A single nucleotide polymorphism in fibronectin 1 determines tumor shape in colorectal cancer

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Abstract. Depressed and flat surface lesions are not easy to identify with routine colonoscopies during screening for colorectal cancer (CRC). Identifying clinically relevant genes that influence tumor shape could be useful when screening for the presence of depressed lesions. Total RNA was extracted from tumor cells collected by laser microdissection from the primary lesions of 146 CRC cases. Microarray analysis was performed to identify genes that were differentially expressed between depressed and elevated tumors. Single nucleotide polymorphism (SNP) analysis of genomic DNA from the peripheral blood of 67 CRC patients was then used to associate polymorphisms with the occurrence of depressed tumors. Microarray analysis revealed significantly higher expression of the fibronectin 1 (*FN1*) gene in 129 depressed-type tumors and lesions compared to 17 elevated-type tumors. *FN1*-abundant CRC tumors were large with a significantly higher incidence of lymphatic permeation. SNP analysis indicated that 44 tumors with a GG genotype at SNP rs6707530 showed significantly higher *FN1* expression than did 23 tumors with GT/TT genotypes ($p<0.05$). The product of the *FN1* gene (located at 2q34) is involved in cell adhesion, migration and metastasis in mesenchymal tumors. Abundant expression of *FN1* may allow cancer cells to invade deeper layers, which would eventually define tumor shape. Identification of this SNP in blood samples may facilitate disease diagnosis and allow prediction of the presence of depressed tumors in the colorectal epithelium before a colon fiberscope examination.

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Introduction

The size and shape of a lesion in colorectal cancer (CRC) are believed to be connected directly to clinical phenotype and to serve as predictors of malignant behavior (1,2). Early superficial CRC lesions can be classified as elevated (polypoid and non-polypoid) or depressed (3,4). Polypoid lesions grow above the surface of the mucosa and the volume of the polypoid component appears to be correlated with malignant behavior. Non-polypoid lesions may grow flat or slightly elevated, eventually progressing, into polypoid lesions or lateral spreading tumors. Finally, depressed lesions (0-IIc, 0-IIc+IIa, 0-IIa+IIc), which comprise only 2.3% of all superficial lesions (5), warrant particular attention due to the difficulty of detection and removal by fiberscope (6,7). Since depressed-type lesions are frequently located in the right colon, they can be difficult to detect. Moreover, depressed lesions, independent of size, have been associated with an increased risk of rapid progression to cancer, as shown in endoscopy and pathology units in Japan (4,5,8,9). Depressed-type lesions and tumors, such as earlier phase lesions (0-IIc, 0-IIc+IIa, 0-IIa+IIc) and/or advanced tumors (types 2 and 3), invade and metastasize to lymph nodes and other distant organs and indicate a poorer prognosis than do type 1 tumors.

Microarray analysis of RNA from the cancer cells of 144 CRC cases revealed that the fibronectin 1 (*FN1*) gene is significantly associated with tumor shape in CRC. FN1 is a glycoprotein that is present in a soluble dimeric form in the plasma, and in a dimeric or multimeric form at the cell surface and in the extracellular matrix (10). It is involved in cell adhesion and migration processes including embryogenesis, wound healing, blood coagulation, host defense and metastasis. The gene has 3 regions that are subject to alternative splicing, with the potential to produce 20 transcript variants. However, the full-length nature of some of these variants has not been determined.

In the present study, we performed a comprehensive analysis to identify genes that determine tumor shape (11). Evaluation of changes in the expression patterns of these genes may allow physicians to make a precise, non-invasive diagnosis of depressed-type lesions in early CRC. The correlation

between *FN1* expression and tumor shape was validated by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) before single nucleotide polymorphism (SNP) analysis was performed to identify polymorphisms in the *FN1* coding region that could be used as predictors of tumor shape (1) in CRC.

