

FANCI knockdown increases the sensitivity of MKN45 cells to oxaliplatin

The loss of FANCI is thought to result in a failure of ICL repair [5], and we found that the FANCI expression was decreased after 5FU treatment, as described above. Therefore, we hypothesized that the decrease in FANCI caused by 5FU treatment contributes to the increase in the sensitivity of gastric cancer cells to oxaliplatin. To verify this hypothesis, siRNA directed against FANCI was transfected into MKN45 and MKN45/F2R cells, and their sensitivity to oxaliplatin and 5FU was analyzed by the MTT assay. Before the sensitivity of the cells was analyzed, the mRNA and protein expression levels of FANCI

were evaluated to confirm that the FANCI gene was knocked down. As shown in Fig. 5a, in the MKN45 cells transfected with the siRNA oligonucleotide against FANCI, the expression of FANCI was decreased to 15.3 % compared to that in the control cells. Similarly, the FANCI expression in MKN45/F2R cells was decreased to 25.1 % compared to that in control MKN45/F2R cells (Fig. 5b). Changes in the mRNA expression levels were also confirmed in these cells (data not shown).

We then performed MTT assays for cells treated with oxaliplatin and 5FU. As expected, the IC₅₀ for oxaliplatin in the MKN45 cells after siRNA transfection decreased, to 0.075 μM from 0.177 μM (Fig. 5c; Table 2). On the other hand, the sensitivity of the MKN45 cells to 5FU was not

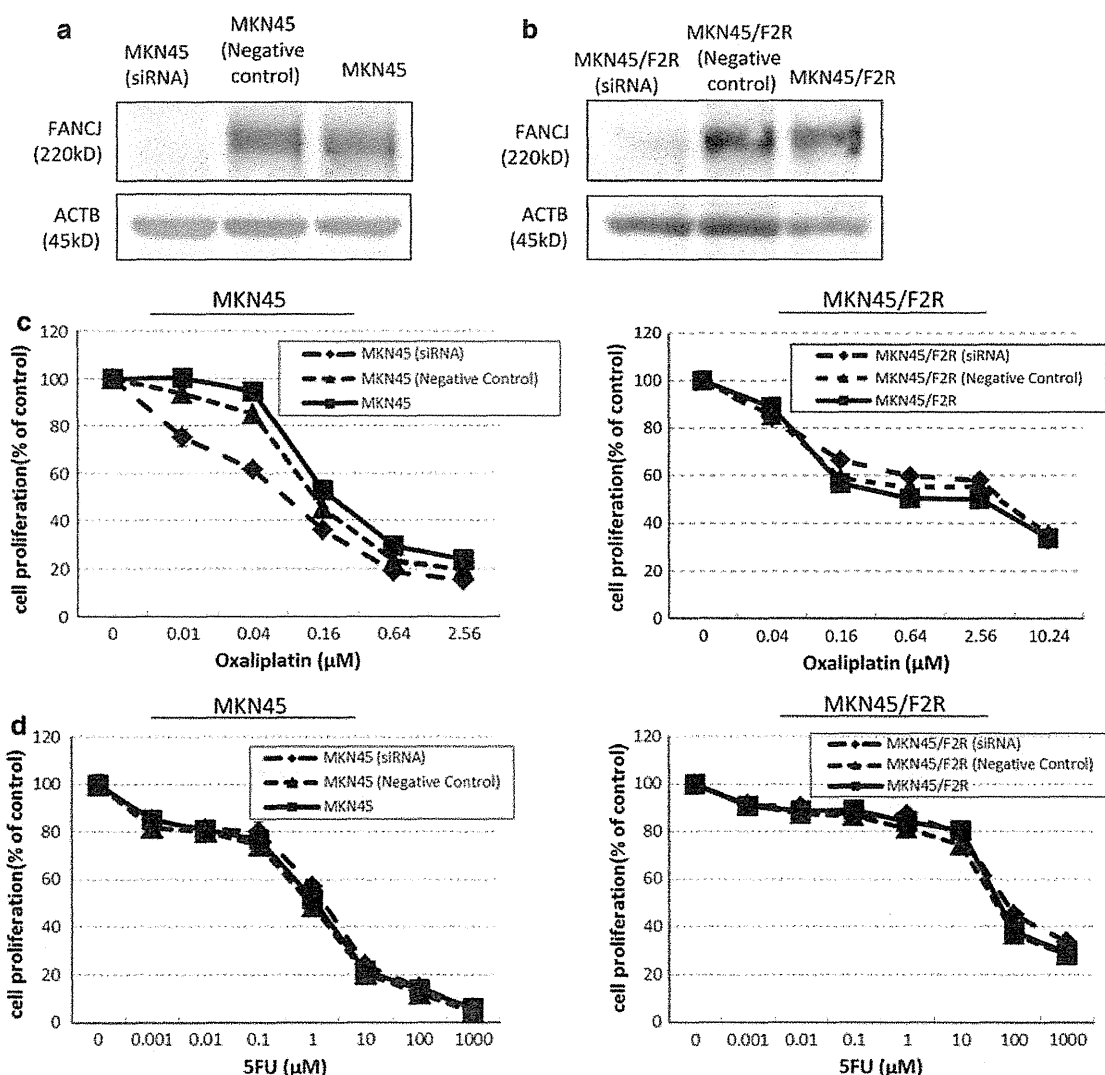


Fig. 5 The downregulation of FANCI after transfection of cells with a small interfering (si) RNA oligonucleotide against FANCI. An siRNA oligonucleotide against FANCI was transfected into **a** MKN45 and **b** MKN45/F2R cells and the expression of FANCI

was evaluated. The in vitro sensitivity to **c** oxaliplatin or **d** 5FU after siRNA transfection demonstrated that the downregulation of FANCI increased the sensitivity of MKN45 cells to oxaliplatin

Table 2 IC50 values for oxaliplatin and 5FU in MKN45 and MKN45/F2R cells after siRNA transfection

Cell line (treatment)	IC50 for oxaliplatin (average \pm SE)	IC50 for 5FU (average \pm SE)
MKN45 (no treatment)	0.177 \pm 0.00992	1.14 \pm 0.888
MKN45 (negative control)	0.135 \pm 0.00175	0.882 \pm 0.281
MKN45 (siRNA)	0.075 \pm 0.0158*	1.65 \pm 0.283
MKN45/F2R (no treatment)	2.58 \pm 0.311	52.4 \pm 8.35
MKN45/F2R (negative control)	3.75 \pm 0.752	44.8 \pm 6.02
MKN45/F2R (siRNA)	3.99 \pm 0.854	72.0 \pm 9.30

MKN45 and MKN45/F2R cells were transfected with a small interfering (si) RNA against FANCI, and the IC50 values were calculated from the results of the MTT assay for oxaliplatin and/or 5FU. The IC50 for oxaliplatin in the MKN45 cells was significantly decreased after siRNA transfection. On the other hand, the IC50 for 5FU in the MKN45 cells was not altered. The IC50 for oxaliplatin and 5FU in the MKN45/F2R cells did not change after siRNA transfection

* $p < 0.05$ based on Student's t -test, compared with untreated MKN45 or MKN45/F2R cells (no treatment)

altered (Fig. 5d; Table 2). The sensitivity of MKN45/F2R cells to oxaliplatin and 5FU did not change after siRNA transfection. These results suggest that decreased FANCI expression increased the sensitivity of MKN45 cells to oxaliplatin, but not to 5FU, while the sensitivity was not altered in 5FU-resistant MKN45/F2R gastric cancer cells.

Discussion

Oxaliplatin, a DACH-containing platinum agent, has a spectrum of activity and mechanisms of action and resistance that appear to be different from those of other platinum-containing compounds, notably cisplatin (CDDP) [22]. Moreover, its anticancer effects are optimized when it is administered in combination with other anticancer agents, such as 5-fluorouracil (5FU) [22], S-1 [23, 24], and capecitabine [25, 26] in gastric and colorectal cancers. There have been several reports about the relationship between the FA pathway and oxaliplatin. For example, it was demonstrated that FANCC- and FANCD2-mutant cells were more sensitive to oxaliplatin and CDDP than FANCA-mutant cells, and mono-ubiquitination of FANCD2, which is mediated by the FANCA- and FANCC-containing FA core complex, was not required for platinum resistance [27]. It was also shown that disruptions of FANCC and FANCG caused a 2-fold increase in the sensitivity of RKO cells to oxaliplatin [28].

With regard to the relationship between FANCI and chemotherapy, Nakanishi et al. reported that there was a correlation between high expression of FANCI and poor

responsiveness of 5FU in colorectal cancer [29]. Our present study is the first to reveal the role of FANCI in the synergism between 5FU and oxaliplatin. However, other reports about the synergistic effects of oxaliplatin or CDDP in combination with 5FU in vitro also exist. For example, Raymond et al. [10] reported that synergistic antiproliferative effects were observed when oxaliplatin was added to 5FU, and the synergistic effects of these combinations were maintained in the 5FU-resistant colon cancer cell line, HT29-5-FU. Scheithauer and Tensch [30] reported that the addition of CDDP to 5FU/leucovorin (LV) yielded synergistic growth inhibition in some human colon cancer cell lines. Our present study revealed that there were synergistic effects of oxaliplatin in combination with 5FU in the MKN45 gastric cancer cell line, and these effects were also observed with CDDP and 5FU (data not shown).

In our study, γ H2AX was increased in MKN45 cells after 5FU treatment. In addition, although BRCA1 protein expression was induced by 5FU treatment, the expression of FANCI was downregulated. This downregulation may have occurred because the FANCI protein was bound to newly synthesized BRCA1 to repair the DSBs caused by 5FU treatment, and FANCI may also have functioned via other mechanisms [31].

In contrast, in the MKN45/F2R 5FU-resistant cells, DSBs did not appear after 5FU treatment, and the expression levels of FANCI and other proteins were not altered after 5FU treatment. These results confirmed that 5FU downregulated the FANCI protein in sensitive cells, and this appears to be important for the activity of 5FU. In the present study, γ H2AX was not detected after treatment with oxaliplatin to the same extent as it was with 5FU (data not shown), suggesting that the induction of DSBs was a phenomenon specifically related to 5FU treatment.

The interaction between FANCI and MutL α (composed of MLH1 and PMS2) is essential for the ICL response [15]. The ICL is first sensed by MutS β , but we examined the MutL α (MLH1-PMS2) complex because FANCI directly binds to MutL α , but not to MutS α or MutS β , and we considered that the interaction between FANCI and MutL α was more directly related to the synergism between 5FU and oxaliplatin. As shown in Fig. 2, the expression levels of MLH1 and PMS2 were not altered after 5FU treatment, while there was decreased FANCI because it was consumed to repair DSBs caused by 5FU treatment. This might have interfered with the repair of ICLs caused by oxaliplatin, thus resulting in the increased sensitivity to oxaliplatin. The involvement of MutS α or β should be examined in the future. A model for the potential involvement of these molecules is illustrated in Fig. 6.

