Overexpression of the signal peptide whirlin isoform 2 is related to disease progression in colorectal cancer patients

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Abstract. We identified that whirlin is localized to chromosome 9q32-33, and is up-regulated in colorectal cancer tissues by using oligonucleotide array techniques and the Sosui system (http://www.tuat.ac.jp/~mitaku/sosui/). The deduced 920-amino acid protein encoded by the whirlin gene contains three PDZ domains and a proline-rich region that separates PDZ2 from PDZ3, which is located at the C terminus. As previously reported, human whirlin gene is alternatively spliced to form a long and a short transcript *in situ* hybridization. The sequence of the encoded protein shows that the short C-terminal isoform contains one PDZ domain and the proline-rich domain (whirlin isoform 2), whereas the long isoform is composed of all three PDZ domains and the proline-rich domain (whirlin isoform 1). The gene expression of whirlin was found to be up-regulated in colorectal cancer tissues compared with matched normal colon tissues by semi-quantitative RT-PCR (P<0.05). Western blotting detected whirlin protein with a molecular mass of 49.3 kDa in colorectal cancer samples, suggesting that the whirlin protein overexpressed in colorectal cancer samples is the short C-terminal isoform 2. Its expression was recognized in colorectal cancer cell lines and was increased in accordance with tumor progression in colorectal cancer patients. Immunohistochemistry showed high levels of staining for whirlin isoform 2 only in the mucosal glands in colon cancers, but this protein was barely detected in normal colonic glands. Immunoelectron microscopic findings showed that whirlin isoform 2 is localized on plasma membranes and endoplasmic reticulum membranes, but not in the nuclei. Tissue microarrays showed that whirlin isoform 2 is abundantly expressed in colon cancers with lymph node metastasis compared with those without lymph node metastasis, and overexpression of this protein was associated with tumor progression. In conclusion, we demonstrated that whirlin isoform 2 is highly expressed in colon cancer tissues and that it is related to tumor progression.

Introduction

Colorectal cancer is the second most common malignancy and the second leading cause of cancer-related mortality in developed countries (1-3). The age-standardized incidence rates have changed little over the last 30 years despite major advances that have been made in understanding its pathogenesis at the molecular level (2). Clinical research in colorectal cancer has focused mainly on prevention, early detection, and optimal selection of patients for adjuvant therapy. Despite these efforts, colorectal cancer still causes significant morbidity and mortality. Recently, molecular target therapy for colorectal cancer has been introduced to the clinical setting (4). However, indications for these therapies have been limited due to the low frequency of target gene expression, unstable effectiveness, and/or severe side effects (5). Thus, there is a crucial need to explore novel cancer-related genes to serve as diagnostic markers and molecular targets for therapies for colorectal cancer.

To identify relevant marker genes that are important in the pathogenesis of colon cancer, we are investigating approaches to identify novel transmembrane proteins or signal peptides that are overexpressed in colorectal tumors compared with normal colonic tissue using oligonucleotide arrays and the Sosui system (6) (http://www.tuat.ac.jp/~mitaku/sosui/), which enables the detection of membrane and soluble proteins together with the prediction of transmembrane helices.

This strategy successfully identified seven differentially expressed genes. We confirmed the up-regulation of one (whirlin gene) of these seven genes in clinical samples, at the RNA and encoded protein (whirlin isoform 2 protein) levels, in human colon cancers and cancer-derived cell lines.

Whirlin is the nearest homologue of the Usher syndrome type 1C (USH1C) protein harmonin, possessing a similar modular structure with, depending on the isoform, up to three PDZ domains and a proline-rich domain. Mutations in the C-terminal half, thus affecting both the long and the short C-terminal isoforms of whirlin (encoded by exons 1-12 and

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6-12, respectively), have previously been reported to cause human non-syndromic deafness in humans (DFNB31; OMIM 607084) and in the whirler mouse (*wi*) (7,8). However, there have been no reports on evaluating the gene expression of whirlin in malignant disease.

Since the protein structure of whirlin isoform 2 includes a PDZ motif, a protein-protein binding domain likely to be involved in protein clustering and scaffolding (9), the up-regulation of this protein with the ability to recruit, bind, and coordinate the activities of other proteins may contribute to tumorigenesis in human colorectal cancer.

In this study, we evaluated the gene expression of whirlin to clarify its relation to the clinical manifestations in patients with colorectal carcinoma.

