gene is paternally expressed and the maternal origin alleles are imprinted by the mechanism of methylation.[9] Loss of imprinting of *IGF-II* gene will lead to biallelic expression and result in 2 to 3-fold increased expression of *IGF-II*,[10] and has been reported in neoplastic diseases such as Wilms' tumour, gastric cancer, and colorectal cancer.[11–13]

The prevalence of LOI of *IGF-II* has been reported to range from 22 to 44% in tumour tissue of patients with colorectal carcinoma in Western populations and in Japanese patients.[13–19] Intriguingly, the degree of LOI in the matched normal tissue was reported to correlate strongly with that in the tumour tissue.[13] About 10% of the general population not having cancer also had LOI of *IGF-II* in their normal colonic mucosa and in their blood.[13] Furthermore, Cui and colleagues reported that 91% of cancer patients with microsatellite instability (MSI) in tumour showed LOI, whereas only 12% of cancer patients without MSI in tumour showed LOI.[13] These data suggest that LOI of *IGF-II* might provide a marker for identifying an important subset of the population with cancer or at risk of developing cancer. However, the role of LOI of *IGF-II* in colorectal tumourigenesis in Chinese patients has not been reported and the clinicopathological features of sporadic colorectal cancers with LOI of *IGF-II* are also rarely reported, except one from Japan. As MSI has been reported to be associated with right-sided colon cancer,[20] we aimed to evaluate whether tumours with LOI of *IGF-II* also have predilections for proximal location of tumour. Besides, as overexpression of *IGF-II* in colon tumour has been reported to be associated with more advanced tumour stage,[21] the development of liver metastasis,[22] and poorer survival,[23] we aimed to examine whether tumours with LOI of *IGF-II* is also associated with more advanced disease.

2. Materials and methods

2.1. Patients

Consecutive Chinese patients with pathologically documented colorectal cancer, who underwent surgery in the Department of Surgery, National Taiwan University Hospital from October 2003 to April 2006, were included in this study. Patients with inflammatory bowel disease or with a known family history of familial adenomatous polyposis (FAP) or HNPCC were excluded. Patients who underwent preoperative radiotherapy or chemotherapy were also excluded. The study protocol was approved by the Institutional Review Board of National Taiwan University Hospital. Tumour location was classified as proximal or distal colon cancer. Proximal colon cancer was defined as tumours at or proximal to the splenic flexure of the colon. Tumors at the descending colon, sigmoid colon, and rectum were classified as distal colon cancers. Written informed consent was obtained from all patients. About 5% of the hospital patients meeting the diagnostic criteria refused enrollment when they were approached. Demographic and clinicopathologic information were collected from each patient on enrollment. Poorly differentiated (high grade) tumour was defined as less than 10% of tumour cell formed glands and mucinous tumour was defined as those containing more than 50% extracellular mucin.[24]

2.2. Nucleic acid preparation

Genomic DNA was extracted from whole blood Buffy coat specimen using Gentra Blood DNA extraction kit (Unimed, USA). RNA was extracted from colorectal cancer tissue using Tri-Solution Reagent Plus (GeneMark Technology, Taiwan) following the manufacturer's instructions. RNA was dissolved in RNA safeguard solution (GeneMark Technology, Taiwan) and stored at −80 °C until use.

2.3. Quantitative analysis of IGF-II impring status

Genomic DNA was amplified with PCR. The sequences of Primer set II were: *IGF-II* /Af 5′-CCT TGG ACT TTG AGT CAA ATT-3′ and *IGF-II*/Br 5′-GGT CGT GCC AAT TAC ATT TCA-3′. The PCR conditions were initial denaturation for 5 min at 94 °C, followed by 35 PCR cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and elongation at 72 °C for 30 s. A 2% agarose gel electrophoresis was used to identify the 293 bp PCR product. The PCR products were then digested with *Apa*I for 3 h at 25 °C. The *Apa*I polymorphism was classified as A/A, B/B, A/B. Heterozygote (A/B) was considered informative for LOI analysis.[13]