Peng et al. [15] reported that, in the absence of the FANCI protein, it was impossible to displace MutL α from recombination intermediates, and consequently, the MutL α

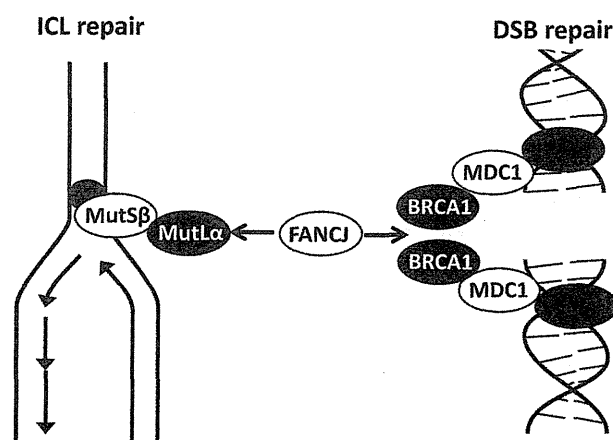


Fig. 6 A model of how FANCI proteins function when cells are treated with 5FU and oxaliplatin. 5FU induces DSBs, while oxaliplatin induces ICLs. Both ICL repair and DSB repair require the FANCI protein. Because there is a lack of FANCI when cells are treated with both drugs, there is synergism between 5FU and oxaliplatin

complex remained stuck to DNA for a longer time period, delaying the exit from the G2/M arrest and enhancing ICL sensitivity [5]. In our study, the level of FANCI in the MKN45 cells was decreased after 5FU treatment. As would be expected based on the report by Peng et al., the sensitivity of the MKN45 cells to oxaliplatin increased when FANCI was knocked down by siRNA. We initially tried to force the expression of FANCI in the cells by transfection, because we wanted to confirm whether the synergism between 5FU and oxaliplatin was reversed by FANCI overexpression. However, there are various other molecules involved in the synergism, such as BRCA1, MLH1, and so on. This led us to examine the direct effects of FANCI using an siRNA knockdown system. Our findings suggest that the decrease in FANCI caused by 5FU treatment leads to an increase in the sensitivity to oxaliplatin, resulting in synergistic cytotoxic effects exerted by the combination of 5FU and oxaliplatin in MKN45 5FU-sensitive cells. In the MKN45/F2R cells, the synergistic effect of oxaliplatin and 5FU was not observed, partly because DSBs did not occur after 5FU treatment in these cells.

In conclusion, the present study provides the first evidence of the role of FANCI in the synergism between 5FU and oxaliplatin, and can be regarded as providing a rationale for using a combination of fluoropyrimidine and platinum agents for the treatment of gastric carcinomas [22].

Acknowledgments This work was supported by Grants-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports, and Culture of Japan and a grant from the Japanese Foundation for Multidisciplinary Treatment of Cancer.

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References

- Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. *CA Cancer J Clin.* 2011;61:69–90.
- Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin.* 2005;55:74–108.
- Koizumi W, Narahara H, Hara T, Takagane A, Akiya T, Takagi M, et al. S-1 plus cisplatin versus S-1 alone for first-line treatment of advanced gastric cancer (SPIRITS trial): a phase III trial. *Lancet Oncol.* 2008;9:215–21.
- Colucci G, Gebbia V, Paoletti G, Giuliani F, Caruso M, Gebbia N, et al. Phase III Randomized Trial of FOLFIRI Versus FOLFOX4 in the Treatment of Advanced Colorectal Cancer: a Multicenter Study of the Gruppo Oncologico Dell'Italia Meridionale. *J Clin Oncol.* 2005;23:4866–75.
- Omura K. Advances in chemotherapy against advanced or metastatic colorectal cancer. *Digestion.* 2008;77(Suppl 1):13–22.
- Cavanna L, Artioli F, Codignola C. Oxaliplatin in combination with 5-fluorouracil (5-FU) and leucovorin (LV) in patients with metastatic gastric cancer (MGC). *Am J Clin Oncol.* 2006;29:371–5.
- Oh SY, Kwon HC, Seo BG, Kim SH, Kim JS, Kim HJ. A phase II study of oxaliplatin with low dose leucovorin and bolus and continuous infusion 5-fluorouracil (modified FOLFOX-4) as first line therapy for patients with advanced gastric cancer. *Acta Oncol.* 2007;46:336–41.
- Takashima A, Yamada Y, Nakajima TE, Kato K, Hamaguchi T, Shimada Y. Standard first-line chemotherapy for metastatic gastric cancer in Japan has met the global standard: evidence from recent phase III trials. *Gastrointest Cancer Res.* 2009;3:239–44.
- Raymond E, Chaney SG, Taamma A, Cvitkovic E. Oxaliplatin: a review of preclinical and clinical studies. *Ann Oncol.* 1998;9:1053–71.
- Raymond E, Buquet-Fagot C, Djelloul S, Mester J, Cvitkovic E, Allain P, et al. Antitumor activity of oxaliplatin in combination with 5-fluorouracil and the thymidylate synthase inhibitor AG337 in human colon, breast, and ovarian cancers. *Anticancer Drugs.* 1997;8:876–85.
- Hiom K. FANCI: solving problems in DNA replication. *DNA Repair.* 2010;9:250–6.
- Wu Y, Brosh RM Jr. FANCI helicase operates in the Fanconi anemia DNA repair pathway and the response to replicational stress. *Curr Mol Med.* 2009;9:470–82.
- Litman R, Peng M, Jin Z, Zhang F, Zhang J, Powell S, et al. BACH1 is critical for homologous recombination and appears to be the Fanconi anemia gene product FANCI. *Cancer Cell.* 2005;8:255–65.
- Moldovan GL, D'Andrea AD. How the Fanconi anemia pathway guards the genome. *Annu Rev Genet.* 2009;43:223–49.
- Peng M, Litman R, Xie J, Sharma S, Brosh RM Jr, Cantor SB. The FANCI/MutL interaction is required for correction of the cross-link response in FA-J cells. *EMBO J.* 2007;26:3238–49.
- Matuo R, Sousa FG, Escargueil AE, Soares DG, Grivicich I, Saffi J, et al. DNA repair pathways involved in repair of lesions induced by 5-fluorouracil and its active metabolite FdUMP. *Biochem Pharmacol.* 2010;79:147–53.
- van der Wilt CL, Kuiper CM, Peters GJ. Combination studies of antifolates with 5-fluorouracil in colon cancer cell lines. *Oncol Res.* 1999;11:383–91.

18. Tsutani Y, Yoshida K, Sanada Y, Wada Y, Konishi K, Fukushima M, et al. Decreased orotate phosphoribosyltransferase activity produces 5-fluorouracil resistance in a human gastric cancer cell line. *Oncol Rep.* 2008;20:1545–51.
19. Tallarida RJ. Drug synergism: its detection and applications. *J Pharmacol Exp Ther.* 2001;298:865–72.
20. Rogakou EP, Pilch DR, Orr AH, Ivanova VS, Bonner WM. DNA double-stranded breaks induce histone H2AX phosphorylation on serine 139. *J Biol Chem.* 1998;273:5858–68.
21. Mah LJ, El-Osta A, Karagiannis TC. GammaH2AX: a sensitive molecular marker of DNA damage and repair. *Leukemia.* 2010;24:679–86.
22. Raymond E, Faivre S, Chaney S, Woynarowski J, Cvitkovic E. Cellular and molecular pharmacology of oxaliplatin. *Mol Cancer Ther.* 2002;1:227–35.
23. Koizumi W, Takiuchi H, Yamada Y, Boku N, Fuse N, Muro K, et al. Phase II study of oxaliplatin plus S-1 as first-line treatment for advanced gastric cancer (G-SOX study). *Ann Oncol.* 2010;21:1001–5.
24. Oh SY, Kwon HC, Jeong SH, Joo YT, Lee YJ, Hee Cho S, et al. A phase II study of S-1 and oxaliplatin (SOx) combination chemotherapy as a first-line therapy for patients with advanced gastric cancer. *Invest New Drugs.* 2010. doi:10.1007/s10637-010-9507-2.
25. Rothenberg ML, Cox JV, Butts C, Navarro M, Bang YJ, Goel R, et al. Capecitabine plus oxaliplatin (XELOX) versus 5-fluorouracil/folinic acid plus oxaliplatin (FOLFOX-4) as second-line therapy in metastatic colorectal cancer: a randomized phase III noninferiority study. *Ann Oncol.* 2008;19:1720–6.
26. Ducreux M, Bennouna J, Hebbar M, Ychou M, Lledo G, Conroy T, et al. Capecitabine plus oxaliplatin (XELOX) versus 5-fluorouracil/leucovorin plus oxaliplatin (FOLFOX-6) as first-line treatment for metastatic colorectal cancer. *Int J Cancer.* 2011;128:682–90.
27. Kachnic LA, Li L, Fournier L, Willers H. Fanconi anemia pathway heterogeneity revealed by cisplatin and oxaliplatin treatments. *Cancer Lett.* 2010;292:73–9.
28. Gallmeier E, Calhoun ES, Rago C, Brody JR, Cunningham SC, Hucl T, et al. Targeted disruption of FANCC and FANCG in human cancer provides a preclinical model for specific therapeutic options. *Gastroenterology.* 2006;130:2145–54.
29. Nakanishi R, Kitao H, Fujinaka Y, Yamashita N, Iimori M, Tokunaga E, et al. FANCI expression predicts the response to 5-fluorouracil-based chemotherapy in MLH1-proficient colorectal cancer. *Ann Surg Oncol.* 2012 (epub ahead of print).
30. Scheithauer W, Temeš EM. A study of various strategies to enhance the cytotoxic activity of 5-fluorouracil/leucovorin in human colorectal cancer cell lines. *Anticancer Res.* 1989;9:1793–8.
31. Xie J, Litman R, Wang S, Peng M, Guillemette S, Rooney T, et al. Targeting the FANCI-BRCA1 interaction promotes a switch from recombination to telomerase-dependent bypass. *Oncogene.* 2010;29:2499–508.



Expression of olfactomedin 4 and claudin-18 in serrated neoplasia of the colorectum: a characteristic pattern is associated with sessile serrated lesion

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Date of submission 5 August 2012

Accepted for publication 13 January 2013

Published online Article Accepted 18 January 2013

Sentani K, Sakamoto N, Shimamoto F, Anami K, Oue N & Yasui W

(2013) *Histopathology* 62, 1018–1027

Expression of olfactomedin 4 and claudin-18 in serrated neoplasia of the colorectum: a characteristic pattern is associated with sessile serrated lesion

Aims: Olfactomedin 4 is a useful marker for stem cells in the intestine and is an independent prognostic molecule for survival in patients with colorectal cancer (CRC). Claudin-18, a component of tight junctions, correlates with poor survival in patients with CRC and is associated with the gastric phenotype. We investigated the possible usefulness of these molecules in serrated neoplasia of the colorectum.