Materials and methods

Patients and tissue samples. A total of 12 colorectal primary cancer tissues and the corresponding normal colonic epithelia were obtained from surgically resected specimens. All patients with tumors were diagnosed as being at an advanced stage, and all normal tissues were histopathologically confirmed to be free of cancer. Six of the 12 samples of primary cancers and normal mucosa were used for microarray analysis. A piece of each tissue sample was immediately frozen upon resection in liquid nitrogen and stored at -80˚C until use. Written informed consent was obtained from each patient. Investigations were performed in accordance with the Helsinki Declaration and were approved by the Institutional Review Board.

Colon cancer cell lines and culture conditions. The human colon cancer cell lines DLD-1 and Caco-2 were obtained from the Japanese Cancer Research Resources Bank (Tokyo). Cells were grown in monolayer cultures in RPMI-1640 (Sigma-Aldrich, Inc., St. Louis, MO, USA) supplemented with fetal bovine serum (FBS) (10%, v/v) (Gibco BRL, Tokyo, Japan), glutamine (2 mM), penicillin (100,000 units/l), streptomycin (100 mg/l), and gentamycin (40 mg/l) at 37˚C in a 5% CO_2 environment. For routine passage, cultures were split 1:10 when they reached 90% confluence, generally every 3 days. For all the experiments, cells at the fifth to ninth passage were used. All experiments were performed with exponentially growing cells.

Total RNA isolation. All mucosa were dissected from underlying tissue and homogenized in Physcotron (Niti-on, Japan) for the isolation of total RNA. Total RNA was extracted with Sepasol-RNA (Nacalai Tesque, Tokyo, Japan), and the quality of the total RNA was judged from the ratio between 28S and 18S RNAs after 1% formalin gel electro-phoresis. Contaminating genomic DNA template was removed by DNase1 treatment (Takara, 2215A).

Preparation of aminoallyl-antisense RNA. Total RNA from each sample (2 µg/slide) was amplified with T7 RNA polymerase (#1752, Ambion) to produce antisense RNA (aRNA), which was labeled with aminoallyl-dUTP (#8439, Ambion) during the amplification reaction. Labeling and production of aRNA of ~1 kb was ensured by analysis with a

Bioanalyzer (Agilent). Five micrograms of each aminoallyl-labeled aRNA was precipitated with ethanol and coupled with either monoreactive Cy3 (PA23001, Amersham Bioscience) or Cy5 (PA25001, Amersham Bioscience). Monoreactive dye was solubilized with 45 µl of DMSO (7898-1, Clontech) before use and stored at -20˚C. Aminoallyl-labeled cDNA was solubilized with 5 µl of 0.2 M sodium bicarbonate buffer (pH 9.0), and then 5 µl of mono-reactive dye in DMSO was added. The solution was mixed by pipetting and incubated for 1 h at 40˚C in the dark. Free dye was removed using P30 columns (732-6223, BioRad). Labeled aRNAs were applied to Microcon-YM30 columns (43409, Millipore), washed twice with 250 µl of sterile distilled water, and concentrated to ≤32 µl. Eight microliters of 5X fragmentation buffer (supplied with AceGene™; Hitachi Software Engineering Co., Ltd.) was added to the purified 32 µl of aRNA and mixed. The mixture was incubated for 15 min at 94˚C and then cooled on ice. After fragmentation, one-twentieth the volume of aRNA was applied to a 3% agarose gel and stained with ethidium bromide to examine the presence and length of aRNA using a transilluminator (fragmented aRNA can be observed as a smear centered at ≤200 bp when compared with a DNA marker). The rest of the purified dye-labeled aRNAs was applied to Microcon-YM10 columns (42406, Millipore), washed twice with 100 µl of sterile distilled water, and concentrated to 5-15.5 µl.

Hybridization. Dye-labeled cDNA was diluted to 15.5 µl, and 12.5 µl of 20X SSC, 2.5 µl of 10% SDS, 4 µl of 50X Denhardt's solution, and 10 µl of hybridization solution (supplied with AceGene) were added. This solution was incubated for 2 min at 95˚C and cooled on ice. Salmon sperm DNA (0.5 µl) and 5 µl of formamide were added to the solution, resulting in a total volume of 50 µl. The solution was returned to room temperature (RT). The target cDNA solution was applied directly to the AceGene slide (AceGene Human Oligo Chip; Hitachi Software Engineering Co., Ltd.), and a cover glass (24x60 mm) was set. Hybridization was performed for 16 h at 45˚C in the dark. After hybridization, the slides were washed with 2X SSC and 0.1% SDS for 5 min at 30˚C, with 2X SSC for 5 min at 30˚C, and with 1X SSC for 5 min at 30˚C. The slides were moved up and down five times at the beginning and the end of each wash step. The slides were then rinsed with 0.1X SSC and dried by air spray.