Materials and methods

CRC patients. The study group comprised 146 patients with primary CRC. The patients ranged in age from 32 to 96 years, with an average age of 66 years. They underwent operations at major hospitals in Japan: Kyushu University, Kitazato University, Tokyo Medical and Dental University, National Defense University, Mie University, Takano Hospital, National Cancer Center and Osaka University from 2004 to 2009. None of the patients received preoperative treatments such as radiation or chemotherapy. Immediately after surgical resection, tumor samples (T) were carefully removed from primary cancerous lesions for cell isolation using laser microdissection (LMD). Clinicopathological patient data were obtained from clinical records. Histopathological assessments were made using the Japanese Classification of Colorectal Carcinoma, 7th edition.

Collection of CRC cells. Tissues from the 146 CRC patients were collected for LMD using the Leica Laser Microdissection System (Leica Microsystems, Wetzlar, Germany). In brief, 5-μm frozen sections were fixed in 70% ethanol for 30 sec, stained with hematoxylin and eosin and dehydrated as follows: 5 sec each in 70, 95 and 100% ethanol and a final 5 min in xylene. The sections were air-dried, then microdissected with the LMD system. Target cells, at least 100 cells/section, were excised and bound to transfer film for total DNA extraction.

Total RNA extraction and first-strand cDNA synthesis. CRC tissue specimens or cultured cell lines at subconfluency were homogenized, and total RNA was extracted using the modified acid-guanidine-phenol-chloroform method. Total RNA (8.0 μg) was reverse transcribed to cDNA using M-MLV RT (Invitrogen Corporation, Carlsbad, CA, USA).

qRT-PCR. The sequences for *FN1* mRNA were: *FN1*, sense primer, 5'-GAACTATGATGCCGACCAGAA-3' and antisense primer, 5'-GGTTGTGCAGATTTCCTCGT-3'. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal control and *GAPDH* primers were: sense primer 5'-TTGGTATCGTGGAAGGACTCTA-3' and antisense primer, 5'-TGTCATATTTGGCAGGTT-3'. Real-time monitoring of PCR reactions was performed using the LightCycler system (Roche Applied Science, Indianapolis, IN, USA) and SYBR-Green I dye (Roche Diagnostics, Tokyo, Japan). Monitoring was performed according to the manufacturer's instructions. In brief, a master mixture containing 1 μl of cDNA, 2 μl of DNA Master SYBR-Green I mix, 50 ng of primers and 24 μl of 25 mM $MgCl_2$ was prepared on ice, and the final volume was adjusted to 20 μl with water. After the reaction mixture was loaded into glass capillary tubes, qRT-PCR was performed with the following cycling conditions: initial denaturation at 95˚C for 10 min, followed by 45 cycles of 95˚C for 10 sec, annealing at 60˚C for 10 sec and extension at 72˚C for 10 sec. After amplification, products were subjected to a temperature gradient from 65˚ to 95˚C at 0.2˚C/sec, under continuous fluorescence monitoring, to produce a melting curve for analysis of primer specificity.

Expression array analysis. For microarray expression analysis, we used a commercially available Whole Human Genome Oligo DNA Microarray kit (Agilent Technologies, Santa Clara, CA, USA). A list of genes on this cDNA microarray is available from http://www.chem.agilent.com/scripts/generic.asp?lpage=5175&indcol=Y& prodcol=Y&prodcol=N&indcol=Y&prodcol=N. Cyanine (Cy)-labeled cRNA was prepared using T7 linear amplification as described in the Agilent Low RNA Input Fluorescent Linear Amplification Kit Manual (Agilent Technologies). Labeled cRNA was fragmented and hybridized to an oligonucleotide microarray (Whole Human Genome 4x44K Agilent G4112F). Fluorescence intensities were determined with an Agilent DNA Microarray Scanner and were analyzed using G2567AA Feature Extraction Software version A.7.5.1 (Agilent Technologies), which uses locally weighted linear regression curve fit (LOWESS) normalization. This microarray study followed the MIAME guidelines issued by the Microarray Gene Expression Data group. Further analyses were performed using GeneSpring version 7.3 (Silicon Genetics, San Carlos, CA, USA).