Methods and results: We performed immunohistochemical analysis of colorectal polyps, including hyperplastic polyps (HP), sessile serrated lesions (SSL), traditional serrated adenomas (TSA) and conventional adenomas (CA). We also investigated the association between expression of these molecules and clinicopathological parameters in serrated adenocarci-

noma (SAC) and non-SAC of the colorectum. Olfactomedin 4 expression was not detected or was decreased in SSL compared with the other polyp types. Claudin-18 expression was higher in SSL than in the other types. Similarly, positivity for olfactomedin 4 in SAC was significantly lower than that in non-SAC, and positivity for claudin-18 in SAC was significantly higher than that in non-SAC. Furthermore, claudin-18-positive SAC showed more advanced N grade and stage than claudin-18-negative SAC.

Conclusions: Reduced expression of olfactomedin 4 and ectopic expression of claudin-18 might be useful markers in the differential diagnosis of serrated polyps.

Keywords: claudin-18, olfactomedin 4, serrated adenocarcinoma, sessile serrated lesion

Introduction

Serrated polyps of the colorectum include the hyperplastic polyp (HP), the traditional serrated adenoma (TSA) and the sessile serrated lesion (SSL). The conventional adenoma (CA)–carcinoma pathway has been accepted widely as the evolutionary paradigm for colorectal cancer (CRC), recognizing molecular

counterparts to a stepwise process. Recently, a ‘serrated polyp–neoplasia pathway’ has been proposed for some CRCs.¹ Most investigators consider HP to be an incidental finding with no potential for neoplastic progression.² In contrast, there is increasing molecular and morphological evidence to support the concept that SSL may be a potential precursor of CRC.^{1,3} The accurate diagnosis of serrated precursor lesions is, therefore, important, and pathologists need to classify these lesions consistently.⁴ The diagnostic features of SSL generally comprise abnormal proliferation and dysmaturation,⁴ including expanded crypt proliferation zones, basilar crypt dilatation and serrated

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architecture, and decreased cell maturation.⁵ However, diagnosis of SSL based solely on histological evaluation is difficult, and is subject to interobserver variability.⁶ In some cases, differentiation of TSA from HP or SSL can also be difficult. Thus, ancillary studies, such as demonstration of a distinct immunophenotype, might prove to be helpful tools if morphological assessment alone is not reliable enough in routine pathology practice. Currently, there are several immunohistochemical markers to assist conventional morphological diagnosis, including p53 and p504S,⁷ MLH1 and MSH2,⁴ cytokeratin 20 and Ki67,⁸ the profile of mucin core proteins such as MUC5AC, MUC2 and MUC6^{9,10} and other molecular markers, including microRNA-181b and microRNA-21.¹¹ Serrated adenocarcinoma (SAC) represents an endpoint in the serrated neoplasia pathway and has been recognized as a distinct entity among CRCs, of which it makes up 7.5%.¹² These tumours are found most commonly in older women, with a female-to-male ratio of approximately 2:1.¹² Most SACs have been reported to arise in association with TSA and SSL.^{3,13}

Olfactomedin 4 was cloned originally from human myeloid cells and encodes a secreted glycoprotein of 510 amino acids. It is expressed normally in the bone marrow, intestine and prostate, and altered expression is observed in various cancers, including those of the stomach, colon, breast and lung.¹⁴ Olfactomedin 4 inhibits apoptosis and may have significant roles in the development of cancer.¹⁵ We have reported previously that it was an independent prognostic molecule for survival in patients with CRC.¹⁶ It has been proposed that olfactomedin 4 may serve as a useful marker for stem cells in the human small intestine and colon.¹⁷

Claudin proteins, a family of proteins comprising at least 24 members, are components of tight junction strands that regulate paracellular transport and lateral diffusion of membrane lipids and proteins.¹⁸ Claudins are expressed in an organ-specific manner, and altered claudin-18 expression has been documented in various diseases. Expression of claudin-18 is increased in both experimental colitis and human inflammatory bowel disease.¹⁹ We have reported previously that ectopic expression of claudin-18 was observed in 4% of 569 CRCs and correlated with poor survival and gastric mucin phenotype.²⁰ In addition, we showed ectopic expression of claudin-18 in signet ring cell carcinoma of CRC.²¹

The present study represents the first detailed analysis of the expression of olfactomedin 4 and claudin-18 in serrated neoplasia of the colorectum. To assess the possible usefulness of these molecules in routine pathology practice, we performed an immunohistochemical

analysis of endoscopically or surgically resected samples. In addition, we also investigated the association between expression of these molecules and clinicopathological parameters in SAC and non-SAC of the colorectum.

Materials and methods

TISSUE SAMPLES AND HISTOLOGICAL EVALUATION

Primary samples were collected randomly from patients who underwent endoscopic or surgical resection at Hiroshima University Hospital or its affiliated hospitals. Of the 215 colorectal polyps collected, 66 were diagnosed histologically as HP (microvesicular subtype), 45 as SSL (without dysplasia), 47 as TSA and 57 as CA. The 254 cases of CRC comprised 36 SACs and 218 non-SACs of the colorectum.

Diagnostic classification of the serrated polyps was based on previously proposed criteria from Snover^{1,22} and Torlakovic *et al.*⁴ The diagnosis of SAC was based exclusively on validated histopathological criteria.^{12,23} In brief, SAC was characterized by evident epithelial serrations composed only of epithelium or epithelium and basement membrane material, clear or eosinophilic and often abundant cytoplasm, vesicular nuclei, absence of or less than 10% necrosis of the total surface area, mucin production, and the presence of serrations and eosinophilic cell globules and rod-like structures floating freely in the mucus. Tumour staging was classified according to the criteria of the AJCC/UICC TNM classification. Histology was according to the WHO classification.²⁴ Because written informed consent was not obtained from the patients, identifying information for all samples was removed before analysis to ensure strict privacy protection. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research enacted by the Japanese Government.

IMMUNOHISTOCHEMISTRY

One or two representative tissue blocks from each patient were examined by immunohistochemistry. A Dako Envision Kit (Dako, Carpinteria, CA, USA) was used for immunohistochemical analysis of all markers. In brief, 5- μ m thick sections were pretreated by microwaving in citrate buffer for 15 min to retrieve antigenicity. After peroxidase activity was blocked with 3% H₂O₂-methanol for 10 min, sections were incubated with normal goat serum (Dako) for 20 min to block non-specific antibody binding sites. Sections were

incubated with the following primary antibodies: anti-olfactomedin 4 (diluted 1:50), anti-claudin-18 (diluted 1:50) and anti-Ki67 (diluted 1:50; clone MIB-1; Dako). The anti-olfactomedin 4 antibody was a monoclonal antibody raised in our laboratory, the specificity of which has been characterized in detail.²⁵ The anti-claudin-18 antibody (C-terminal) was purchased from Invitrogen/Zymed Laboratories, Inc. (San Francisco, CA, USA); this antibody was the same as that used in our previous study^{20,26} and recognizes only claudin-18a2. Sections were incubated with primary antibody for 1 h at 25°C, followed by incubations with biotinylated anti-rabbit/mouse IgG and peroxidase-labelled streptavidin for 10 min each. Staining was completed with a 10-min incubation with the substrate-chromogen solution. The sections were counterstained with 0.1% haematoxylin. Negative controls were created by omission of the primary antibody.

Olfactomedin 4, claudin-18 and Ki67 staining was classified according to the percentage of stained cells. Expression of olfactomedin 4 in colorectal polyps was considered to be negative if <1% of cells were observed to be stained. Expression of claudin-18 and Ki67 in colorectal polyps, and olfactomedin 4 in SAC or non-SAC, was considered to be negative if <10% of cells were stained, and positive when at least 10% were stained. In addition, we divided the crypts of the colorectal polyps into three parts, and distribution patterns of positive cells were analysed according to the following classification: confined to lower one-third of the crypts, extending to two-thirds of the crypts, and diffuse. To reduce interobserver variation, the results of immunostaining were evaluated independently by two investigators (K.S. and F.S.), and when the evaluations differed a decision was made by consensus while investigators reviewed the specimen simultaneously using a multihead microscope. Percentage values were rounded to a whole number.

STATISTICAL METHODS

Correlations between clinicopathological parameters and immunohistochemical staining were analysed by Fisher's exact test. A *P*-value of <0.05 was considered statistically significant.

Results

EXPRESSION PATTERNS OF OLFACTOMEDIN 4 AND CLAUDIN-18 IN COLORECTAL POLYPS

The distribution of colorectal polyps was as follows: 66 HP (28 right-sided, 38 left-sided), 45 SSL (32

right-sided, 13 left-sided), 47 TSA (14 right-sided, 33 left-sided) and 57 CA (25 right-sided, 32 left-sided). We performed immunostaining of olfactomedin 4 or claudin-18 in these polyps, and also in the mucosa of 85 normal colorectal specimens. The results of immunohistochemistry are summarized in Table 1.

Olfactomedin 4 staining showed a cytoplasmic expression pattern in these colorectal polyps and normal colorectal mucosa (Figure 1). In the normal mucosa olfactomedin 4 expression was limited to the basal portions of the crypts.¹⁶ Expression in HP was confined predominantly to the lower one-third of the crypts. In the 45 SSL cases, olfactomedin 4 was located in the lower one-third of the crypts in 24 (53%) cases and extended beyond the lower one-third of the crypts in 10 (22%) cases. In the remaining 24% of SSL cases, however, fewer than 1% of stained cells were observed (Table 1 and Figure 1). Olfactomedin 4 expression was detected in all 66 HP cases and 34 (75%) of 45 SSL cases, and positivity for olfactomedin 4 in SSL was significantly lower than that in HP (*P* < 0.0001). In both TSA and CA olfactomedin 4 was observed mainly throughout the full length of the epithelium, both at the surface and at the base of the polyp. SSL was more likely to be located in the right colon compared with HP, TSA or CA; however, olfactomedin 4 expression in SSL showed no significant regional difference (data not shown).

We also assessed the distribution of positive cells for the proliferation marker Ki67. In normal colorectal mucosa, Ki67 was expressed in the lower one-third of the crypts. The majority of HP (86%) and SSL (80%) cases showed Ki67 staining confined to the basal third of the crypts, whereas expression of Ki67 in the majority of TSA (87%) and all CA (100%) cases was distributed throughout the crypts (Figure 1). Distributions of olfactomedin 4 and Ki67 expression in HP, TSA and CA appeared to be roughly similar. However, Ki67-positive cells were located in the lower one-third of the crypts in 36 (80%) of 45 SSL cases and extended beyond the lower one-third of the crypts in 9 (20%) of these 45 cases. Positivity for olfactomedin 4 in SSL was significantly lower than that of Ki67 (76% versus 100%, *P* < 0.0001).