Seeking novel genes. Fluorescent signals were detected on a confocal laser scanner (HB GeneArray Scanner; Affymetrix) and analyzed with the Denasis Array (Hitachi Software Engineering, Japan). Signal intensity information of the Denasis Array was exported to Excel (Microsoft, Redmond, CA) as needed.

Semi-quantitative RT-PCR and full-length sequence of human whirlin isoform 2 cDNA. We designed the following primers on the basis of the predicted full-length whirlin isoform 2 sequence: 5'-<u>GAATTC</u>ATGCATGGTTCTCTTGAAGCTT T-3' (forward) and 5'-<u>GAATTC</u>TAGAGCATCACACATTGAA CTCAG-3' (reverse); underlined are the *Eco*R restriction sequences.

cDNA was synthesized from 3 µg of total RNA from 12 colorectal cancer tissues and the corresponding normal mucosa, and subjected to PCR using specific primers and ß-actin as a standard for sample normalization. The PCR reactions included 2X GC buffer, 2.5 mM dNTP mix, 80 ng of each primer, and LA taq DNA polymerase. Each cycle consisted of 30 sec of denaturation at 94˚C, 30 sec of annealing at 58˚C, and 2 min of elongation at 72˚C; 30 cycles were performed. Amplified products were separated electrophoretically in 1% agarose gels, visualized, and photographed under UV light after ethidium bromide staining.

PCR products were cloned into pCR4Blunt-TOPO Vector (Toyobo). Some colonies were picked up and sequenced with a CEQ 8000 DNA Analysis system (Beckman Coulter, Inc.). The determined sequences were identical to the hypothesized full-length sequence without any amino acid alteration.

Western blotting. Mouse monoclonal antibodies against both whirlin isoform 1 (long transcript) and isoform 2 (short transcript) were generated using partial recombinant protein as the immunogen, corresponding to the predicted amino acid sequence 808 to 908 of whirlin isoform 1 (NP-056219) (Novus Biologicals, Inc., Littleton, CO, USA). To verify that the whirlin antibody specifically recognized the whirlin isoform 2 protein, the resulting PCR product was subcloned into a pGEX-2T plasmid with a GFP fusion protein, sequence verified, and expressed in *Escherichia coli* DH5 cells as a positive control.

Tissue specimens and cells were homogenized in lysis buffer (Tris-buffered saline, pH 7.5, containing 1% Triton X-100) for 5 min on ice. After spinning at 15,000 rpm for 15 min at 4˚C, supernatants were collected and frozen at -20˚C until use. The protein concentration was measured by the BCA protein assay (Pierce, Rockford, IL). Lysates containing 10 µg of total protein were mixed with an equal volume of 2X Laemmli loading buffer containing 2-mercaptoethanol and heated at 100˚C for 5 min. Samples were electrophoretically separated on 12.5% polyacrylamide gels containing 0.1% SDS at 25 mA for 2 h followed by semi-dry transfer to an Immun-Blot PVDF membrane (Bio-Rad Lab., Hercules, CA) at 12 V for 2 h. The membranes were blocked for 2 h at RT using 5% skim milk in Tris-buffered saline, pH 7.5, supplemented with 0.1% Tween-20 (TBS-T). The blots were then incubated with anti-whirlin mouse monoclonal antibody at a 1:100 dilution in 5% skim milk in TBS-T overnight at 4˚C. After having been washed three times in TBS-T, the blots were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Promega Corp., Madison, WI) at a 1:500 dilution in 5% skim milk in TBS-T for 1 h at RT. Following treatment with an enhanced chemiluminescence detection solution, the blots were exposed to an X-ray film for autoradiographic visualization of the bands. The relative quantities of protein on scanned images were analyzed by densitometry using CS Analyzer version 2.0 (Atto Corporation, Japan).

Immunohistochemistry. Histopathological examination of the 10% formalin-fixed, paraffin-embedded specimens was performed by a pathologist at the Pathology Division. After dewaxing and dehydration, the sections were washed with deionized water and phosphate-buffered saline (PBS), and then incubated for 20 min in 20% Block Ace (Dainippon Pharmaceutical, Suita, Japan) in 0.1 M phosphate buffer containing 0.005% saponin (Merck, Darmstadt, Germany, referred to as buffer A). They were then washed three times with PBS and subsequently blocked in 20% Blockace in buffer A. They were then incubated with either anti-whirlin mouse monoclonal antibody (1:100) in 0.1 M phosphate buffer containing 4% Blockace and 0.001% saponin at 4˚C overnight. Fluorescein-conjugated goat anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody (1:1000) with Alexa Fluor 594 phalloidin (Invitrogen, Tokyo, Japan). The specimens were examined using Radiance 2000 confocal laser scanning microscopy (Bio-Rad, Hercules, CA, USA).

