Association between aberrant dynein cytoplasmic 1 light intermediate chain 1 expression levels, mucins and chemosensitivity in colorectal cancer

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Received September 24, 2019; Accepted March 19, 2020

DOI: 10.3892/mmr.2020.11086

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Abstract. Dynein transport along the cytoskeletal microtubules towards the minus end is essential for cell division, cell migration and other basic cellular functions. Dynein cytoplasmic 1 light intermediate chain 1 (DYNCL1LI1) has been previously associated with pancreatic ductal adenocarcinoma, hepatocellular carcinoma and prostate cancer. Cytoskeletal structures are involved in the regulation of the mucosal barrier integrity. Thus, improving our understanding of the molecular mechanisms that regulate the mucosal barrier is critical for cancer management and treatment. The present study aimed to investigate DYNCL1LI1 expression in colorectal cancer (CRC) tissues. The American Joint Committee on Cancer Stage II CRC cell line LS 174T was used to determine the association between the cellular expression levels of DYNCL1LI1 and different types of mucin (MUC) by reverse transcription-quantitative PCR. The role of DYNCL1LI1 in cell chemosensitivity and proliferation was also evaluated in the presence of the DNA analog 5-fluorouracil (5-FU) or the platinum-based drug, oxaliplatin by the MTT assay. LS 174T cells with decreased expression levels of DYNCL1LI1 were discovered to be more sensitive to 5-FU compared with LS 174T cells with endogenous DYNCL1LI1 expression levels. Moreover, LS 174T cells transfected with short hairpin RNA targeting DYNCL1LI1 were associated with low MUC1 and high MUC2, MUC4 and MUC5AC expression levels. Notably, the CRC cells with low MUC1 expression levels and high expression levels of the other MUCs (MUC2, MUC4 and MUC5AC) were shown to benefit from 5-FU treatment. In conclusion, the findings of the present study have suggested that DYNCL1LI1 expression may be significantly associated with MUC expression levels and may be used to predict the chemotherapeutic efficiency. However, additional functional studies and clinical reports are required for an improved understanding of the significance of these molecular interactions in tumorigenesis.

Introduction

Cytoskeletal rearrangement is required for cell migration and invasion, which are also important steps for the process of cancer metastasis (1,2). Dynein transport along the cytoskeletal microtubules towards the minus end is essential for cell division, migration and cytoskeletal motors (3-5).

The cytoplasmic dynein complex is implicated in intracellular vesicular transport (6). Numerous studies have reported that microtubule-dependent vesicular trafficking serves a central role in cell function and may impair tumor invasion (7-9). The expression levels of cytoplasmic dynein, particularly dynein light chains, have been associated with several types of cancer in humans; for example, melanoma cell apoptosis was associated with dynein light chain LC8-type (DYNLL1) and DYNLL2 expression (10); and upregulation of dynein light chain roadblock-type (DYNLRB1) and down-regulation of DYNLRB2 expression levels were detected in hepatocellular carcinoma (HCC) (11). Moreover, in another study investigating HCC, aberrant expression levels of dynein...
with short hairpin RNA (shRNA) to target DYNC1LI1. Lentiviral knockdown of DYNC1LI1. plKO_Trc005-dYnc1li1 (clone id: Trcn0000299843), of dYnc1li1. The control plKO_Trc005-luciferase (luc; shdYnc1li1), was used to knockdown the expression level observed (12). dYnc1li1 expression has also been detected in human colorectal cancer cell lines (18,19). Both MUCs have been discovered to be intimately involved in the inflammatory response and tumorigenesis (19). In addition, the cytoskeletal structures have also been found to be involved in the regulation of the mucosal barrier integrity and its breakdown during inflammation (20). Therefore, molecular detection methods that can improve our understanding of the mucosal barrier are critical to both cancer management and treatment. In the present study, the expression levels of DYNC1LI1 were investigated in colorectal cancer (CRC) tissues and in the human epithelial colon cell line LS 174T, which is considered by the American Joint Committee on Cancer (AJCC) to be a model of stage II CRC and to express different MUCs (21). A series of reverse transcription-quantitative PCR (RT-qPCR) experiments were performed to determine the association between the expression levels of DYNC1LI1 and different MUCs in LS 174T cells.