Next, we performed immunohistochemical analysis using claudin-18 antibody. Claudin-18 staining was seen exclusively on the cell membrane.²⁰ Claudin-18 was expressed in 44% of SSL cases, which was significantly higher than that for TSA cases (12%, *P* = 0.001). Similarly, significant differences were observed in claudin-18 staining between TSA and HP

Table 1. Localization of cells positive for olfactomedin 4, claudin-18, and Ki67 in 215 colorectal polyps

	Localization of positive cells in the crypts ($\geq 1\%$)				<i>P</i> value
	Lower one-third	Up to two-thirds	Diffuse	Negative (1%>)	
Olfactomedin 4					
HP (<i>n</i> = 66)	64 (97%)	2 (3%)	0	0	–
SSL (<i>n</i> = 45)	24 (53%)	8 (18%)	2 (4%)	11 (24%)	<0.0001
TSA (<i>n</i> = 47)	2 (4%)	4 (9%)	41 (87%)	0	–
CA (<i>n</i> = 57)	0	0	57 (100%)	0	–
Normal (<i>n</i> = 85)	85 (100%)	0	0	0	–
	Localization of positive cells in the crypts ($\geq 10\%$)				<i>P</i> value
	Lower one-third	Up to two-thirds	Diffuse	Negative (10%>)	
Ki67					
HP (<i>n</i> = 66)	57 (86%)	9 (14%)	0	0	–
SSL (<i>n</i> = 45)	36 (80%)	8 (18%)	1 (2%)	0	NS
TSA (<i>n</i> = 47)	0	6 (13%)	41 (87%)	0	–
CA (<i>n</i> = 57)	0	0	57 (100%)	0	–
Normal (<i>n</i> = 85)	85 (100%)	0	0	0	–
Claudin-18					
HP (<i>n</i> = 66)	0	0	0	66 (100%)	–
SSL (<i>n</i> = 45)	15 (33%)	3 (7%)	2 (4%)	25 (56%)	<0.0001
TSA (<i>n</i> = 47)	2 (4%)	3 (6%)	1 (2%)	41 (87%)	–
CA (<i>n</i> = 57)	0	0	0	57 (100%)	–
Normal (<i>n</i> = 85)	0	0	0	85 (100%)	–

HP, Hyperplastic polyp; SSL, sessile serrated lesion; TSA, traditional serrated adenoma; CA, conventional adenoma; NS, not significant. *P* values of <0.05 were considered statistically significant (SSL versus HP, TSA and CA).

or CA, the latter two being negative for claudin-18 ($P = 0.0042$ and $P = 0.0071$, respectively). There were no HP and CA cases with more than 1% of cells stained, and 14 (31%) of 45 SSL cases and 4 (9%) of 47 TSA cases with more than 20% of cells stained. Claudin-18 expression in SSL or TSA showed no significant regional differences (data not shown).

On the basis of these results, we presumed that SSL might show loss of expression of olfactomedin 4 and/or increased expression of claudin-18 compared with that in HP, TSA or CA. The sensitivity and specificity of olfactomedin 4 for SSL were 24% and 100%, respectively. The sensitivity and specificity of claudin-18 for SSL were 44% and 96%, respectively. Furthermore, the sensitivity and specificity of olfactomedin 4

combined with claudin-18 were 53% (24 of 45) and 96% (164 of 170), respectively. The combination of olfactomedin 4 and claudin-18 elevated the diagnostic sensitivity, and the results suggest that these molecules have potential utility in the diagnosis of SSL.

RELATION BETWEEN EXPRESSION OF OLFACTOMEDIN 4 OR CLAUDIN-18 AND CLINICOPATHOLOGICAL PARAMETERS IN SAC OR NON-SAC

Next, we performed immunostaining for olfactomedin 4 or claudin-18 in 36 colorectal SACs and 218 non-SACs. In general, staining for olfactomedin 4 was detected in the cytoplasm of tumour cells. In

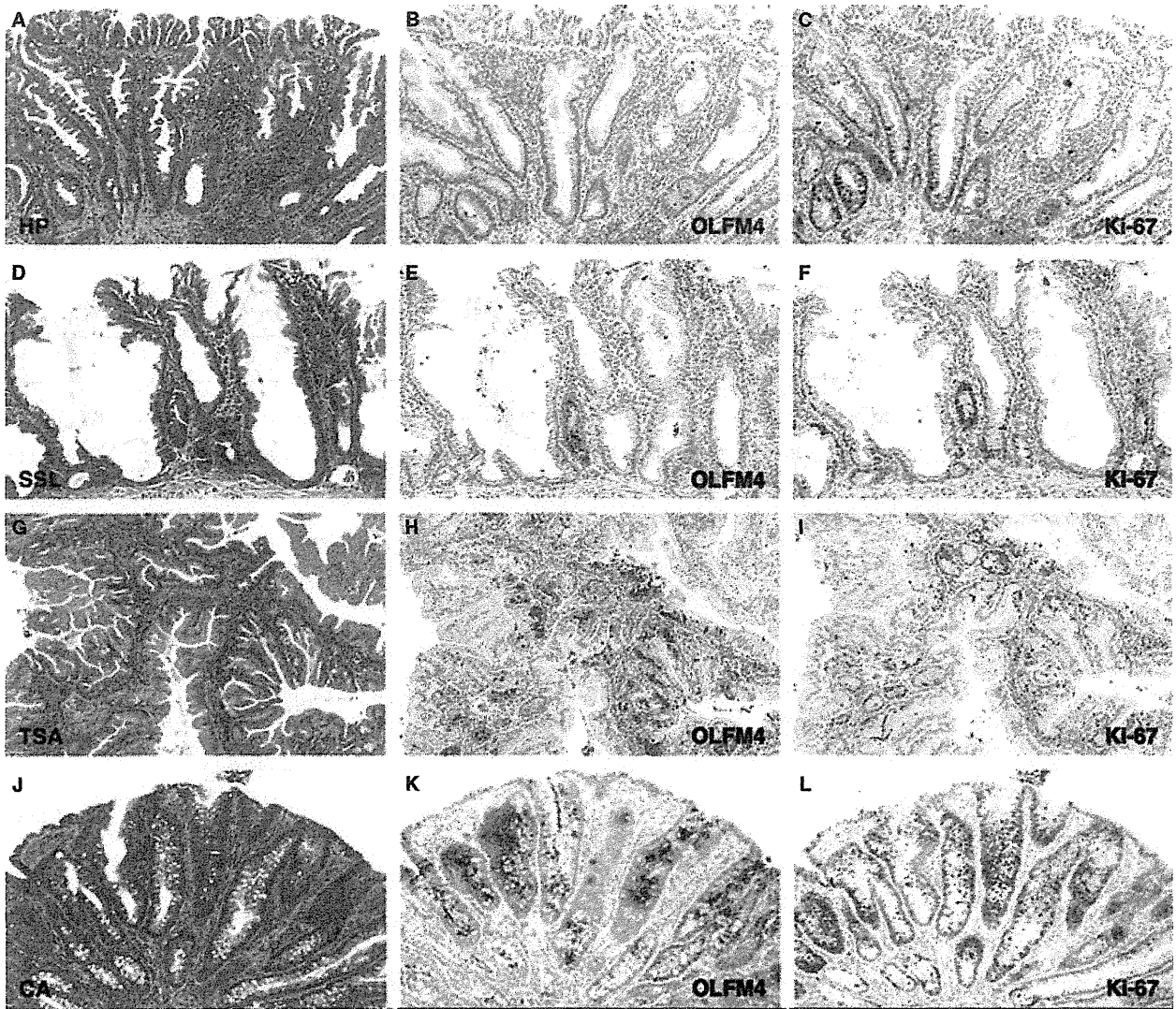


Figure 1. Immunohistochemical staining for olfactomedin 4 (OLFM4) and Ki67 in hyperplastic polyp (HP; A–C), sessile serrated lesion (SSL; D–F), traditional serrated adenoma (TSA; G–I) and conventional adenoma (CA; J–L). A,D,G,J, H&E staining. B,C, Expression of OLFM4 and Ki67 in HP was confined predominantly to the lower one-third of the crypts. E,F, Some SSL included <1% of OLFM4-positive cells, whereas Ki67-positive cells were located in the lower one-third of the crypts. H,I and K,L, Expression of OLFM4 and Ki67 in the majority of TSA and CA was distributed throughout the crypts.

peritumoral mucosa of CRC, almost all epithelial cells showed olfactomedin 4 staining regardless of whether staining was seen in the tumour cells (Figure 2). Olfactomedin 4 staining decreased gradually, moving away from the CRC tissue, and in mucosa distant from the tumour tissue was limited to the basal crypt. Olfactomedin 4 expression was detected in three (8%) of 36 SAC cases and 75 (34%) of 218 non-SAC cases ($P = 0.0014$). Olfactomedin 4-positive non-SAC cases showed earlier N grade ($P = 0.041$) and stage ($P = 0.031$) than did olfactomedin 4-negative

non-SAC cases, but olfactomedin 4 staining did not correlate with any clinicopathological parameters in the SAC cases (Table 2).

Next, the relation of claudin-18 staining with clinicopathological characteristics was investigated (Table 3). Claudin-18 expression was detected in 10 (28%) of 36 SAC cases and 11 (5%) of 218 non-SAC cases ($P = 0.0001$). There were no differences in claudin-18 expression levels between intratumoral areas and infiltrative margins or in the presence or absence of vessel infiltration. Some coexisting serrated