Immunoelectron microscopy. Confluent DLD-1 cells on cover glasses were fixed for 2 h at RT with 4% para-formaldehyde (Merck, Darmstadt, Germany) in 0.1 M PB at pH 7.5 containing 20 mM (*p*-amidinophenyl) methane-sulfonyl fluoride hydrochloride. They were then washed with PB three times for 10 min each time, and subsequently incubated in 20% Blockace in 0.1 M PB containing 20 mM (*p*-amidinophenyl) methane-sulfonyl fluoride hydrochloride and 0.005% saponin (referred to as buffer B) for 15 min to block non-specific antibody binding. The cells were then incubated overnight at 4˚C with anti-whirlin mouse monoclonal antibody diluted 1:100 in buffer B containing 5% Blockace. After having been washed with buffer B four times, they were next incubated for 2 h at RT with goat anti-mouse IgG labeled with 1-nm gold particles (Nanogold; Nanoprobes, Stony Brook, NY) diluted 1:100 in buffer B containing 5% Blockace. After three washes with buffer B, they were post-fixed for 15 min with 1% glutaraldehyde (Nacalai Tesque, Kyoto, Japan) in PB, and then tissue-bound gold particles were enhanced by a 6-min incubation with a silver developer (HQ silver, Nanoprobes) in the dark. They were post-fixed again with 0.5% OsO$_4$ in PB for 90 min at 4˚C after having been washed with PB, and thereafter stained with 4% uranyl acetate for 30 min at RT and dehydrated by passage through a graded series of ethanol. Cells were then embedded in Epon 812 (Nacalai Tesque) and removed from the culture slide. Ultra-thin sections were made on an LKB Ultratome (Amersham Pharmacia Biotech), plated on specimen grids coated with polyvinyl formal resin (Nissin EM, Tokyo, Japan), and observed under an electron microscope (JEM-1200EX; Jeol, Tokyo, Japan) after staining with uranyl acetate and lead citrate.

Statistical analysis. The results were expressed as the means ± SD. The Mann-Whitney U test was used for comparisons among the unpaired groups. P-values <0.05 were considered statistically significant.

Results

To eliminate data with low reliability, genes with a hybridization signal intensity of 0 and each average intensity, calculated as [(Cy3-Cy3 background) + (Cy5-Cy5 background)]

Table I. Gene transcripts displaying a 2-fold higher increase in expression level.

| ID	Description	Accession no.	Ratio
AGhsB020616	Hypothetical protein xp_054209; loc113402	XM_054209	2.515121
AGhsB091215	Bicarbonate transporter related protein 1; btr1	XM_045419	2.463204
AGhsB020305	kiaa0995 protein; kiaa0995	AB023212	2.420191
AGhsB220405	Pancreatic polypeptide-2; ppy2	AF222903	2.26012
AGhsB030912	Pro0282p	AF090912	2.191931
AGhsB071207	**Hypothetical protein xp_027010; dkfzp434n014**	**XM_027010**	**2.070385**
AGhsB150403	Hypothetical protein xp_045605; loc94586	XM_045605	2.024242
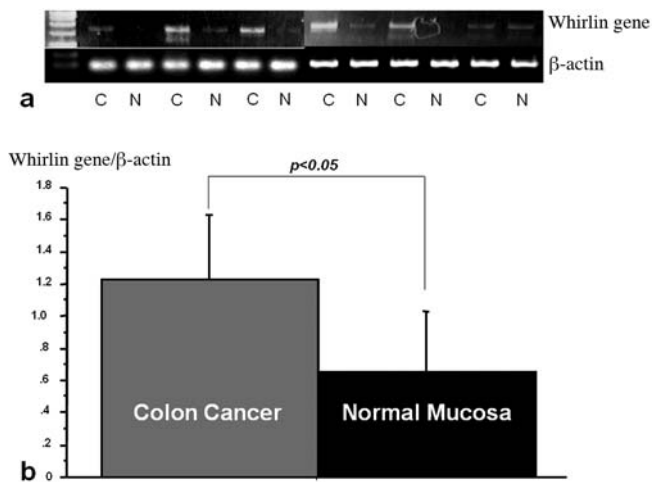
Figure 1. RT-PCR analysis of whirlin gene expression in normal colonic mucosa and colon cancer tissue. (a) Results of RT-PCR performed on six paired colon cancer (C) and adjacent normal mucosa (N) samples. ß-actin was used as a control for cDNA synthesis. (b) Semi-quantification of whirlin gene expression (whirlin/ß-actin) in 12 paired colon cancer and adjacent normal mucosa samples using Scion Image software.
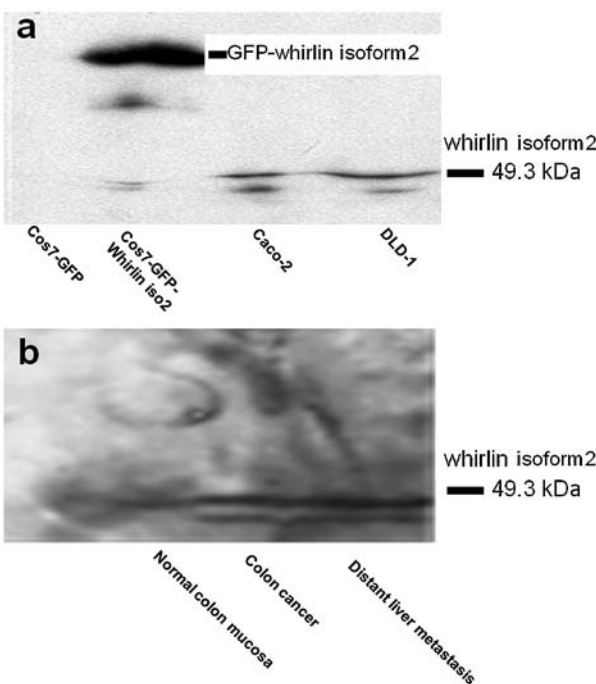