RNA samples were treated with DNAse at 37 °C before RT-PCR to avoid DNA contamination. All reactions were done in duplicate with and without reverse transcriptase. The primer sequences of Primer set I used in RT-PCR were: *IGF-II*/Zf 5′-CCT CCG ACC GTG CTT CCG GAC-3′ and *IGF-II*/Er 5′-GGA TGG GAA TTG AGA TGT AAG-3′. The RT-PCR conditions were

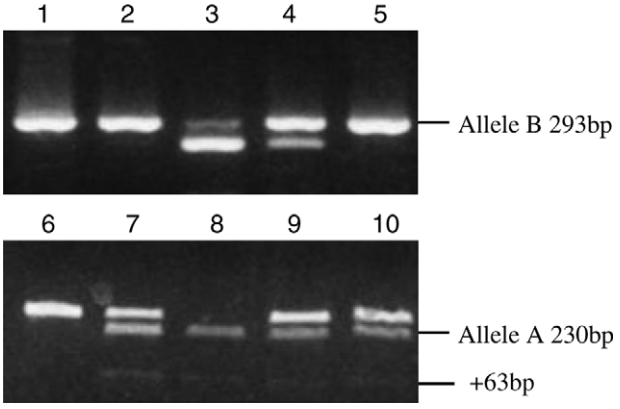

Fig. 1 – Electrophoresis of RT-PCR products. The allele A contains *Apa*I site and two bands (230bp and 63bp) would be observed after *Apa*I digestion. The allele B lacks *Apa*I site and only one band (293bp) would be observed after *Apa*I digestion. Patients 1, 2, 5, and 6 carried an allele B/B expression. Patient 8 had only allele A/A status. Patient 3 had a LOI scoring threshold of about 4-fold difference between two alleles, and thus was considered as allele A expression. Patients 4, 7, 9, and 10 had a less than 3-fold difference between the two alleles, and were regarded as biallelic expression (loss of imprinting).

the same as in PCR for genomic DNA. Then, PCR was performed using Primer set II. The PCR products were also digested with *Apa*I enzyme. LOI was scored semi-quantitatively by 2% agarose gel electrophoresis. The threshold for scoring LOI was defined as a ratio of less than 3-fold difference in expression between two alleles.[14] For patients with LOI of the *IGF-II* gene, both allele A and B appeared after *Apa* I digestion (Fig. 1). For quality control purposes, genotyping was repeated in 10% of the randomly selected samples and revealed no discrepancies. The success rates in extracting DNA and RNA were greater than 99%.

2.4. Microsatellite instability analysis

MSI was assessed using five microsatellite markers at distinct chromosomal loci (BAT-25, BAT-26, D5S346, D2S123, D17S250) by comparing tumour and matched normal genomic DNA, as recommended by the National Cancer Institute consensus.[25] The PCR primers were purchased from Research Genetics (Huntsville, AL). All the five markers were amplified with PCR for 35 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s. The products were electrophoresed on 6% polyacrylamide gels containing 8 M urea and analysed by autoradiography. Tumoural MSI for a given primer set was defined as the appearance of one or more PCR products either smaller or larger than those produced from normal mucosa, as previously described.[26] The MSI status of tumours was classified as MSS (0 of the 5 showed MSI), MSI-L (1 of the 5 showed MSI) and MSI-H (⩾2 of the 5 showed MSI). For analyses, tumours with MSS or MSI-L were together categorised as MSI-H (–).

2.5. Statistical analysis

The prevalence of LOI in patients with colon cancer was described as a proportion. The demographic and clinicopathological characteristics in LOI positive and LOI negative patients were compared and tested using the Chi-Square test or Fisher's exact test when necessary. Logistic regression analyses were used to compute the odds ratios (ORs) and 95% confidence interval (CI). Independent sample *t*-test was used to compare the mean age differences between LOI-positive and -negative patients. All *p*-values were two-tailed with 0.05 specified as statistical significance. All statistical analyses were performed with statistical software (SPSS version 10.0 for Windows, SPSS, Inc., Chicago III.).