Materials and methods

Cell lines and reagents. The goblet-cell-like CRC cell line, LS 174T (AJCC stage II; CL-188) and two other CRC stage II cell lines, SW480 (CCL-228) and HCT 116 (CCL-247), were purchased from the American Type Culture Collection. The clinical status of the cell lines and the culture conditions are available on the ATCC website (www.atcc.org). Briefly, LS 174T cells were cultured in minimum essential medium (cat. no. 41500-034; Thermo Fisher Scientific, Inc.). All the culture mediums were supplemented with 10% fetal calf serum (cat. no. A6806-35; QBB International Biological Corp.) and 1% antibiotic/antimycotic solution (cat. no. 15240-062; Thermo Fisher Scientific, Inc.). LS 174T cells and HCT 116 cells were maintained in a humidified chamber with 95% air and 5% CO2. SW480 cells were maintained in a humidified incubator with 100% air at 37°C. The Human Reference cDNA (HRC; cat. no. 636692; Takara Bio, Inc.) was used as an expression control for DYNC1LI1.

Lentiviral knockdown of DYNC1LI1. The lentiviral construct, pKO_TRC005-DYNCLI1 (clone ID: TRCN0000231719) vector targeting LUC was used as the negative control (shLUC-LS 174T) for the shDYNC1LI1. Both the lentiviral constructs were obtained from the RNA Technology Platform and Gene Manipulation Core (Sinica, Taiwan). A total of 1.25x10^5 LS 174T cells/well were cultured in a 6-well plate for 24 h and the subsequent lentiviral infections (multiplicity of infection=3) were performed to knock down the expression of DYNC1LI1 in LS 174T cells (shDYNC1LI1-LS 174T). Subsequently, medium containing 2 mg/ml puromycin (Thermo Fisher Scientific, Inc.) was used to select for and maintain stable clones. Following incubation for 48 h, the infection efficiency of DYNC1LI1 knockdown in LS 174T cells compared with the shLUC-LS 174T cells was determined using RT-qPCR and western blotting.

Western blotting. Total protein was extracted from shLUC-LS 174T and shDYNC1LI1-LS174T cells using the PRO-PREP Protein Extraction solution (Intron Biotechnology, Inc.) according to the manufacturer's protocol, supplemented with a protease inhibitor (cat. no. P8340; Merck KGaA, Inc.). Total protein was quantified using Bio-Rad Protein Assay reagent (cat. no. 500-0006; Bio-Rad Laboratories, Inc.) and 15 μg protein/lane was denatured in 1X NuPAGE LDS sample buffer (Thermo Fisher Scientific, Inc.) for 10 min at 95°C, and separated via 12% SDS-PAGE. The separated proteins were subsequently transferred onto a PolyScreen 2 PVDF Transfer membrane (0.2 μm; PerkinElmer, Inc.). The membranes were blocked with 3% bovine serum albumin (cat. no. ALB001.100; BioShop Canada Inc.) for 1 h at room temperature and incubated with the following primary antibodies for 1 h at room temperature: Anti-DYNC1LI1 (1:1,000; cat. no. ab154251; Abcam) and anti-β-actin (1:1,000; cat. no. MAB1501R; Merck KGaA). Following the primary antibody incubation, the membranes were washed with 1X TBST wash buffer (20 mM Tris, 0.15 M NaCl, 0.1% Tween-20; pH 7.4) three times (15 min/time) at room temperature. Subsequently, the membranes were incubated with an alkaline phosphatase-conjugated anti-rabbit antibody (1:5,000; cat. no. A3812; Merck KGaA) or alkaline phosphatase-conjugated anti-mouse antibody (1:5,000; cat. no. A9316; Merck KGaA) for 60 min at room temperature. Protein bands were visualized using a VECTASTAIN® ABC-AmP Duolux chemiluminescent/fluorescent substrate kit (cat. no. AK-6000; Vector Laboratories, Inc.), according to the manufacturers' protocol, and a FluorChem FC2 system (Cell Biosciences, Inc.).