Figure 2. Western blot analysis of whirlin isoform 2 protein expression in colon cancer-derived cell lines and human samples. (a) Protein was prepared from COS7 cells transfected with GFP-fusion protein as a negative control (lane 1), COS7 cells transfected with whirlin isoform 2-GFP fusion protein (lane 2), and two colon cancer cell lines (Caco-2, lane 3 and DLD-1, lane 4). (b) Protein was prepared from adjacent normal colonic mucosa (lane 1), colon cancer tissue (lane 2), and a liver metastasis (lane 3).

<2000, were excluded from all data sets. Genes with average intensity ratios that showed a >2-fold change were selected. Among the remaining genes, 2-fold average intensity ratio genes with each intensity ratio <1 on array tests were excluded. We further selected membrane proteins and signal peptides using the system SOSUI. Table I lists the gene transcripts displaying a 2-fold increase in expression level in colorectal cancer tissue. Among the genes overexpressed in colon cancers, we selected one gene termed DKFZP434N014 (GenBank Accession No. XM_027010) as a candidate target gene, but this record was removed as a result of standard genome annotation processing. Therefore, we attempted to predict the gene with strong homology. BLAST analysis (http://www.ncbi.nih.gov/BLAST) with the XM_027010 sequence as a query gave us the human genomic DNA sequence for whirlin as a result.

To verify the array results, the expression levels of the genes shown to be differentially expressed by oligonucleotide array analysis were examined by RT-PCR using an additional 12 pairs of samples (colon cancer tissues and distant normal colon mucosa).

Differential expression of the whirlin gene in colorectal cancer. The differential expression of the whirlin gene in colorectal cancer was first confirmed using original primers in a semi-quantitative RT-PCR experiment. Expression levels were normalized to that of ß-actin (Fig. 1a). Gel density was calculated with Scion Image (downloaded from Scion's web site: www.scioncorp.com) and gene expression was quantified. As shown in Fig. 1b, whirlin gene expression in colorectal cancer tissue was significantly up-regulated compared with that in normal colon mucosa (p=0.0209).