3. Results

3.1. Demographic data

Of the 324 patients eligible for enrollment, eight patients underwent concurrent chemoradiation therapy prior to surgery and were excluded. Of the 316 tumours analysed, 149 were heterozygous and thus informative for *IGF-II* LOI analyses. Loss of imprinting of *IGF-II* was detected in 47% (70/149) of tumors. The demographic characteristics of patients with or without LOI of *IGF-II* were shown in Table 1. There were no differences in the mean age, sex ratio, diabetes mellitus (DM), cigarette smoking, alcohol consumption, body mass index (BMI), and family history of CRC between the two groups.

Table 1 – Demographic data of patients with and without IGF-II LOI

	LOI (+) N = 70	LOI (–) N = 79	*p*-value
Gender			0.596
Male	42 (60%)	44 (55.7%)	
Female	28 (40%)	35 (44.3%)	
Mean age, yr (SD)	64.7 (12.6)	65.7 (11.4)	0.611
Family history of CRC	8/70 (11.4%)	9/79 (11.4%)	0.994
DM	11/70 (15.7%)	10/79 (12.7%)	0.593
Cigarette smoking	22/70 (31.4%)	21/79 (22.6%)	0.515
Alcohol consumption (>10 g/day)	9/70 (12.9%)	7/79 (8.9%)	0.432
BMI, mean (SD)	23.4 (3.4)	22.8 (3.7)	0.308
BMI			0.674
⩾27	10 (14.3%)	8 (10.1%)	
23–26.9	31 (44.3%)	34 (43%)	
<23	29 (41.4%)	37 (46.8%)	

IGF-II: insulin growth factor II; LOI: loss of imprinting; SD: standard deviation; CRC: colorectal cancer; DM: diabetes mellitus; BMI: body mass index.

Table 2 – Association of clinicopathological features and MSI-H with IGF-II LOI status

	LOI (+) (*n* = 70)	LOI (–) (*n* = 79)	*p*-value
Distribution of cancer			0.013
Distal	45 (64.3%)	65 (82.3%)	
Proximal	25 (35.7%)	14 (17.7%)	
Histological differentiation (well,mod/poor, muc)	8/62	7/72	0.603
Lymph node metastasis	33 (47.1%)	37 (46.8%)	0.978
Liver metastasis	8 (11.4%)	10 (12.7%)	0.818
Lymphatic invasion	36 (51.4%)	41 (51.9%)	0.954
Venous invasion	26 (37.1%)	24 (30.4%)	0.383
Depth of invasion			0.081
T1+T2	9 (%)	19 (%)	
T3+T4	61 (%)	60 (%)	
Duke's stage			0.056
A and B1	7 (%)	17 (%)	
⩾B2	63 (%)	62 (%)	
Tumour Size			0.379
<2 cm	3 (%)	2 (%)	
2–5 cm	33 (%)	46 (%)	
>5 cm	34 (%)	31 (%)	
MSI-H			0.812
Positive	7 (10%)	7 (8.9%)	
Negative	63 (90%)	72 (91.1%)	

IGF-II: insulin growth factor II; LOI: loss of imprinting; MSI: microsattelite instability.

3.2. Clinicopathological characteristics and MSI-H according to LOI status

Of the 149 tumours, 110 tumours were located at the distal colon and 39 tumours were located at the proximal colon, as shown in Table 2. Proximal colon cancer (64%) are more likely to have LOI of *IGF-II* in tumours than distal cancers ($p = 0.013$). There were no differences in the histological differentiation, lymph node metastasis, liver metastasis, lymphatic or venous invasion, and depth of invasion between the LOI (+) and LOI (–) groups. The LOI positive rate was higher in patients with more advanced tumour stage (⩾ Duke's B2) than in patients with earlier stage (Dukes' A and B1), but the difference was not statistically significant ($p = 0.056$). MSI-H was detected in 9.4% (14/149) of colon cancer tissues. The positive rates of MSI-H were not different in colon cancer with (10%) and without (8.9%) LOI of *IGF-II* ($p = 0.812$).