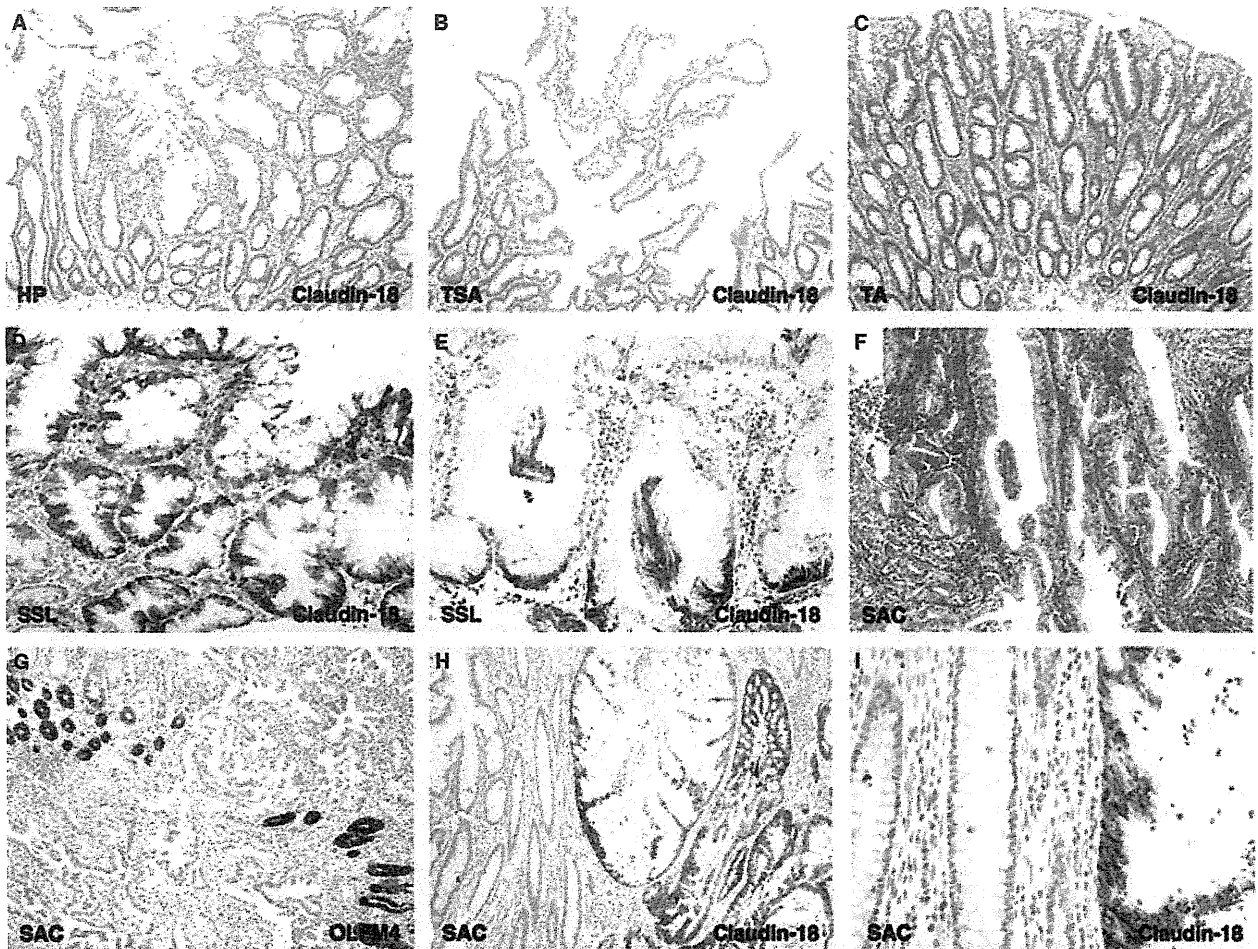


Figure 2. Immunohistochemical staining for claudin-18 or olfactomedin 4 (OLFM4) in various colorectal polyps and serrated adenocarcinoma (SAC). A–E, Some sessile serrated lesions (SSL) showed membranous staining for claudin-18, compared with other colorectal polyps. F, H&E staining of SAC. G–I, Loss of expression of OLFM4 and/or increased expression of claudin-18 was observed in SAC.

polyps expressed claudin-18, whereas corresponding non-neoplastic colorectal mucosa did not express claudin-18. Claudin-18-positive SAC cases showed more advanced N grade ($P = 0.022$) and stage ($P = 0.022$) than did claudin-18-negative SAC cases, but claudin-18 staining did not correlate with any clinicopathological parameters in the non-SAC cases.

Discussion

There is growing evidence that a subset of serrated colorectal polyps may serve as precursor lesions for CRC resulting from the serrated pathway.^{27,28} Of these polyps, SSL bear a closer histological resemblance to the traditional HP than to TSA or CA, which are identified easily on H&E-stained slides. Morphological criteria have been proposed to distinguish

SSL from traditional HP.^{4,5} These criteria take into account the location and size of the polyp and the architectural and cytological features of the crypt. The application of morphological criteria leads inevitably to intraobserver and interobserver variability, and specimen orientation and cautery artefacts can also interfere with interpretation. A biomarker that delineates conventional benign serrated polyps from SSL objectively and reproducibly would help practising pathologists in assessing equivocal cases. In the present study, we demonstrated clearly that olfactomedin 4 and claudin-18 are useful immunohistochemical markers of SSL.

Various serrated lesions have characteristic abnormalities of the proliferative zone and resultant architectural changes.²² In SSL, as-yet unknown factors lead to alterations in the location of the proliferative

Table 2. Olfactomedin 4 expression and clinicopathological parameters in 36 colorectal serrated adenocarcinomas and 218 non-serrated adenocarcinomas

	Serrated (<i>n</i> = 36)		<i>P</i> value	Non-serrated (<i>n</i> = 218)		<i>P</i> value
	Positive	Negative		Positive	Negative	
Total	3 (8%)	33	–	75 (34%)	143	–
Age (years)						
≤ 65	0	13	NS	39 (34%)	76	NS
>65	3 (13%)	20	–	36 (35%)	67	–
Sex						
Male	3 (21%)	11	NS	41 (34%)	81	NS
Female	0	22	–	34 (35%)	62	–
Tumour location						
Right/transverse	0	15	NS	18 (32%)	38	NS
Left/sigmoid/rectum	3 (14%)	18	–	57 (35%)	105	–
T grade						
Tis/T1/T2	1 (20%)	4	NS	30 (42%)	42	NS
T3/T4	2 (6%)	29	–	45 (31%)	101	–
N grade						
N0	2 (13%)	14	NS	52 (40%)	78	0.041
N1/N2	1 (5%)	19	–	23 (26%)	65	–
M grade						
M0	2 (6%)	31	NS	67 (35%)	122	NS
M1	1 (33%)	2	–	8 (28%)	21	–
Stage						
0/I/II	2 (13%)	14	NS	51 (40%)	75	0.031
III/IV	1 (5%)	19	–	24 (26%)	68	–
Histological type						
Well/moderately	3 (10%)	27	NS	73 (35%)	136	NS
Poorly/mucinous	0	6	–	2 (22%)	7	–

NS, Not significant.

zone away from its usual location in the base of the crypts, resulting in maturation which may develop towards the base of the crypts and lead to distortion of the crypt architecture, commonly with dilated or L-, inverted T- or anchor-shaped crypts with mature cells where the proliferative zone is normally located.⁸ In this study, the proliferation marker Ki67 showed variable staining in colorectal serrated polyps. However, Ki67 does not appear helpful in distinguishing SSL from HP in pathology practice, as reported previously.²⁹ There were roughly similar distributions of

olfactomedin 4 and Ki67 expression in HP, TSA and CA. This result appeared to be convincing when the localization and role of olfactomedin 4 in proliferation and cell cycle progression was considered.¹⁷ However, positivity for olfactomedin 4 in SSL was significantly lower than that for Ki67. There are, to our knowledge, no previous reports of the reduction or loss of olfactomedin 4 in SSL. However, reduced expression of olfactomedin 4 was reported to be regulated at the transcriptional or post-transcriptional level rather than at the genomic level in CRC.³⁰ The most

Table 3. Claudin-18 expression and clinicopathological parameters in 36 colorectal serrated adenocarcinomas and 218 non-serrated adenocarcinomas

	Serrated (<i>n</i> = 36)		<i>P</i> value	Non-serrated (<i>n</i> = 218)		<i>P</i> value
	Positive	Negative		Positive	Negative	
Total	10 (28%)	26	–	11 (5%)	207	–
Age (years)						
≤ 65	3 (23%)	10	NS	5 (4%)	110	NS
>65	7 (30%)	16	–	6 (6%)	97	–
Sex						
Male	6 (43%)	8	NS	5 (4%)	117	NS
Female	4 (18%)	18	–	6 (6%)	90	–
Tumour location						
Right/transverse	4 (27%)	11	NS	4 (7%)	52	NS
Left/sigmoid/rectum	6 (29%)	15	–	7 (4%)	155	–
T grade						
Tis/T1/T2	0	5	NS	4 (6%)	68	NS
T3/T4	10 (32%)	21	–	7 (5%)	139	–
N grade						
N0	1 (6%)	15	0.022	6 (5%)	124	NS
N1/N2	9 (45%)	11	–	5 (6%)	83	–
M grade						
M0	8 (32%)	25	NS	8 (4%)	181	NS
M1	2 (67%)	1	–	3 (10%)	26	–
Stage						
0/I/II	1 (6%)	15	0.022	5 (4%)	121	NS
III/IV	9 (45%)	11	–	6 (7%)	86	–
Histological type						
Well/moderately	8 (27%)	22	NS	11 (5%)	198	NS
Poorly/mucinous	2 (33%)	4	–	0	9	–

NS, Not significant.

common CRCs arising via the serrated pathway are prone to methylation of the CpG island promoter regions, resulting in epigenetic silencing of a number of genes. It is presumed that the specific genes silenced may be random events. The most well-characterized epigenetic silencing in these lesions is that of the *hMLH1* gene, which is silenced in sporadic microsatellite instability carcinomas. Aberrant hypermethylation of bone morphogenic protein 3 was also observed frequently in colorectal tumours progressing via the serrated pathways.³¹ On the basis of the

results of the present study, methylation of olfactomedin 4 might occur in SSL. In the present study, expression of olfactomedin 4 in SAC was also significantly lower than that in non-SAC. Furthermore, right-sided SAC did not express olfactomedin 4.

Claudin family members are crucial components of tight junctions and show highly tissue-specific patterns.³² We have reported previously that retained claudin-18, which is expressed in normal stomach, correlates with a survival benefit in gastric cancer.²⁶ Conversely, ectopic expression of claudin-18, which is

not expressed in normal colon, correlates with poor survival in CRC.²⁰ However, little is known about the biological significance of claudin-18 in colorectal serrated polyps. In this study, we showed that claudin-18 was expressed in 44% of SSL, 12% of TSA and 28% of SAC cases. Both SSL and TSA have been reported to display the gastric mucin phenotype.³³ CRC with gastric phenotype show frequent lymphatic permeation and lymph node metastasis,³⁴ and a close association with specific genetic subtypes such as microsatellite instability (MSI). *CDX2* was also reported to be mutated in CRC with MSI-H.³ We have reported previously that the positive expression of claudin-18 correlated significantly with the positive expression of MUC5AC and the negative expression of *CDX2*.²⁰ *SOX2* is an HMG-box transcription factor expressed in gastric mucosa but not in intestine. Park *et al.*³⁵ reported that aberrant expression of *SOX2* up-regulated MUC5AC in CRC. In addition, Tsukamoto *et al.*³⁶ reported that the immunohistochemical expression patterns of *SOX2* and *CDX2* were related inversely in the human stomach. In the present study, non-neoplastic colorectal mucosa and colorectal polyps other than SSL and TSA did not express claudin-18.

In summary, we have performed an immunohistochemical analysis of olfactomedin 4 and claudin-18 in serrated neoplasia of the colorectum. A characteristic expression pattern of these molecules was observed in serrated polyps, especially in SSL. Reduced expression of olfactomedin 4 and ectopic expression of claudin-18 might be associated with the serrated pathway of colorectal carcinogenesis, and these markers may be useful in the differential diagnosis of serrated polyps.