CRC cDNA array and RT-PCR. DYNC1LI1 expression levels were quantified using a cDNA array of colon tissues covering four CRC stages (n=40) and normal colon tissues (n=8; cat. no. HCRT104; OriGene Technologies, Inc.). Total RNA from the cultured cells was extracted using the Easy Pure Total RNA Mini kit (Bioman Scientific Co. Ltd.), according to the manufacturer's protocol. Subsequently, total RNA (1 g) was reverse transcribed into single-stranded cDNA using an oligo(dT)12 primer with a High Capacity cDNA Reverse Transcriptase kit (cat. no. 4368813; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. DYNC1LI1 and MUC2 mRNA expression levels were subsequently quantified by qPCR using the TaqMan Master mix (Roche Diagnostics GmbH). The following thermocycling
conditions were used for qPCR: Initial denaturation at 95°C for 10 min; followed by 60 cycles of 95°C for 10 sec and 60°C for 20 sec (22). The primer pairs and universal TaqMan probes used for the qPCR are presented in Table I. Expression levels of Muc1, Muc4, MUC5AC and MUC6 were determined as previously described by Ohuchida et al (23). Expression levels were quantified using the 2−ΔΔCq method and normalized to the internal reference gene GAPDH.

Determining the chemosensitivity and proliferative rate of shLUC-LS 174T and shDYNC1LI1-LS 174T cells. The chemosensitivity and proliferative rate of shLUC-LS 174T and shDYNC1LI1-LS 174T cells were determined using a MTT assay (cat. no. M5655; Sigma-Aldrich; Merck KGaA). Briefly, 5x10^3 shLUC-LS 174T or shDYNC1LI1-LS 174T cells/well were cultured in 96-well plates for overnight at 37°C before drug treatments. The chemosensitivity of cells to the DNA analog 5-fluorouracil (5-FU; Merck KGaA) or the platinum-based drug oxaliplatin (Merck KGaA) at various concentrations (0.01 µM to 37.5 mM for 5-FU and 2 nM to 1,000 µM for oxaliplatin) was plotted after 72 h of incubation at 37°C and the half-maximal inhibitory concentration (IC50) for each compound was calculated using Gen5™ Data Analysis (version 2.04; BioTek Instruments, Inc.).

To determine the cell proliferative rate, 5x10^3 shluc-lS 174T and sh dYnc1li1-lS174T cells/well were plated into 96-well plates and incubated alone, or with the IC50 dose of 5-FU or oxaliplatin for the 1-7 days at 37°C. For both assays, the cells were treated with 10 µl MTT reagent in the dark for 4 h at room temperature and then 100 µl DMSO was added/well to dissolve the purple formazan formed by the live cells. The absorbance at 540 nm of each well was measured using a Synergy HT Multi-Mode microplate reader (BioTek Instruments, Inc.). Data were obtained from three independent experiments.

Statistical analysis. Statistical analysis was performed using SPSS version 15.0 software (SPSS, Inc.). Statistical differences between 2 groups were determined using a Student’s t-test, whereas statistical differences between >2 groups were determined using a one-way ANOVA, followed by a Bonferroni’s post hoc test for multiple comparisons. Data was presented as the mean ± SD. P≤0.05 was considered to indicate a statistically significant difference.

Results

DYNC1LI1 expression levels in CRC tissues. The differential expression levels of DYNCL1I1 in normal and tumor colonic tissues from a CRC cDNA array were determined using quantitative PCR. Gene expression was normalized to GAPDH. The solid black line denotes the median for each group and the box depicts the 25 and 75th percentile ranges. Capped error bars represent the 95% range of expression levels. Circles represent outliers and solid circles represent extreme values. Data were analyzed using a one-way ANOVA, followed by Bonferroni’s post hoc test. *P<0.01. DYNCL1I1, dynein cytoplasmic 1 light intermediate chain 1; CRC, colorectal cancer.

Differential MUC expression levels in DYNC1LI1 knock-down LS 174T cells. DYNCL1I1 mRNA expression levels were quantified in three CRC cell lines: HCT 116, SW480...
and LS174T. Among these CRC cell lines (AJCC stage II), LS 174T cells had significantly increased expression levels of DYNC1LI1 compared with the HRC control and other CRC cell lines (P<0.01; Fig. 2); therefore, LS 174T cells were used in subsequent experiments. The knockdown efficiency of shDYNC1LI1 in LS 174T cells was subsequently determined using RT-qPCR and western blotting, and it was established to be successful (Fig. 3A and B). Notably, MUC mRNA expression levels were discovered to be differentially altered in the shDYNC1LI1-LS 174T cells (Fig. 3C). Briefly, MUC1 expression levels were significantly decreased by 0.06-fold, whereas the expression levels of MUC2, MUC4 and MUC5AC were all significantly increased by 2.70, 4.17 or 2.75-fold, respectively, in shDYNC1LI1-LS 174T cells compared with the shLUC-LS 174T cells (Fig. 3C). No significant differences were observed in the expression levels of MUC6 between the shDYNC1LI1-LS 174T and shLUC-LS 174T cells.