3.3. LOI of IGF-II and MSI-H positive rates according to tumour location

The positive rate of LOI of *IGF-II* was significantly higher in patients with proximal colon than in patients with distal colon (64.1% versus 40.9%, $p = 0.013$), as shown in Table 3. However, the positive rate of MSI-H was comparable in patients with proximal or distal colon cancer (7.7% versus 10%, $p = 0.671$). We further demonstrated that tumours with LOI of *IGF-II* are independently associated with increased risk (OR = 2.60, 95%CI = 1.21–5.56, $p = 0.014$), of proximal located colon cancer than those without LOI, as shown in Table 4.

3.4. Factors associated with more advanced stage disease

In the multivariate analysis, positive LOI of *IGF-II* (OR = 2.90, 95%CI = 1.05–8.04, $p = 0.04$) and elevated CEA (OR = 6.52,

Table 3 – Association of IGF-II LOI and MSI-H with tumour location

Location		LOI (+) rate			MSI-H (+) rate		
		Segment	Overall	*p*-value	Segment	Overall	*p*-value
Distal	Rectum	40.4% (19/47)	40.9% (45/110)	0.013	6.4% (3/47)	10% (11/110)	0.671
	Sigmoid	42.3% (23/54)			13% (7/54)		
	Descending	33.3% (3/9)			11.1% (1/9)		
Proximal	Splenic flexure	100% (3/3)	64.1% (25/39)		33.3% (1/3)	7.7% (3/39)	
	Transverse	50% (2/4)			0% (0/4)		
	Ascending	64.3% (18/28)			7.1% (2/28)		
	Cecum	50% (2/4)			0% (0/4)		

IGF-II: insulin growth factor II; LOI: loss of imprinting; MSI: microsatellite instability.

Table 4 – Odds ratio for proximal location of cancer

Variable	% patients with proximal cancer	Crude OR for proximal cancer (95% CI)	Adjusted OR for proximal cancer (95% CI)
Age (for each year older)		1.001 (0.97–1.03)	1.005 (0.97–1.03)
Sex			
Female	27% (17/63)	1	1
Male	25.6% (22/86)	0.93 (0.45–1.95)	0.93 (0.38–2.25)
Cigarette smoking			
Non-smoker	24/106	1	1
Smoker	11/43	0.96 (0.43–2.15)	0.81 (0.28–2.39)
Alcohol			
No	34/133	1	1
Yes	5/16	1.32 (0.43–4.10)	1.42 (0.37–5.40)
DM			
Non-DM	33/128	1	1
DM	6/21	1.15 (0.41–3.2)	1.12 (0.39–3.25)
IGF-II LOI status			
Normal imprinting	17.7% (14/79)	1	1
LOI (+)	35.7% (25/70)	2.58* (1.63–5.08, $p = 0.014$)	2.60* (1.21–5.56, $p = 0.014$)
Microsatellite instability			
MSI-H (–)	36/135	1	1
MSI-H (+)	3/14	0.75 (0.2–2.84)	0.73 (0.18–3.03)

OR: odds ratio; CI: confidence interval; DM: diabetes mellitus; *IGF-II*: insulin growth factor II; LOI: loss of imprinting; MSI: microsatellite instability.
* $p < 0.05$.