Evaluation of representative SNPs in the FN1 coding region. Genomic DNA was extracted from the peripheral blood of 64 patients with primary CRC using a QIAamp DNA Mini kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). SNPs in the exonic and intronic regions of *FN1* were evaluated simultaneously using an Affymetrix genome-wild SNP array to determine the association between *FN1* expression and the genotype of each SNP in the *FN-1* gene: rs6707530, rs11651, rs7594168, rs10498037, rs33996776, rs1250214, rs7568287, rs10201850, rs41347752, rs1250204, rs7588661, rs2577302, rs1968510, rs34255697, rs10172425, rs7572169, rs2372545, rs1250264, rs12105173, rs10199059, rs1437799, rs1250270, rs11693652, rs7567647, rs1898536, rs10202483, rs1250247, rs1250233, rs6753702 and rs1250252. However, rs2372545 and rs1250264 were excluded due to low-quality data. The Ethics Committee of each institute approved this project.

Results

Differential expression of FN-1 in cells isolated from CRC primary tumors. Microarray analysis revealed a significant difference in the expression of the *FN1* gene between depressed-type tumors and lesions (0-IIb, 0-IIc, 0-IIa+IIc, 0-IIc+IIa, type 2 and type 3 tumors) and elevated-type tumors (0-Ip, 0-Isp, 0-Is, 0-IIa and type 1 tumors). The microarray contained three probes for *FN1*, all of which were significantly upregulated in depressed-type lesions (n=129) compared to elevated-type tumors (n=17). The 3 probes were also used to validate these findings in representative samples of depressed (n=19) and elevated (n=9) tumors and normal tissues (n=9).

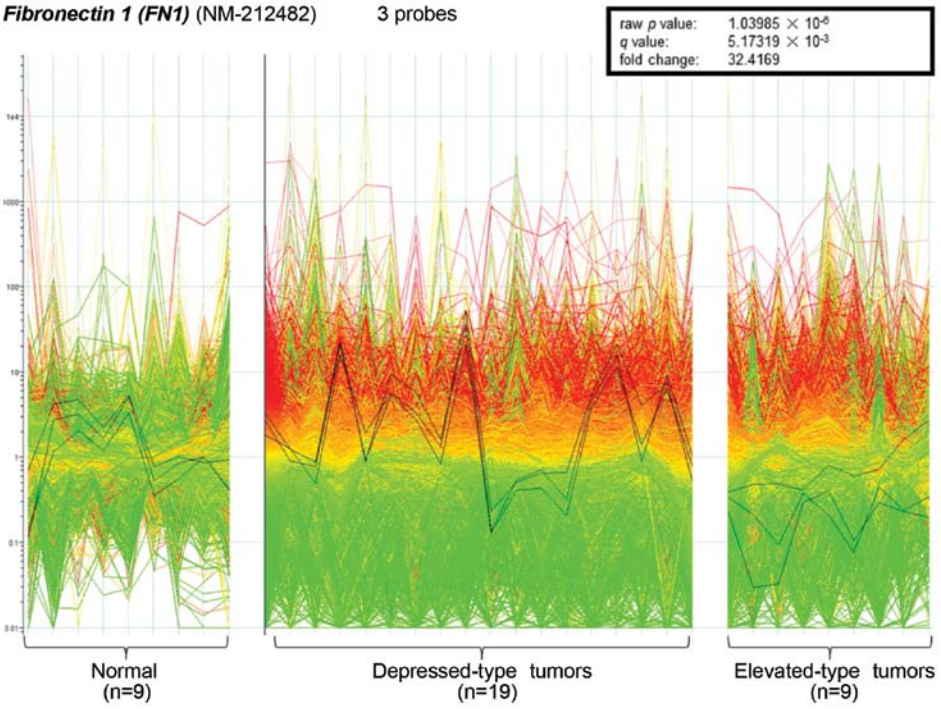

Figure 1. Differences in the growth type in Tis-T2 CRC tumor. Three probes covered the coding region of *FN1* (NM-212482) on the Agilent expression array. *FN1* expression detected by each probe is represented by the black lines in each column. Left column, expression in normal colorectal epithelial cells (n=9); middle column, expression in depressed-type tumors (n=19); right column, expression in elevated-type tumors (n=9). The upregulated (red lines) and downregulated (green lines) expression levels of whole genes in the microarray analysis are also denoted.