**Chemotherapeutic effects in shLUC-LS 174T and shDYNC1LI1-LS 174T cells.** 5-FU, leucovorin, and oxaliplatin (FOLFOX4) is a chemotherapeutic reagent used for the treatment of CRC (24,25). It was discovered that the genetic knockdown of DYNC1LI1 in LS 174T cells increased the susceptibility of the cells to 5-FU and oxaliplatin treatment; the IC_{50} of 5-FU (9.7 μM) and oxaliplatin (0.4 μM) was significantly decreased in the shDYNC1LI1-LS 174T cells compared with the shLUC-LS 174T cells (23.3 μM and 1.06 μM, respectively; Fig. 4A and B). The differences in the cell proliferative rate of shLUC-LS 174T and shDYNC1LI1-LS 174T cells began at day 4 without any drug treatment (Fig. 5A), and at day 3 with 5-FU (Fig. 5B) or oxaliplatin chemotherapy (Fig. 5C). Moreover, the shDYNC1LI1-LS 174T cells exhibited a significantly reduced proliferative rate at day 7 compared with the shLUC-LS 174T cells following both 5-FU or oxaliplatin chemotherapy (P<0.05; Fig. 5B and C).

**Discussion**

Tumorigenesis involves cytoskeletal reorganization (26) and as demonstrated by van Rees et al. (27), cytoplasmic dyneins can regulate centrosome dynamics. Thus, any changes in their function have been hypothesized to lead to cancer formation. For example, it was previously reported that in patients with advanced CRC, the expression levels of the cytoplasmic dynein 1 heavy chain 1 (DYNC1H1) were decreased (28). This aberrant expression of DYNC1H1 was suggested to impede the assembly of complete cytoplasmic dynein, resulting in inadequate microtubule dynamics.

In the present study, increased expression levels of DYNC1LI1 were observed in late-stage, metastatic CRC tissues. Therefore, the tumorigenic potential of DYNC1LI1 in CRC warrants further metastatic investigations and research. The goblet-cell-like LS 174T cells, which exhibit mucinous secretory granules, have previously provided an excellent in vitro model for studying the expression levels of MUCs in CRC (21). The present study revealed that genetic knockdown of DYNC1LI1 induced decreased expression levels of MUC1 and increased expression levels of MUC2, MUC4 and MUC5AC in LS 174T cells. The differentially expressed levels of DYNC1LI1 in LS 174T cells have been suggested to change the MUC profile, thereby generating differences in the mucosal barrier (29). Thus, it can be further hypothesized that DYNC1LI1-induced changes in the mucosal barrier may be positively associated with tumor formation. The integrity of the gut mucosal barrier, which is composed of a specific profile of MUCs, has been found to be crucial for protecting epithelial cells from inflammatory or cancerous responses (30,31).

A previous study provided molecular evidence to indicate that the changes in the expression levels of cytoskeleton-related genes may worsen the prognosis of patients with CRC and lead to the formation of an aberrant mucosal barrier with a different MUC profile (19). A previous study indicated that several MUCs, such as increased MUC1 and decreased MUC2, are associated with CRC (18). Based on the results of the present study and the aforementioned studies, it was hypothesized that increased DYNC1LI1 expression levels may be associated with the advanced stages of CRC. In LS 174T cells, the expression levels of MUC1, a transmembrane glycosylated phosphoprotein, and DYNC1LI1 displayed a similar expression trend in the present study; this result is consistent with that observed in the CRC cell lines, HCT 116 and SW480. A previous study indicated that cells with lower DYNC1LI1 expression levels also had decreased expression levels of MUC1 (32,33). MUC1, a membrane-bound MUC, was also found to be overexpressed in numerous types of human cancer, such as endometrioid endometrial carcinoma and CaP (34,35). Moreover, patients with increased MUC1 expression levels were suggested to have a poorer prognosis (36). Bette et al. (17) concluded that MUC1 expression levels had no effect on the clinical outcomes, although more than half of the studied patients with CRC exhibited MUC1 overexpression. Other previous studies investigating the prognostic value of MUCs in cancers have also reported contradictory results (37-39). These discrepancies in the results of different studies may be attributable to the differences in cytoskeletal gene expression. Thus, DYNC1LI1...
expression levels, and its effects, must be further understood in order to predict patient prognosis.