Table 5 – Odds ratios (multivariate) for more advanced disease

	Duke's stage ⩾B2			Deeper invasion (⩾T3)		
	OR	95% CI	*p*-value	OR	95% CI	*p*-value
Age (each yr increase)	1.01	0.97–1.06	0.661	0.996	0.96–1.04	0.855
Gender (male versus female)	1.94	0.72–5.20	0.188	1.595	0.62–4.08	0.330
LOI of *IGF-II* (positive versus negative)	2.90	1.05–8.04	0.040	2.563	0.99–6.62	0.052
MSI-H (positive versus negative)	0.38	0.06–2.36	0.300	0.484	0.08–2.94	0.430
CEA ⩾5 ng/ml versus <5 ng/ml	6.52	1.92–22.19	0.003	7.976	2.43–26.14	0.001

OR: odds ratio; CI: confidence interval; LOI: loss of imprinting; *IGF-II*: insulin growth factor II; MSI: microsatellite instability.

95%CI = 1.92–22.2, $p = 0.003$) were independently associated with increased risk of more advanced disease (Duke's stage ⩾B2), as shown in Table 5. Positive LOI of *IGF-II* (OR = 2.56, 95%CI = 0.993–6.62, $p = 0.052$) and elevated CEA (OR = 7.98, 95%CI = 2.43–26.1, $p = 0.001$) were also associated with increased risk of T3 or T4 disease.

4. Discussion

In the present study, we have demonstrated that LOI of *IGF-II* is present in 47% of colon cancer tissue in Chinese patients, and that proximal colon cancers are more likely to have LOI of *IGF-II* in tumour tissues than distal colon cancers (OR = 2.60, 95%CI = 1.21–5.56, $p = 0.014$). Patients with LOI of *IGF-II* in their tumour also had more advanced disease than those without (OR = 2.90, 95%CI = 1.05–8.04, $p = 0.04$). To the best of our knowledge, this is the first study to report on the LOI of *IGF-II* in colon cancer in Chinese patients and its positive correlation with proximal location of tumour and more advanced disease.

In the present study, the frequency of *IGF-II* LOI (+) colon cancers in Chinese (47%) is slightly higher than those reported from Western countries (22–44%) and Japan (27.4%). Possible explanations included different methods used in patient enrollment, in obtaining informative cases and identifying *IGF-II* LOI status, as well as ethnic difference. Firstly, patients included in the studies by Cui,[13] Nishihara,[17] and Nakagawas and colleagues[18] were not designed as a cohort. In the study by Sasaki and colleagues[19] and the present study, patients were enrolled consecutively and the case numbers were also larger. Secondly, Cui and colleagues[13] used DNA from tumour tissue to identify informative genomic polymorphism of *IGF-II* gene. However, we used DNA from peripheral blood leucocytes instead of tumour tissue to identify informative cases. Therefore, some cases with loss of heterozygosity (LOH) might be regarded as LOI of *IGF-II* in the study by Cui and colleagues[13], whereas some cases with LOH might be regarded as normal imprinting in the present study. In contrast, Sasaki and colleagues[19] identified informative *IGF-II* genomic polymorphism from normal mucosa and he further analysed genomic DNA from tumour tissue to exclude those with LOH. Thirdly, Sasaki and colleagues[19] investigated *IGF-II* LOI status by direct sequencing of RT-PCR products. In contrast, we used the allele-specific restriction enzyme digestion technique to identify LOI status, similar to that reported by Cui and colleagues.[13] The methods used in the study by Sasaki and colleagues[19] were more accurate but were more costly and time consuming. Of course, ethnic difference is another possibility. The frequency of MSI-H (+) colon cancers in the present study is also slightly lower (9.7%) than those reported from Western countries and Japan (10–15%).[27,28]