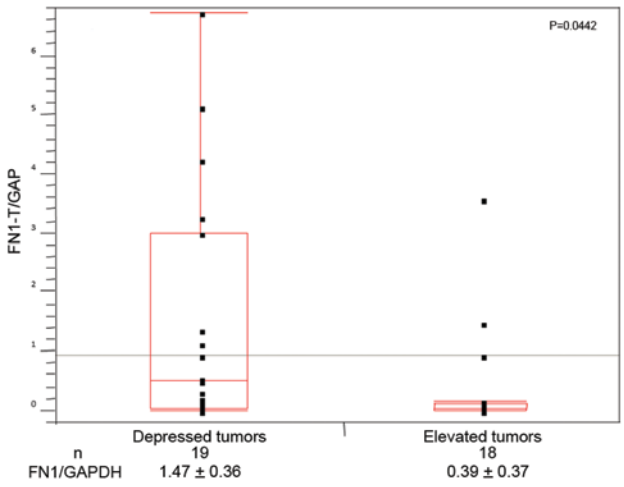

Figure 2. Validation of the association between tumor shape and *FN1* expression. The expression of *FN1* in the subgroup of depressed tumors (n=19; 1.47±0.36) vs. the subgroup of elevated tumors (n=18; 0.39±0.37; p<0.05). *FN1*, fibronectin 1.

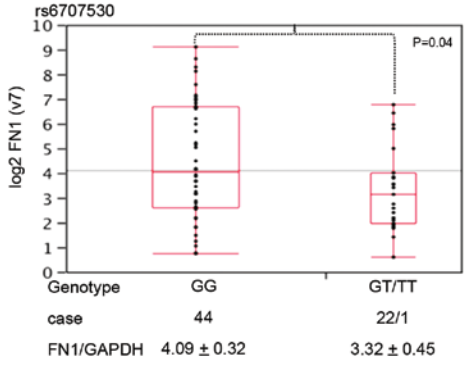

Figure 3. *FN1* expression is associated with a single nucleotide polymorphism. The GG and GT/TT genotypes of rs6707530 were identified in 44 and 23 CRC cases, respectively. The expression of *FN1* was significantly higher in GG cases of CRC than in GT/TT cases. *FN1*, fibronectin 1.

The 19 depressed tumors showed significantly higher expression than the other groups (raw p-value, 1.03985×10^{-6}; q-value, 5.17319×10^{-3}; fold-change, 32.4169; Fig. 1). In comparison to that of normal samples, the average expression of *FN1* in depressed type lesions was upregulated, while expression in elevated-type lesions was downregulated.

qRT-PCR supported the finding that the expression of *FN1* in the subgroup of depressed tumors (19 tumors; 1.47±0.36) was significantly higher (p<0.05) than that in the subgroup of elevated tumors (n=18; 0.39±0.37; Fig. 2).

Clinicopathological significance of FN1 in CRC cases. In addition to tumor shape, 2 other clinicopathological factors were associated with *FN1* expression (Table I). Higher expression was observed in larger tumors (>5 cm; n=71) than in smaller tumors (n=75; p=0.0024). There was also a significant difference in *FN1* expression between lymphatic permeation negative (n=65; 4.38±0.24) and positive (n=61; 3.70±0.22) tumors (p=0.036).

FN1 expression is associated with 1 SNP. Of the 30 SNPs in the *FN1* region, rs6707530 was associated with tumor shape in CRC (Fig. 3). The expression of *FN1*/GAPDH was higher in CRC samples with a GG genotype (n=44; 4.09±0.32) at this locus rather than a GT or TT genotype (n=23; 3.32±0.45).

Table I. Clinicopathologic significance of the expression of fibronectin-1/GAPDH in CRC cases.