Secreted gel-forming MUCs, another family of MUCs, include both MUC2 and MUC5AC (18). Betge et al (17) reported that the loss of MUC2 and MUC5AC expression was associated with favorable CRC outcomes; however, only reduced expression levels of MUC2, and not of MUC5AC, were detected in colonic tumors by Byrd and Bresalier (18). Previously, MUC2 was suggested to be a crucial component of the MUC profile in the mucosal barrier, but served no role in carcinogenesis (40). In addition, an animal model indicated that Muc2-knockout mice had a poorly defined mucosal barrier (16). Collectively, these results indicated that DYNC1LI1, which upregulates the expression levels of transmembrane MUCs, such as MUC1, and downregulates the expression levels of secreted gel-forming MUCs, such as MUC2 and MUC5AC, may be critical for assessing the composition of the mucosal barrier in patients with CRC. Compared with normal prostate or benign tissue, CaP tissue exhibited a similar MUC profile, with increased MUC1 and decreased MUC2, MUC4, MUC5AC and MUC6 expression levels (34). In addition, the difference of immunohistochemical staining patterns of MUC1 and MUC2 were caused by the carcinomatous transformation of urothelial neoplastic and preneoplastic lesions (41). Taken together, these findings suggested that different types of cancer, including those of the urological and gastrointestinal systems, exhibit diverse MUC profiles.

However, the possibility that disrupting the microtubule dynamics in shDYNC1LI1-LS 174T cells may influence cell proliferation cannot be excluded. For example, Even et al (42) reported that DYNC1LI1 contributed to the proliferative overgrowth of CRC cells, which is consistent with the present study results, which found that shDYNC1LI1-LS 174T cells had a slower proliferative rate compared with shLUC-LS 174T cells without any drug treatment. Adjuvant chemotherapy, such as 5-FU or oxaliplatin, which are the ingredients of FOLFOX4, was discovered to further inhibit the proliferation of LS 174T cells with low DYNC1LI1 expression levels. Thus, these findings suggested that DYNC1LI1 may be a marker molecule for prescribing 5-FU as a first-line chemotherapy treatment for patients with CRC. In our in vitro cell study, shDYNC1LI1-LS

Figure 3. Relative mRNA expression levels of MUCs in shDYNC1LI1-LS 174T cells. (A) Relative mRNA and (B) protein expression levels of DYNC1LI1 were determined by RT-qPCR or western blotting, respectively, in shLUC-LS 174T and shDYNC1LI1-LS 174T cells. (C) Expression levels of MUCs were determined in shLUC-LS 174T and shDYNC1LI1-LS 174T cells. All mRNA expression levels were normalized to GAPDH and protein expression levels were normalized to β-actin. The Student's t-test was used to determine the efficiency of the shDYNC1LI1 infection in parts A and C. Each experiment was independently performed in duplicate or triplicate. Numerical data are expressed as the mean ± SD. ∗P<0.05 and ∗∗P<0.01. MUC, mucin; shRNA, short hairpin RNA; DYNC1LI1, dynein cytoplasmic 1 light intermediate chain 1; LUC, luciferase.
174T cells were more sensitive to 5-FU treatment compared with cells with endogenous DYNC1LI1 expression levels. As previously discussed, shDYNC1LI1-LS 174T cells had low MUC1, and high MUC2, MUC4 and MUC5AC expression levels, implying that CRC cells with low MUC1 expression levels and high expression levels of other MUCs may benefit from 5-FU-based therapy. Furthermore, this result was similar to findings in pancreatic cancer cells reported by Kalra and Campbell (43), where it was demonstrated that increased MUC1 expression levels potentially impeded the cytotoxic effects of 5-FU against the proliferation of pancreatic cancer cells.

In conclusion, based on the findings of the present results and those of other previous studies, it was suggested that DYNC1LI1, which is significantly associated with MUC...
expression levels, may affect the efficiency of chemotherapy. Additional functional studies and clinical reports are required for understanding its molecular significance in tumorigenesis.

Acknowledgements
Not applicable.

Funding
The current study was supported by grants from the Cathay General Hospital and Taipei Medical University (grant nos. 104CGH-TMU-05 and 105CGH-TMU-04) and the Cathay General Hospital (grant no. CGH-MR-A10316).

Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Authors' contributions
CCC and KCC performed the experiments and wrote the manuscript. CJH performed the statistical analysis and helped revise the manuscript. CSH contributed to the interpretation of the data. YCW conceived and designed the study. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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