References

1. Snover DC. Serrated polyps of the large intestine. *Semin. Diagn. Pathol.* 2005; 22: 301–308.
2. Rashid A, Houltham PS, Booker S, Petersen GM, Giardiello FM, Hamilton SR. Phenotypic and molecular characteristics of hyperplastic polyposis. *Gastroenterology* 2000; 119: 323–332.
3. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. *Histopathology* 2007; 50: 113–130.
4. Torlakovic E, Skovlund E, Snover DC, Torlakovic G, Nesland JM. Morphologic reappraisal of serrated colorectal polyps. *Am. J. Surg. Pathol.* 2003; 27: 65–81.
5. Goldstein NS, Bhanot P, Odish E, Hunter S. Hyperplastic-like colon polyps that preceded microsatellite-unstable adenocarcinomas. *Am. J. Clin. Pathol.* 2003; 119: 778–796.
6. Farris AB, Misraji J, Srivastava A *et al.* Sessile serrated adenoma: challenging discrimination from other serrated colonic polyps. *Am. J. Surg. Pathol.* 2008; 32: 30–35.
7. Ngo NT, Tan E, Tekkis P, Peston D, Cohen P. Differential expression of p53 and p504s in hyperplastic polyp, sessile serrated adenoma and traditional serrated adenoma. *Int. J. Colorect. Dis.* 2010; 25: 1193–1200.
8. Torlakovic EE, Gomez JD, Driman DK *et al.* Sessile serrated adenoma (SSA) vs. traditional serrated adenoma (TSA). *Am. J. Surg. Pathol.* 2008; 32: 21–29.
9. Owens SR, Chiosea SI, Kuan SF. Selective expression of gastric mucin MUC6 in colonic sessile serrated adenoma but not in hyperplastic polyp aids in morphological diagnosis of serrated polyps. *Mod. Pathol.* 2008; 21: 660–669.
10. Gibson JA, Hahn HP, Shahsafaei A, Odze RD. MUC expression in hyperplastic and serrated colonic polyps: lack of specificity of MUC6. *Am. J. Surg. Pathol.* 2011; 35: 742–749.
11. Schmitz KJ, Hey S, Schinwald A *et al.* Differential expression of microRNA 181b and microRNA 21 in hyperplastic polyps and sessile serrated adenomas of the colon. *Virchows Arch.* 2009; 455: 49–54.
12. Tuppurainen K, Mäkinen JM, Junttila O *et al.* Morphology and microsatellite instability in sporadic serrated and non-serrated colorectal cancer. *J. Pathol.* 2005; 207: 285–294.
13. Noffsinger AE. Serrated polyps and colorectal cancer: new pathway to malignancy. *Annu. Rev. Pathol.* 2009; 4: 343–364.
14. Yasui W, Oue N, Sentani K, Sakamoto N, Motoshita J. Transcriptome dissection of gastric cancer: identification of novel diagnostic and therapeutic targets from pathology specimens. *Pathol. Int.* 2009; 59: 121–136.
15. Zhang X, Huang Q, Yang Z, Li Y, Li CY. GW112, a novel antiapoptotic protein that promotes tumor growth. *Cancer Res.* 2004; 64: 2474–2481.
16. Seko N, Oue N, Noguchi T *et al.* Olfactomedin 4 (GW112, hGC-1) is an independent prognostic marker for survival in patients with colorectal cancer. *Exp. Ther. Med.* 2010; 1: 73–78.
17. van der Flier LG, Haegebarth A, Stange DE, van de Wetering M, Clevers H. OLFM4 is a robust marker for stem cells in human intestine and marks a subset of colorectal cancer cells. *Gastroenterology* 2009; 137: 15–17.
18. Tsukita S, Furuse M, Itoh M. Multifunctional strands in tight junctions. *Nat. Rev. Mol. Cell Biol.* 2001; 2: 285–293.
19. Zwiers A, Fuss JJ, Leijen S, Mulder CJ, Kraal G, Bouma G. Increased expression of the tight junction molecule claudin-18 A1 in both experimental colitis and ulcerative colitis. *Inflamm. Bowel Dis.* 2008; 14: 1652–1659.
20. Matsuda M, Sentani K, Noguchi T *et al.* Immunohistochemical analysis of colorectal cancer with gastric phenotype: claudin-18 is associated with poor prognosis. *Pathol. Int.* 2010; 60: 673–680.
21. Sentani K, Oue N, Tashiro T *et al.* Immunohistochemical staining of Reg IV and claudin-18 is useful in the diagnosis of gastrointestinal signet ring cell carcinoma. *Am. J. Surg. Pathol.* 2008; 32: 1182–1189.
22. Snover DC. Update on the serrated pathway to colorectal carcinoma. *Hum. Pathol.* 2011; 42: 1–10.
23. García-Solano J, Pérez-Guillermo M, Conesa-Zamora P *et al.* Clinicopathologic study of 85 colorectal serrated adenocarcinomas: further insights into the full recognition of a new subset of colorectal carcinoma. *Hum. Pathol.* 2010; 41: 1359–1368.
24. Hamilton SR, Aaltonen LA (eds). *World Health Organization classification of tumours. Pathology and genetics of tumours of the digestive system.* Lyon: IARC Press, 2000; 104–111.
25. Oue N, Sentani K, Noguchi T *et al.* Serum olfactomedin 4 (GW112, hGC-1) in combination with Reg IV is a highly sensi-

- tive biomarker for gastric cancer patients. *Int. J. Cancer* 2009; **125**; 2383–2392.
26. Sanada Y, Oue N, Mitani Y, Yoshida K, Nakayama H, Yasui W. Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. *J. Pathol.* 2006; **208**; 633–642.
 27. O'Brien MJ, Yang S, Clebanoff JL *et al.* Hyperplastic (serrated) polyps of the colorectum: relationship of CpG island methylator phenotype and K-ras mutation to location and histologic subtype. *Am. J. Surg. Pathol.* 2004; **28**; 423–434.
 28. Huang CS, O'Brien M J, Yang S, Farraye FA. Hyperplastic polyps, serrated adenomas, and the serrated polyp neoplasia pathway. *Am. J. Gastroenterol.* 2004; **99**; 2242–2255.
 29. Gray D, Obermann EC, Evans M, Hartmann A, Cooper K, Blaszyk H. MIB-1 and MCM-2 immunohistochemical analysis does not aid in identification of serrated colorectal polyps with abnormal proliferation. *Am. J. Clin. Pathol.* 2006; **125**; 407–412.
 30. Liu W, Liu Y, Zhu J, Wright E, Ding I, Rodgers GP. Reduced hGC-1 protein expression is associated with malignant progression of colon carcinoma. *Clin. Cancer Res.* 2008; **14**; 1041–1049.
 31. Loh K, Chia JA, Greco S *et al.* Bone morphogenic protein 3 inactivation is an early and frequent event in colorectal cancer development. *Genes Chromosom. Cancer* 2008; **47**; 449–460.
 32. Hewitt KJ, Agarwal R, Morin PJ. The claudin gene family: expression in normal and neoplastic tissues. *BMC Cancer* 2006; **6**; 186.
 33. Fujita K, Hirahashi M, Yamamoto H *et al.* Mucin core protein expression in serrated polyps of the large intestine. *Virchows Arch.* 2010; **457**; 443–449.
 34. Yao T, Tsutsumi S, Akaiwa Y *et al.* Phenotypic expression of colorectal adenocarcinomas with reference to tumor development and biological behavior. *Jpn. J. Cancer Res.* 2001; **92**; 755–761.
 35. Park ET, Gum JR, Kakar S, Kwon SW, Deng G, Kim YS. Aberrant expression of SOX2 upregulates MUC5AC gastric foveolar mucin in mucinous cancers of the colorectum and related lesions. *Int. J. Cancer* 2008; **122**; 1253–1260.
 36. Tsukamoto T, Inada K, Tanaka H *et al.* Down-regulation of a gastric transcription factor, Sox2, and ectopic expression of intestinal homeobox genes, Cdx1 and Cdx2: inverse correlation during progression from gastric/intestinal-mixed to complete intestinal metaplasia. *J. Cancer Res. Clin. Oncol.* 2004; **130**; 135–145.

The Search for Secreted Proteins in Prostate Cancer by the *Escherichia coli* Ampicillin Secretion Trap: Expression of NBL1 Is Highly Restricted to the Prostate and Is Related to Cancer Progression

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Key Words

CAST · NBL1 · Prostate cancer

Abstract

Aims: Genes expressed only in cancer tissue or specific organs will be useful molecular markers. To identify genes that encode secreted proteins present in prostate cancer (PCa), we generated *Escherichia coli* ampicillin secretion trap (CAST) libraries from PCa and normal prostate (NP). **Methods and Results:** We identified 15 candidate genes that encode secreted proteins present in PCa and NP. Quantitative RT-PCR analysis revealed that *MSMB*, *NBL1* and *AZGP1* were expressed with much higher specificity in PCa and NP than in 14 other kinds of normal tissue. We focused on *NBL1*, which was originally identified as a putative tumor suppressor gene. Western blot analysis revealed that NBL1 protein was highly expressed in both cell lysate and culture media of the DU145 PCa cell line. Immunohistochemical analysis showed that NBL1 expression was highly detected in and restricted to NP and PCa and was significantly down-regulated in PCa. NBL1 expression was significantly reduced according to the tumor stage, Gleason grade and preoperative prostate-specific antigen (PSA) value. **Conclusion:** NBL1 is a secreted pro-

tein that is highly restricted to the prostate. Underexpression of NBL1 correlated with PCa progression. NBL1 might be a candidate tumor marker for PCa in addition to PSA.

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Introduction

Prostate cancer (PCa) is one of the most common human male cancers. Cancer develops as a result of multiple genetic and epigenetic alterations [1]. Better knowledge of changes in gene expression that occur during prostatic carcinogenesis may lead to improvements in its diagnosis, treatment and prevention [2]. Genes encoding secretory proteins expressed specifically in cancers or specific organs may be ideal biomarkers for cancer diagnosis. To identify novel genes that encode secreted protein present in PCa, we used the *Escherichia coli* ampicillin secretion trap (CAST) method. We have previously identified several PCa-specific genes encoding transmembrane proteins with the CAST method and reported their high potential as therapeutic targets [3, 4]. To our knowledge, however, CAST analysis of secreted protein in PCa has not been reported.

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1015–2008/13/0802–0060\$38.00/0

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Prostate-specific antigen (PSA), a serine protease found in semen, is the most widely used serum marker for detecting and monitoring PCa [5, 6]. The rapid incorporation of aggressive PSA testing has resulted in dramatically earlier identification of PCa and is attributed with the decrease in mortality from PCa [7]. However, there are limitations to the use of PSA. PSA levels are also increased in benign prostatic hyperplasia (BPH) and general inflammatory responses. PSA testing has the potential disadvantage of low specificity and has led to a tremendous increase in the number of unnecessary prostate biopsies [8]. Furthermore, blood PSA level is not significantly increased in patients with poorly differentiated PCa and patients receiving androgen deprivation therapy [9, 10]. Moreover, the prognosis of castration-resistant PCa remains unsatisfactory [11]. Therefore, increasing emphasis has been placed on the need to determine new protein biomarkers for use in the diagnosis of PCa.

In the present study, to identify genes that encode secreted proteins, we generated CAST libraries from 2 PCa cell lines, LNCaP and DU145, and normal prostate (NP). CAST is a signal sequence trap method developed by Ferguson et al. [12]. Signal peptides target secreted and transmembrane proteins to their appropriate subcellular localization [13]. A consensus sequence for the signal peptide has not been identified, and, thus, standard molecular techniques are not well suited to identify such proteins. CAST exploits the ability of mammalian signal sequences to confer ampicillin resistance to a mutant β -lactamase lacking the endogenous signal sequence [14]. We report here the identification of several genes that encode secreted proteins expressed in PCa and NP. Among these, we focused on the *NBL1* gene because this gene is highly restricted in PCa and NP. *NBL1* (neuroblastoma suppression of tumorigenicity 1) was originally identified as a putative tumor suppressor gene in a transformed fibroblast rat model [15]. We confirmed the expression of *NBL1* in cell lysate and culture media of PCa cells. We examined the expression and distribution of *NBL1* in human PCa and NP by immunohistochemistry and compared them with clinicopathological characteristics. We also studied the expression of *NBL1* in normal systemic organs because *NBL1* has the possibility of being a serum marker for PCa.