Clinicopathologic analysis	N	Fibronectin-1/ GAPDH log	P-value
Gender			
Male	82	4.27+0.28	NS
Female	64	3.67+0.25	
Location of tumor			
Rectum	52	4.40+0.27	NS
Proximal colon	43	3.57+0.30	
Distal colon	51	3.97+0.28	
Size of tumor (cm)			
Large (>5)	71	4.51+0.23	0.0024
Small (<5)	75	3.53+0.22	
Type of tumor[a]			
Depressed	129	4.16+0.17	0.0098
Elevated	17	2.84+0.47	
Histologic differentiation[b]			
Well	82	4.02+1.92	NS
Mod	61	4.03+2.11	
Poor	2	2.76+0.13	
Muc	1	3.62	
Depth of tumor invasion			
pTis-pT1(pM-pSM)	8	2.92+0.70	NS
pT2 (pMP)	19	4.25+0.46	
pT3 (pSS/pA)	88	4.12+0.21	
pT4(pSE-PSO/pAI)	31	3.82+0.36	
Lymphatic permeation			
Negative	65	4.38+0.24	0.036
Positive	81	3.70+0.22	
Venous permeation			
Negative	54	4.15+0.27	NS
Positive	91	3.93+0.21	
Lymph node metastasis			
Negative	75	4.01+0.23	NS
Positive	70	3.98+0.24	
Peritoneal dissemination			
Negative	140	4.00+0.17	NS
Positive	6	3.933+0.81	
Liver metastasis			
Negative	126	4.01+0.18	NS
Positive	20	3.98+0.45	
Dukes			
A	21	3.86+0.44	NS
B	49	4.09+0.29	
C	53	4.09+0.28	
D	23	3.77+0.42	

[a]Shape of tumors: depressed-type tumor; 0-IIc, type 2; type 2. Elevated type tumor: 0-Isp, 0-Ip; type 1. [b]Histologic differentiation: well, well differentiated adenocarcinoma; mod, moderately differentiated adenocarcinoma; poor, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma.

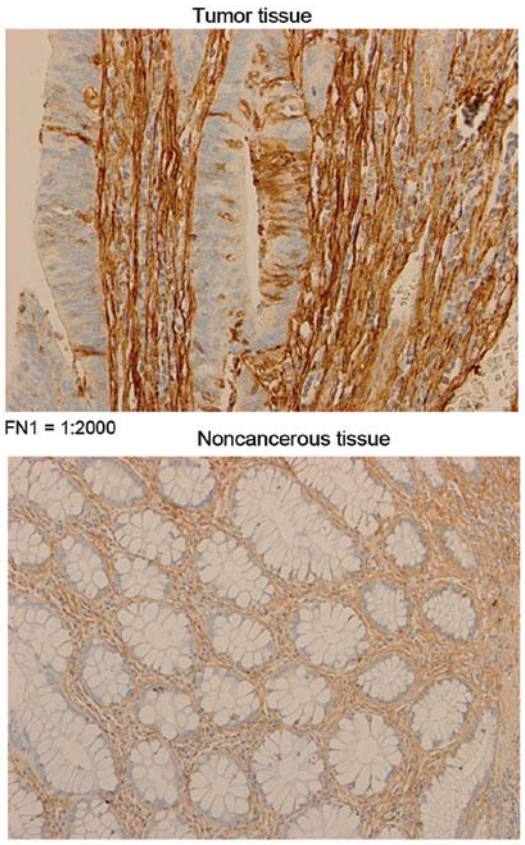

Figure 4. *FN1* expression in representative CRC tissues. *FN1* expression was much stronger in the interstitial tissues of tumors than in the corresponding normal tissues. *FN1*, fibronectin 1; CRC, colorectal cancer.

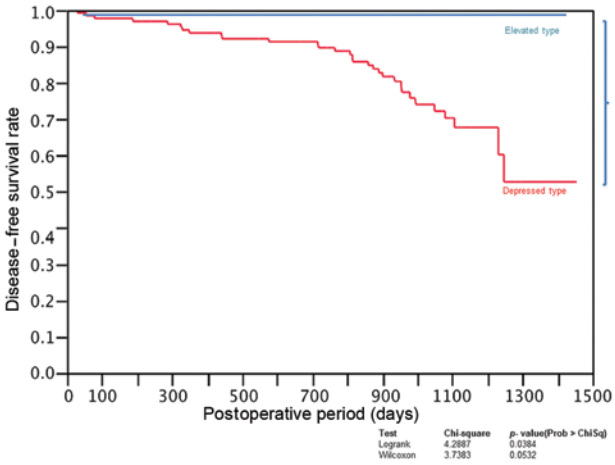

Figure 5. Comparison of disease-free survival rates based on Dukes classification and tumor shape. A Dukes D classification was indicative of the highest recurrence rate among this collection of cases. CRC cases with depressed tumor (n=129) had a much higher incidence of recurrence than those with elevated tumors (n=17; log-rank test; $p<0.05$). CRC, colorectal cancer.