Western blotting. We next examined whether whirlin isoform 1 and 2 proteins are expressed in human colorectal cancer cell lines and resected samples from patients with colorectal
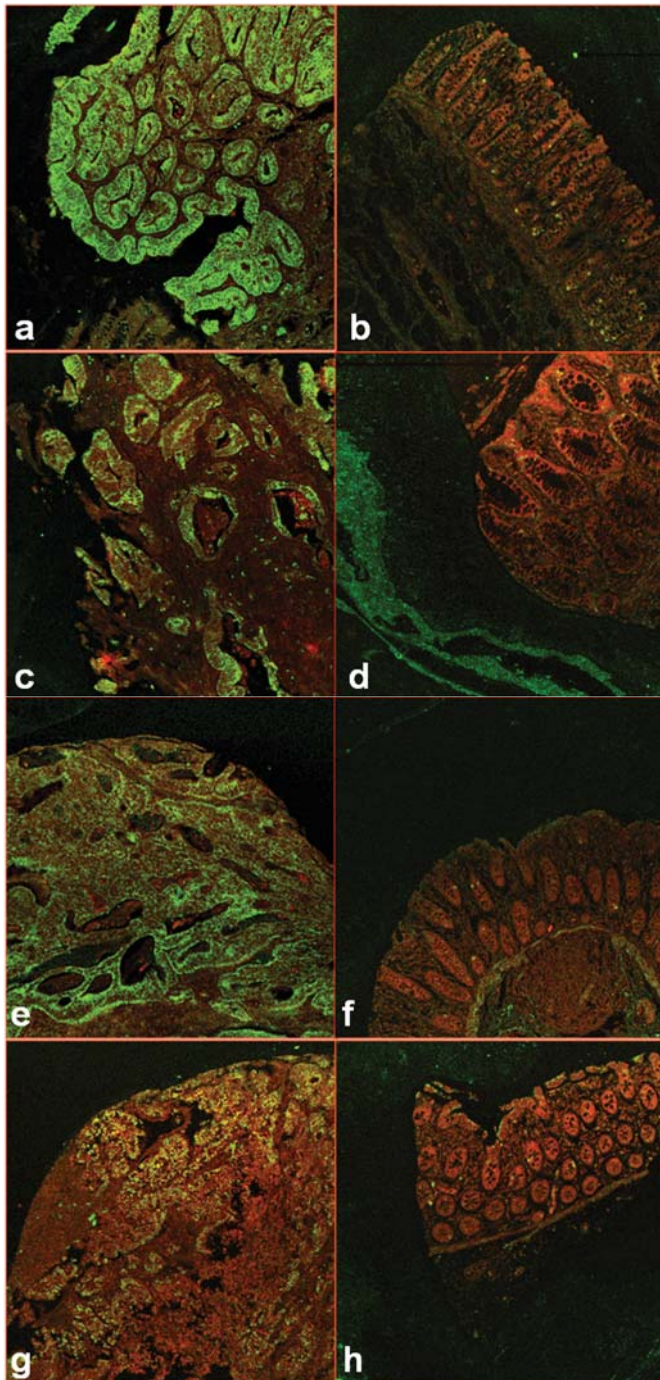
Figure 3. Whirlin isoform 2 expression was evaluated on a high-density tissue microarray of human colon tissues by immunohistochemistry. (a) Sigmoid colon cancer, well differentiated adenocarcinoma (40-year-old female, T3N2M0 stage 3C), intensity grade 3. (b) Adjacent normal colonic mucosa, intensity grade 0. (c) Ascending colon cancer, moderately differentiated adenocarcinoma (49-year-old female, T3N1M0 stage 3B), intensity grade 3. (d) Adjacent normal colonic mucosa, intensity Grade 0. (e) Cecum colon cancer, mucinous adenocarcinoma. (65-year-old female, T3N2M0 stage 3C), intensity grade 2. (f) Adjacent normal colonic mucosa, intensity grade 0. (g) Transverse colon cancer, well-differentiated adenocarcinoma (36-year-old male, T3N2M0 stage 3C), intensity grade 1. (h) Adjacent normal colonic mucosa, intensity grade 0.
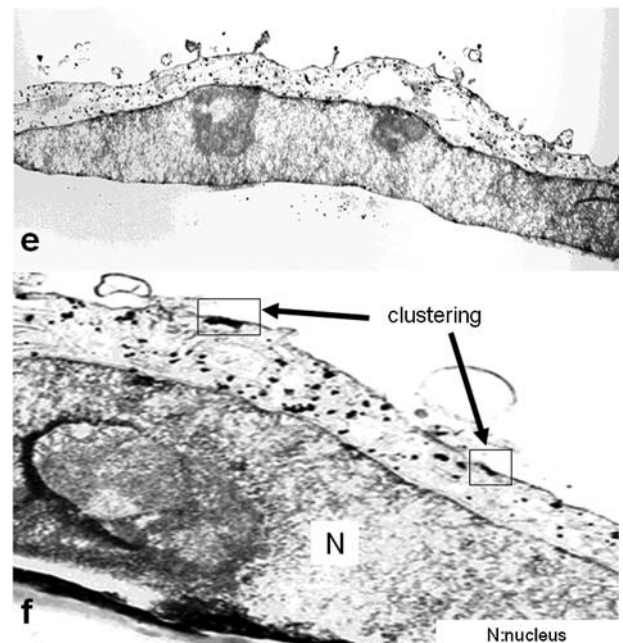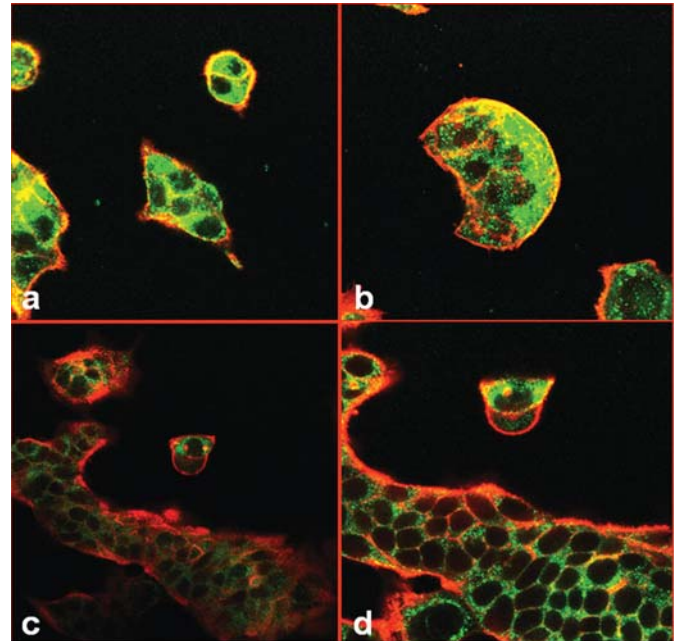