Materials and Methods

CAST Library Construction

Plasmid CAST (pCAST) was designed to contain the kanamycin resistance gene and the β -lactamase gene lacking the first 69 nucleotides encoding the endogenous signal peptide. *EcoRI* and

Table 1. Characteristics of the PCa patients (n = 181)

Age	<70 years	92 (51%)
	\geq 70 years	89 (49%)
PSA at diagnosis	<20	108 (60%)
	20 to <100	31 (17%)
	\geq 100	42 (23%)
Gleason score	6	64 (35%)
	7	44 (24%)
	8	40 (22%)
	9–10	33 (18%)
pStage	B	57 (31%)
	C	79 (44%)
	D	45 (25%)
Treatment	prostatectomy	127 (70%)
	hormone and/or radiation	54 (30%)

*Bam*HI sites were placed upstream of the mutant β -lactamase gene for directional cloning. CAST library construction was performed as described previously [3, 12]. In brief, CAST cDNA libraries were generated from 2 μ g of mRNA with a random primer containing a *Bam*HI restriction site for reverse transcription (SuperScript Choice System; Invitrogen, Carlsbad, Calif., USA). The *EcoRI*-adapted cDNA was digested with *Bam*HI, size fractionated, ligated into pCAST and plated onto Luria-Bertani/ampicillin medium. Individual colonies were picked and grown in 1.0 ml of this medium with kanamycin in a 96-well format. Plasmid DNA was sequenced in a 96-well format using a primer located within the β -lactamase gene.

Tissue Samples

In total, 219 primary tumor samples were collected from patients diagnosed with PCa and those with NP. Patients were treated at the Hiroshima University Hospital or an affiliated hospital. Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government.

For quantitative RT-PCR, 16 PCa samples and 9 non-neoplastic samples were used. Samples were frozen immediately in liquid nitrogen and stored at -80°C until use. We confirmed microscopically that the tumor specimens consisted mainly (>50%) of cancer cells. Noncancerous samples of heart, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, peripheral leukocytes, spleen, skeletal muscle, brain and spinal cord were purchased from Clontech (Palo Alto, Calif., USA).

For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 127 PCa patients treated by radical prostatectomy, 54 PCa patients who underwent prostate biopsy, and 13 patients treated by suprapubic prostatectomy for BPH. Tumor staging was in accordance with the TNM classification system [16], and histological classification of PCa was made in accordance with the World Health Organization classification [17]. The clinical characteristics of the PCa patients are shown in table 1. In addition, we used archival formalin-fixed, paraffin-embedded tissues from normal systemic organs including 5 speci-

Table 2. List of genes encoding secreted protein from the CAST libraries

	Sample name		
	LNCaP	DUI45	Normal Prostate
Genes encoding secreted protein	6	8	7
Gene name	TFPI SPP1 FN1 COL4A5 CALU SFTPA1B	TFPI CCDC126 CLU DMKN NBL1 ARMETL FGB NTN4	MSMB AZGP1 SPP1 TFPI C1RL CLU SRGN

mens each of the brain, spinal cord, heart, lung, esophagus, stomach, small intestine, colon, liver, spleen, pancreas, kidney, adrenal, ureter, bladder, testis, skin, skeletal muscle and blood vessels.

Quantitative RT-PCR and Western Blot Analysis

Quantitative RT-PCR was performed with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, Calif., USA) as described previously [18]. Quantitation of *NBL1* mRNA levels was done by real-time fluorescence detection as reported in a previous study [19]. The *NBL1* primer sequences were 5'-TCAACAAGCTGGCACTGTTC-3' and 5'-GCAGGAGTCA-CAGTGAACCA-3' (for more information, see online suppl. material at www.karger.com/doi/10.1159/000341396).

For Western blot analysis, tissue samples or cells were lysed as described previously [20]. The primary antibody against *NBL1* (R&D Systems, Inc., Minneapolis, Minn., USA) was used (see online suppl. material).

Evaluation of Specificity of Gene Expression

To evaluate the specificity of expression in each gene, a specificity index was calculated as follows: first, we identified the 14 normal tissues, which are indispensable for survival, in which the target gene expression was highest among tissues analyzed by quantitative RT-PCR. We then identified PCa among the 16 PCa samples in which the target gene expression was highest by quantitative RT-PCR (mRNA expression level in this tissue was denoted as A). We next identified NP among the 9 NP samples in which the target gene expression was highest by quantitative RT-PCR (mRNA expression level in this tissue was denoted as B). The target mRNA expression levels were standardized to normal organ with highest expression set as 1. A and B were defined as the PCa and NP specificity indices, respectively. When the specificity index of the target gene in A and/or B was ≥ 10 , the gene was considered to show high specificity. When the specificity index of the target gene was < 10 and ≥ 5 , the gene was considered to show low specificity. When the specificity index of the target gene was < 5 , the gene was considered to show no specificity.

Immunohistochemistry

Immunohistochemical analysis was performed with a Dako Envision+ mouse peroxidase detection system (Dako Cytomation, Carpinteria, Calif., USA). The following antibody dilutions were used: goat polyclonal anti-*NBL1*, 1:100 (R&D Systems; see supplementary material). We used the percentage of *NBL1*-positive cells in the total cells as an immunohistochemical scoring system. Evaluation of immunoreactivity was scored independently by two pathologists (K.S. and T.H.). The sections from the 127 patients treated by radical prostatectomy were evaluated in both cancerous and non-cancerous areas. The sections from the 54 prostate biopsy patients were evaluated in the cancerous area. The percentage of *NBL1* expression in each region was scored as follows: no expression, 0; $< 10\%$, 1+; 10–30%, 2+; 31–50%, 3+, and $> 50\%$, 4+. We used *NBL1* expression in nerve cells as a positive control based on previously reported *NBL1* immunoreactivity in nerve cells [21].

RNA Interference and Cell Growth and in vitro Invasion Assays

RNA interference was performed to knock down the endogenous *NBL1*. siRNA oligonucleotides for *NBL1* and a negative control were purchased from Invitrogen (Carlsbad, Calif., USA). The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] and modified Boyden chamber assays were performed to examine cell growth and invasiveness, respectively (see online suppl. material) [22, 23].

Statistical Method

Associations between clinicopathological variables and *NBL1* expression were analyzed by Mann-Whitney U test. The comparison of cell growth and invasive activity was analyzed by t test. A value of $p < 0.05$ was considered statistically significant.

Results

Identification of Genes with Higher Expression in the Prostate than in 14 Normal Tissues

To identify genes that encode secreted proteins present in PCa, we generated CAST libraries from 2 PCa cell lines (LNCaP and DUI45) and NP, as previously described [3, 4]. We identified 6, 7 and 8 genes encoding secreted proteins from the respective cell lines and NP. The names of these 21 genes are shown in table 2. We performed quantitative RT-PCR to identify genes expressed specifically in PCa and NP. Representative results are shown in figure 1. We then identified the PCa among 16 tissues in which the target gene expression was highest (mRNA expression levels are shown as A; table 3) and NP among 9 tissues in which the target gene expression was highest (mRNA expression levels are shown as B; table 3). Next, the PCa specificity index (A/B ratio) for each gene was calculated. We could not find a gene specific only to PCa, but we could detect genes specific to both PCa and NP. Because

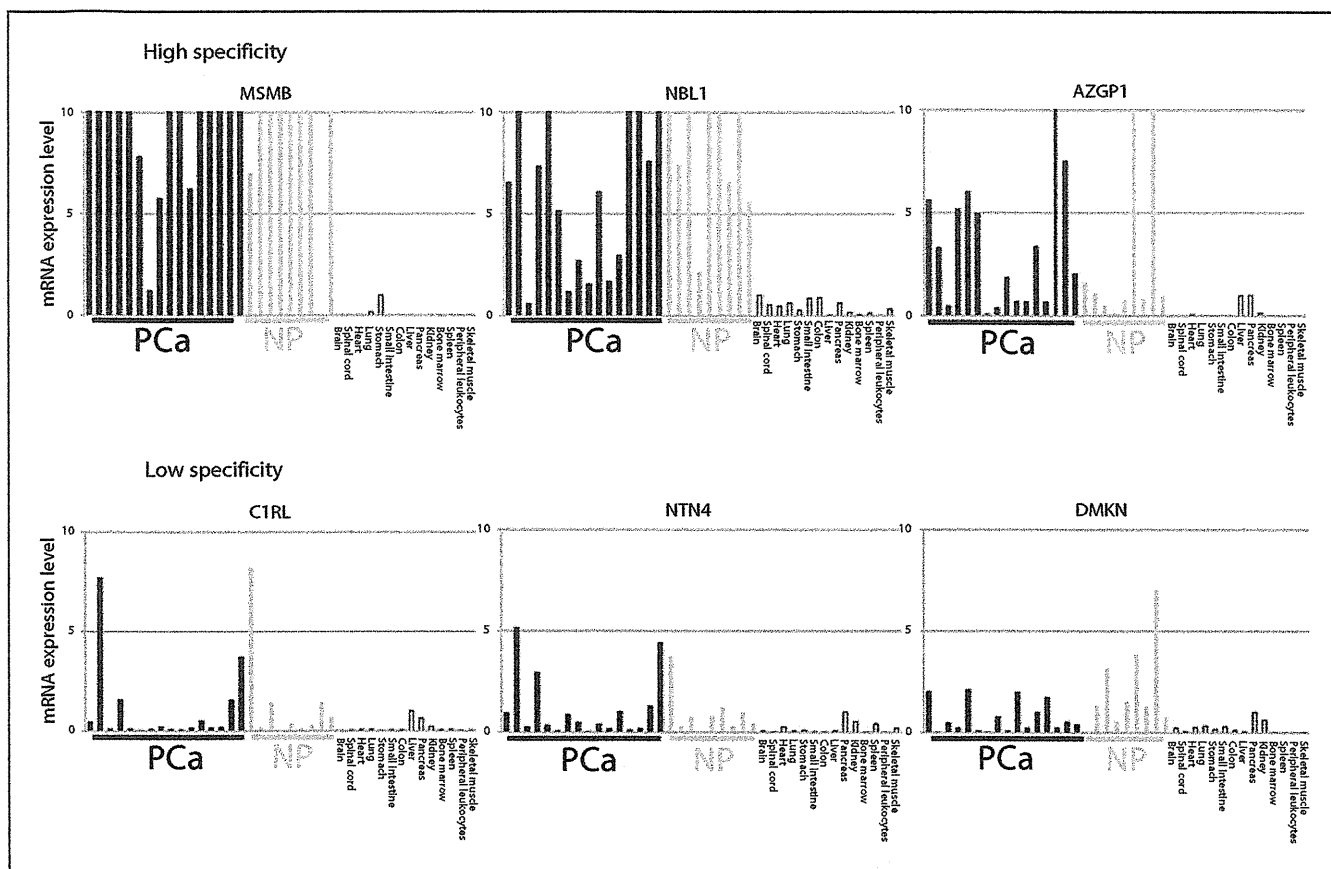


Fig. 1. Quantitative RT-PCR analysis of candidate genes in 16 PCa samples, 9 NP samples, and 14 kinds of normal tissues. MSMB, NBL1 and AZGP1 were found to show high specificity for the prostate. C1RL, NTN4 and DMKN were found to show low specificity for the prostate.