Several studies have shown that the mechanisms in the tumourigenesis of proximal and distal colon cancer are different.[20,29] In sporadic colon cancers, tumours with microsatellite instability (MSI) tended to occur in the proximal colon, whereas tumours with chromosomal instability (CIN) tended to be distributed in the distal colon.[20] It has been reported that 91% of colon cancers with MSI showed *IGF-II* LOI, whereas only 12% of colon cancer without MSI showed LOI.[13] Therefore, the association of the *IGF-II* LOI and predilection for proximal tumour location might be related to the strong correlation of LOI and MSI. However, our study failed to find a correlation between MSI-H and LOI of *IGF-II*. Instead, we found that proximal colon cancer is independently associated with higher positive LOI rate, consistent with a recent report from Japan.[19] The carcinogenesis of MSI tumours in proximal colon has been reported to be related to methylating agents, whereas CIN tumours in distal colon are related to bulky-adduct-forming carcinogens.[29] Hypomethylation of the differentially methylated region (DMR) upstream of exon 3 has been demonstrated as the mechanism that leads to loss of imprinting of *IGF-II*.[30] Therefore, it is possible that *IGF-II* LOI play a role in the tumourigenesis of proximal colon cancer through epigenetic modification of DMR. However, more large scale studies in different ethnic populations are warranted to examine whether *IGF-II* LOI is involved in conventional pathways or in a new pathway of colorectal carcinogenesis.

A recent study has revealed that patients with CRC and colorectal neoplasia had 17.8 and 5.47 times increased odds, respectively, of expressing LOI of *IGF-II* in their peripheral blood leucocytes compared with those without colorectal neoplasia.[26] Therefore, it is reasonable to offer colon cancer screening programmes for those expressing LOI of *IGF-II*. Our results further support that screening with colonoscopy might be more appropriate because proximal colon cancers are more likely to express *IGF-II* LOI than distal cancers. Future studies regarding the impact of *IGF-II* LOI on the colon cancer screening strategies are warranted.

IGF-II can act as an autocrine growth factor in human colorectal cancer cell lines by binding to *IGF-I* receptors (*IGF-IR*).[5,31] Enhanced expression of *IGF-II* might allow the tumour to proliferate and grow more rapidly. *IGF-II* expression was reported to be associated with poor prognosis, including more advanced stage, poorer differentiation, and large residual lesions in ovarian cancer.[32] Expression of *IGF-II* in colorectal cancer was reported to correlate with tumour size, depth of

invasion, and survival rate.[21,23] *IGF-II* was also reported to play a role in the development of liver metastasis from colorectal cancer.[22] Our study showed that LOI of *IGF-II*, which is associated with 2–3-fold increased expression of *IGF-II*, is associated with more advanced stage disease (⩾Duke's B2). Sasaki and colleagues reported that the *IGF-II* LOI-positive rate was significantly higher in patients with poorly differentiated or mucinous carcinoma cases than in patients with well or moderately differentiated carcinoma.[19] However, our study failed to find such an association. *In vitro* studies have revealed that the growth of colon cancer xenografts can be inhibited by blockade of the *IGF-II/IGF-IR* axis by soluble *IGF-IR*.[33] Soluble *IGF-II* receptor, which acts as a specific inhibitor of *IGF-II* ligand, can also rescue the progression of intestinal adenoma, as reported in a recent report.[34] Therefore, these agents are promising in treating patients with *IGF2* LOI in their tumour, which is associated with more advanced disease.

There were some limitations in the present study. Firstly, our study failed to find an association between MSI-H and LOI of *IGF-II*, as well as a tendency for proximal location of MSI-H (+) tumours. We considered that the relatively small case number (14 patients) with MSI in our study population might lead to inadequate study power to detect the difference. Secondly, the *IGF-II* genomic polymorphism in tumour tissue was not examined. Therefore, some cases with LOH were not excluded from analysis. These patients are considered as normal imprinting rather than LOI in our analysis. Therefore, the prevalence of LOI was underestimated in our study population. Nevertheless, our result was consistent with other studies that LOI of *IGF-II* is also important in the colorectal carcinogenesis in Chinese patients.

In summary, high frequency of *IGF-II* LOI is also present in patients with colon cancer in Chinese in Taiwan, a newly developed country. The predilection for proximal location of colon cancer in LOI-positive tumours indicates the necessity of using total colonoscopy as a screening tool for *IGF-II*-positive persons. The association of *IGF-II* LOI and more advanced stage disease also supports the role of *IGF-II* LOI in the disease progression of colon cancer.

Conflict of interest statement

None declared.

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