Figure 4. Whirlin isoform 2 was localized in the cytoplasm and on the plasma membrane. Colon cancer cell lines [DLD-1 (a and b) and Caco-2 (c and d)] were stained for ß-tubulin III (red) and whirlin isoform 2 (green). Bar, 100 μM. (e and f) Sections were cut perpendicularly to the base of the culture dish. Immunoreactivity for whirlin isoform 2 (gold particles) revealed that particles were lined up along the plasma membrane and clustered. N, nucleus; arrow, clustering.

cancer. To test the specificity of the antibody to whirlin isoform 2, all of the whirlin isoform 2 sequence was subcloned as part of a GFP fusion protein construct, and expressed in *Escherichia coli* DH5 cells as a GFP-fusion protein.

Western blot analysis using affinity-purified whirlin-specific antibody demonstrated that this protein is expressed in Caco-2 and DLD-1 human colon tumor-derived cultured cells (Fig. 2a). Western blotting showed that the molecular mass of this protein is 49 kDa (Fig. 2b), and it is speculated that this short C-terminal isoform contains one PDZ domain and one proline-rich domain.

Immunohistochemistry. To detect whirlin isoform 2 expression in primary human colon adenocarcinomas, paired colon cancer samples and adjacent normal mucosa samples were screened by immunohistochemistry (Fig. 3a-d). Expression of whirlin isoform 2 was barely detectable in epithelial cells of normal mucosa. By contrast, whirlin isoform 2 expression was confined to epithelial cells and absent from the underlying stroma in colorectal cancer samples.

Subcellular localization of whirlin isoform 2

   Confocal microscopy. To examine the subcellular localization of whirlin isoform 2, immunohistochemistry was performed on cultured human DLD-1 and Caco-2 colon cancer cell lines. Immunoreactivity for whirlin isoform 2 was diffuse in the cytoplasm and on the plasma membrane, but not detected in the nuclei (Fig. 4a and b).

   Electron microscopy. Furthermore, we examined the localization of whirlin isoform 2 at sites of the plasma membrane and cytoplasm of colon cancer cells by immuno-electron microscopy. Gold particles indicating the presence of whirlin isoform 2 were lined up along the plasma membrane and, importantly, showed clustering (Fig. 4c).

Relationship between whirlin isoform 2 expression and clinicopathological variables. The expression of whirlin isoform 2 protein was next evaluated in normal and colon cancer tissue by immunohistochemical analysis of a tissue microarray. The tissue microarray comprised 50 tissue elements from 40 primary colon cancer specimens and 10 adjacent normal colonic mucosa. Staining intensity was evaluated for each tissue element on a scale of 0-3 corresponding to absent, 0; weak, 1; moderate, 2 or strong, 3 staining. A portion of the array is shown in Table II. This analysis demonstrated that the levels of whirlin isoform 2 increased in accordance with stage progression. Furthermore, whirlin isoform 2 is more abundantly expressed in colon cancers with lymph node metastasis compared with those without lymph node metastasis.