PSA is also specific for prostate and not specific for PCa, we examined these prostate-specific genes. Of the 15 candidates, 3 genes, *MSMB*, *NBL1* and *AZGP1*, were found to show high specificity for the prostate, and 3 genes, *C1RL*, *NTN4* and *DMKN*, were found to show low specificity for the prostate. Of the 3 genes showing high specificity, *MSMB* (microseminoprotein- β) and *AZGP1* (zinc- α_2 -glycoprotein) have already been studied in PCa, and their utility as serum tumor marker and therapeutic target have been reported [24–27]. Therefore, we focused on *NBL1*. *NBL1* has been reported as one of the genes whose expression is reduced in PCa compared with NP by profiling of expressed sequence tags and quantitative RT-PCR analysis [28, 29]. However, there is no report, to our knowledge, in which *NBL1* expression in human systemic organs is compared and *NBL1* expression in PCa and NP determined by immunohistochemical analysis has not been reported.

We compared *NBL1* mRNA expression between prostate and systemic normal organs. The average *NBL1* mRNA expression levels in NP and PCa were 19.9- and 8.1-fold greater, respectively, than in brain, in which *NBL1* is most highly expressed of the systemic normal organs. In comparison with that in the other systemic organs, the expression of *NBL1* mRNA is highly restricted to the prostate.

NBL1 Protein Expression in Cell Lysate and Culture Medium

To study whether *NBL1* is a secreted protein, we performed Western blot analysis in 3 PCa cell lines. In cell lysate, moderate *NBL1* expression was detected in DU145 cells as a band of approximately 27 kDa. LNCaP cells showed low *NBL1* expression, and *NBL1* expression was absent in PC3 cells (fig. 2a). In culture medium, very high *NBL1* expression was noted in DU145 cells, low *NBL1* ex-

Table 3. Summary of quantitative RT-PCR analysis of candidate genes specifically expressed in PCa and NP samples

Gene name	Name of normal organ with highest expression	PCa with highest mRNA expression level (A)	NP with highest mRNA expression level (B)	PCa specificity index (A/B)	PCa cases with mRNA expression level ≥ 10 -fold normal organ with highest expression	NP cases with mRNA expression level ≥ 10 -fold normal organ with highest expression
<i>High specificity</i>						
MSMB	Stomach	>100	>100	0.13	12	8
NBL1	Brain	29.6	71.2	0.42	5	5
AZGP1	Pancreas	21.5	22.4	0.96	1	2
<i>Low specificity</i>						
C1RL	Liver	7.7	8.2	0.94	0	0
NTN4	Pancreas	5.2	3.7	1.4	0	0
DMKN	Pancreas	2.1	7.0	0.3	0	0
<i>No specificity</i>						
FNI	Liver	0.78	0.37	2.1	0	0
COL4A5	Stomach	0.72	0.69	1.0	0	0
CCDC126	Skeletal muscle	0.58	0.07	8.3	0	0
SPP1	Kidney	0.56	0.34	1.6	0	0
CALU	Heart	0.32	0.26	1.2	0	0
CLU	Liver	0.3	1.0	0.3	0	0
SRGN	Bone marrow	0.27	0.67	0.4	0	0
FGB	Liver	<0.01	<0.01	-	0	0
TFPI	Kidney	<0.01	0.022	-	0	0
SFTPA1B	Lung	0.057	<0.01	-	0	0
ARMETL	Skeletal muscle	<0.01	<0.01	-	0	0

Target mRNA expression levels were standardized to 1.0 μ g total RNA from the normal organ with the highest expression set as 1.0 (16 PCa and 9 NP samples).

pression was seen in LNCaP cells, and no expression of NBL1 was noted in PC3 cells. In the CAST analysis, colonies containing the NBL1 gene were detected in the DU145 CAST library, indicating that Western blot analysis of NBL1 protein was consistent with CAST analysis. Next, we examined transition of NBL1 expression by Western blot analysis of cell extracts of DU145 transfected with NBL1-specific siRNAs. Two types of siRNAs (siRNA1 and siRNA2) and negative control siRNA were transfected into DU145 cell extracts. Expression of NBL1 in DU145 was suppressed by treatment with siRNA1 and siRNA2 in both cell lysate and culture medium (fig. 2b).

Immunohistochemical Analysis of NBL1 Expression in Normal Systemic Organs

We performed immunohistochemical analysis of NBL1 in 19 kinds of non-cancerous systemic tissues in 5 samples of each tissue. NBL1 expression was detected only in epithelium of the small intestine and colon, islets of the

pancreas, and nerve cells in brain and spinal cord (fig. 3a-d). We did not detect the expression of NBL1 in heart, lung, esophagus, stomach, liver, spleen, pancreas, kidney, adrenal, ureter, bladder, testis, skin, skeletal muscle and vessel. When the level of NBL1 expression is compared in small intestine and colon, islets of the pancreas, and nerve cells in brain and spinal cord, expression in the brain nerve cells is higher than in the other tissues. These results are consistent with our quantitative RT-PCR results.

Immunohistochemical Analysis of NBL1 Expression in PCa and NP

We also performed immunohistochemical analysis of NBL1 in a total of 194 prostate samples, which included BPH in 13 patients and PCa in 181 patients. The clinical characteristics of the PCa patients are summarized in table 1. NBL1 staining was observed in the cytoplasm of both NP epithelium and cancer cells. In some acini, NBL1 expression was stronger at the apical side of luminal cells

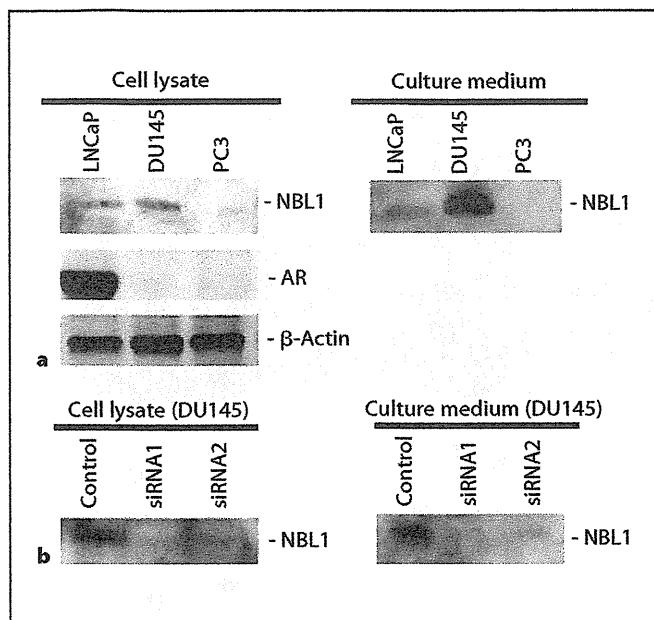


Fig. 2. Western blot analysis of NBL1 protein in PCa cell lines. NBL1 expression in DU145 and LNCaP cell lines was observed in cell lysate. In culture medium, high NBL1 expression was noted in DU145 cells (a). NBL1 expression in DU145 was suppressed by treatment with siRNA1 and siRNA2 in both cell lysate and culture medium (b).

and was detected in the prostatic ducts. Expression of NBL1 was not detected in stromal cells. Although NBL1 expression in PCa cells has some heterogeneity, NBL1 expression was detected in 1–95% of all samples (fig. 3e–h). All prostate samples were considered NBL1 positive if any cell stained positive. These immunohistochemical results suggest that NBL1 was highly expressed in and restricted to the prostate.

Next, we compared clinicopathological parameters with NBL1 expression scores in the prostate samples (fig. 4a–d). Mean NBL1 expression score was significantly higher in BPH samples and NP adjacent to PCa than in PCa itself ($p < 0.0001$). The mean NBL1 expression score was significantly higher in PCa classified as stage B than in PCa classified as stages C and D ($p = 0.0014$) and was significantly higher in PCa with Gleason score 6 than in PCa with Gleason score 7–10 ($p = 0.0024$). The mean NBL1 expression score was also significantly higher in PCa with PSA level ≤ 20 than in PCa with PSA level > 20 ($p < 0.0001$).

Effect of NBL1 Inhibition on Cell Growth and Invasive Activity of PCa Cells

We studied the biological role of NBL1 using the DU145 cell line because of the high expression of NBL1 in this cell line. To investigate the possible proliferative effects of NBL1 knockdown, we performed an MTT assay 4 days after siRNA transfection (fig. 4e). Cell viability was not significantly different between NBL1 siRNA-transfected DU145 and negative control siRNA-transfected DU145. Next, to determine the possible role of NBL1 in invasiveness, a transwell invasion assay was performed (fig. 4f). Invasion ability 1 day after siRNA transfection was not significantly different between NBL1 siRNA-transfected DU145 and negative control siRNA-transfected DU145.

Discussion

In the present study, we identified several genes that encode secreted proteins present in PCa and NP from CAST libraries. Quantitative RT-PCR revealed that *MSMB*, *NBL1* and *AZGP1* were expressed to a much higher extent in PCa and NP than in 14 types of normal tissues. *MSMB*, prostatic acid phosphatase and PSA are the three most abundant proteins found in semen. It was reported that *MSMB* also has high specificity for the prostate and has utility as a serum biomarker for PCa [24]. In addition, a single-nucleotide polymorphism of *MSMB* has been reported to increase the risk of developing PCa [25]. It was also reported that immunohistochemical staining of *AZGP1* was a predictor of tumor recurrence and could be used as a specific serum biomarker for PCa [26, 27]. Although we could not detect PSA in our CAST library, we think that PSA might be one of the genes difficult to ligate into the pCAST vector. In contrast, little is known about NBL1 expression in PCa. NBL1 can stimulate differentiation of neuroblastoma cells in culture in the presence of retinoic acid [30], and its growth-suppressive activity has been noted in sarcoma cells [31]. NBL1 may play an important role in preventing cells from entering into the S phase [32]. *NBL1* mRNA was reported to be down-regulated in PCa compared with corresponding NP by quantitative RT-PCR analysis [28, 29].

Because the genes in the present study were identified by CAST analysis of PCa cell lines and quantitative RT-PCR analysis of bulk PCa and NP tissues, immunohistochemical analysis was required to determine which cells expressed these genes. With this analysis, we confirmed that NBL1 was highly expressed in the epithelium of PCa