Discussion

The tumorigenesis of depressed tumors progresses along a different pathway from the conventional path for elevated tumors that was advocated by Vogelstein *et al* (4). It is believed

that malignant cells in serrated and adenomatous lesions proliferate and grow laterally and top-down from the surface of the lesion (1-3). However, little is known about how the molecular biology of certain cancer cells is determined. In the present study, we focused on clarifying the mechanism responsible for the differentiation of the shapes of malignant cells. In order to do this, we extracted cancer cells from primary tumor tissues, avoiding contamination with interstitial cells or non-malignant cells with the use of LMD. Microarray analysis following the extraction of total RNA and purified mRNA identified 1 gene, FN1 (10). *FN1* was more highly expressed in the interstitial tissues than in the superficial glands in the section (Fig. 4). We propose that the abundant expression of *FN1* may allow for the generation of traction forces through its surface receptors. However, further *in vitro* and *in vivo* studies are required to answer this question.

CRC characterized by depressed tumors has a higher incidence of recurrence than CRC cases characterized by elevated tumors (Fig. 5). Considering practical clinical applications, reliable markers that can be used to predict tumor shape may facilitate the early diagnosis of malignancy prior to a colonoscopy. In the present study, we identified 1 SNP in *FN1* that was significantly associated with tumor shape.

In conclusion, we found that the majority of CRC cases with depressed tumors had a higher frequency of elevated *FN1* expression. In addition, we could predict the presence of depressed tumors by evaluation of 1 SNP (rs6707530) in the *FN1* region in germline DNA from peripheral blood. This discovery will beneficial in the clinical setting, providing a method for the early diagnosis of depressed-type tumors by colon fiberscope.

References

1. Jass JR, Baker K, Zlobec I, *et al*: Advanced colorectal polyps with the molecular and morphological features of serrated polyps and adenomas: concept of a 'fusion' pathway to colorectal cancer. Histopathology 49: 121-131, 2006.
2. Jass JR, Biden KG, Cummings MC, *et al*: Characterisation of a subtype of colorectal cancer combining features of the suppressor and mild mutator pathways. J Clin Pathol 52: 455-460, 1999.
3. Snover DC, Jass JR, Fenoglio-Preiser C and Batts KP: Serrated polyps of the large intestine: a morphologic and molecular review of an evolving concept. Am J Clin Pathol 124: 380-391, 2005.
4. Vogelstein B, Fearon ER, Hamilton SR, *et al*: Genetic alterations during colorectal-tumor development. N Engl J Med 319: 525-532, 1988.
5. Kudo S, Lambert R, Allen JI, *et al*: Nonpolypoid neoplastic lesions of the colorectal mucosa. Gastrointest Endosc 68 (Suppl 4): S3-S47, 2008.
6. Kudo S, Kashida H, Tamura S and Nakajima T: The problem of 'flat' colonic adenoma. Gastrointest Endosc Clin N Am 7: 87-98, 1997.
7. Kudo S, Tamura S, Hirota S, *et al*: The problem of de novo colorectal carcinoma. Eur J Cancer 31A: 1118-1120, 1995.
8. The Paris endoscopic classification of superficial neoplastic lesions: esophagus, stomach, and colon: November 30 to December 1, 2002. Gastrointest Endosc 58 (Suppl 6): S3-S43, 2003.
9. Update on the Paris classification of superficial neoplastic lesions in the digestive tract. Endoscopy 37: 570-578, 2005.
10. Goossens K, Van Soom A, Van Zeveren A, Favoreel H and Peelman LJ: Quantification of fibronectin 1 (FN1) splice variants, including two novel ones, and analysis of integrins as candidate FN1 receptors in bovine preimplantation embryos. BMC Dev Biol 9: 1, 2009.
11. Sato Y, Yoshizato T, Shiraishi Y, *et al*: Integrated molecular analysis of clear-cell renal cell carcinoma. Nat Genet 45: 860-867, 2013.