Discussion

We showed, for the first time, that the whirlin gene, encoded as AK022854, is highly expressed in colon cancer tissues compared with normal colonic mucosa by microarray analysis. Semi-quantitative RT-PCR also showed significant increases in the expression levels of this gene in primary colon cancer tissues compared with paired distant normal colon tissues, consistent with the microarray data (P<0.05).

   Previously, Mburu *et al* demonstrated that the human whirlin gene is alternatively spliced to form a long and a short transcript (7). Northern blot analysis identified 2 transcripts of approximately 1.8 and 4 kb in the liver and testis and a single 4-kb transcript in the kidney, brain, heart, and lung. The sequence of the encoded protein showed that the short C-terminal isoform contains one PDZ domain and one proline-rich domain, whereas the long isoform is composed of three PDZ domains and one proline-rich domain.

   In the present study, Western blot analysis using a whirlin-specific antibody with a high affinity for both short and long isoforms detected whirlin protein with a molecular

Table II. Correlation of whirlin isoform 2 protein expression with clinical and pathological variables.

Stage	No.	Whirlin isoform 2 staining score (mean ± SD)	P-value
Normal mucosa	10	0.200±0.632	<0.0001
Colon cancer stage II	14	1.357±0.929	
Colon cancer stage III	19	2.474±0.697	
Colon cancer stage IV	7	2.571±0.535	
LN meta (+)	27	2.370±0.550	0.0080
LN meta (-)	13	1.462±1.050	
Distant meta (+)	7	2.571±0.535	0.1437
Distant meta (-)	33	2.000±0.931	

mass of 49.3 kDa, equivalent to the size of the 465 C-terminal amino acids of whirlin, and revealed overexpression of the short C-terminal whirlin isoform 2 protein in colorectal cancer. In addition, whirlin isoform 2 was shown to be highly expressed in cancer samples, and the expression level increased in accordance with disease progression.

   The PDZ domain within the whirlin isoform 2 sequence may mediate binding interactions between whirlin isoform 2 and other cellular proteins. PDZ domains may occur in multiple copies, with each PDZ domain having similar or different binding specificities. PDZ domain-mediated protein-protein interactions have been implicated in the organization of protein complexes in signal transduction cascades, in coupling channels and transmembrane receptors to down-stream signaling elements, in clustering transmembrane receptors and channels, in recruiting cytosolic proteins to membrane complexes, in organizing large two-dimensional complexes such as cell junctions and plasma membrane domains, and in interactions with the cortical cytoskeleton (10,11).

   In the present study, immunohistochemical analysis showed that whirlin isoform 2 protein was diffusely expressed in the cytoplasm and plasma-membrane in human colon cancer cells. In addition, immunoelectron microscopy showed that whirlin isoform 2 proteins were lined up along the plasma membrane and, importantly, showed clustering, a characteristic in common with other PDZ domain-containing proteins.

   Among many PDZ-associated functions, signal trans-duction, clustering, and scaffolding may play important roles in tumorigenesis. One recent study reported up-regulation of a PDZ domain-containing protein, PCD1, in several human tumor types, including malignant prostate tissue, colorectal cancer and breast cancer (12-14). Another study demonstrated that the APC protein binds to the second of five PDZ domains of the protein tyrosine phosphatase protein PTP-BL, suggesting that this binding interaction might modulate the phos-phorylation of associated proteins, and thereby play a role in several cellular activities that are dysfunctional in cancer cells, including cell division, migration and adhesion (15).

   Although the binding specificities of the PDZ domain within whirlin isoform 2 have not been fully investigated,

future studies will identify which cellular proteins bind the PDZ domain of whirlin isoform 2 and how these interactions may contribute to colonic tumorigenesis. Since whirlin isoform 2 was not expressed in the normal colonic mucosa, but expressed in stage III and IV rather than stage II colon cancers, whirlin isoform 2 may be a useful cancer diagnostic marker once its biological function becomes clearer.

In conclusion, although the function of whirlin isoform 2 is still unclear, whirlin isoform 2 is highly expressed in colorectal cancers and its expression is associated with disease progression. Whirlin isoform 2 may play an important role as a target in anticancer drug development or in the search for cancer diagnostic markers. The study of the function of whirlin isoform 2 in colon cancer is underway, and the elucidation of its biological function and its role in carcinogenesis will lead to a further understanding of the importance of whirlin isoform 2 in cancer therapy or diagnosis.

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