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ORIGINAL ARTICLE – TRANSLATIONAL RESEARCH AND BIOMARKERS

WiNTRLINC1/ASCL2/c-Myc Axis Characteristics of Colon Cancer with Differentiated Histology at Young Onset and Essential for Cell Viability

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ABSTRACT

Background. WiNTRLINC1 is a long non-coding RNA (lncRNA) that positively regulates the Wnt pathway via achaete-scute complex homolog 2 (ASCL2) in colorectal cancer. ASCL2 was recently reported to play a critical role in chemoresistance, however clinical relevance of the WiNTRLINC1/ASCL2 axis remains obscure in colon cancer.

Patients and Methods. WiNTRLINC1/ASCL2 expression was investigated at messenger RNA (mRNA) level in 40 primary colon cancer tissues and the corresponding normal mucosa tissues, together with Wnt-related genes (c-Myc/PRL-3) and other lncRNAs (H19, HOTAIR, and MALAT1). Knock-down experiments of WiNTRLINC1 clarified its role in their expression and chemoresistance.

Results. Real-time quantitative reverse transcriptase–polymerase chain reaction confirmed definite overexpression of WiNTRLINC1 mRNA in primary colon cancer compared with the corresponding normal colon mucosa tissues ($p = 0.0005$), such as ASCL2, c-Myc, and PRL-3 ($p < 0.0001$). The four gene expression signatures were tightly associated in the center of the ASCL2 gene ($r = 0.72$, $p < 0.0001$) in clinical samples. WiNTRLINC1 was not significantly associated with prognostic factors in colon cancer and other lncRNAs, while the WiNTRLINC1/ASCL2/c-Myc signatures were unique to young-onset colon cancer with differentiated histology. On the other hand, undifferentiated histology was significantly associated with H19 expression. Knockdown of the WiNTRLINC1 gene reduced the expression of ASCL2/c-Myc, but rather augmented PRL-3 at mRNA level, and robustly affected cell viability in colon cancer cell lines.

Conclusion. The enhanced WiNTRLINC1/ASCL2/c-Myc axis involved in Wnt pathway activation is a common pathway essential for differentiated colon tumorigenesis, especially with young onset, and may be essential for a viable phenotype of colon cancer.

The prevalence of colorectal cancer (CRC) is increasing and has one of the highest cancer morbidities reported worldwide.[1] Prognosis becomes poor once CRC has progressed to lymph node metastasis (LNM) or distant organ metastasis.[2,3] Thus, multimodality anticancer therapies, including radiation therapy, have been rigorously established for advanced CRC;[4,5] however, some cases exhibit treatment failure and/or severe adverse effects associated with the anticancer therapies. Hence, the mechanisms involved in drug resistance must be clarified to develop elaborate therapeutic strategies, using useful biomarkers, for patient selection.

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We recently built an exploration model to identify genes involved in drug resistance to anticancer therapy using the histone deacetylase inhibitor phenyl butyrate. In breast cancer, this model discovered the ZEB1 gene,[6] which has been confirmed to be associated with anticancer therapy resistance in breast cancer.[7,8] On the other hand, in CRC, and using the same model, achaete-scute complex homolog 2 (ASCL2) was newly identified as being associated with drug resistance.[9]

ASCL2 has been proposed as a marker of dynamic stem cells in the goblet of the colonic mucosa, which was ubiquitously co-expressed with Lgr5[10,11] but not in + 4 stem cells, which are quiescent stem cells positive for HOPX[12,13]. ASCL2/HOPX are thus considered to be involved in Wnt pathway regulators as cancer-initiating cells,[14] and ASCL2 expression has recently been reported to be regulated by the novel long non-coding RNA (lncRNA) WiNTRLINC1, rather than by genomic amplification in CRC.[9,15,16]

There have been no reports describing the clinical significance of WiNTRLINC1 in primary CRC. In this study, for the first time, we examined its clinical relevance together with ASCL2 and other Wnt pathway-related genes, as well as major lncRNAs in primary colon cancer.

MATERIALS AND METHODS

Patients and Sample Collection

We collected tumor specimens and corresponding normal mucosal specimens from 40 consecutive colon cancer patients who underwent colectomy at the Department of Surgery, Kitasato University Hospital, Japan, from February 2018 through March 2019 (electronic supplementary Table S1). TNM classification was defined according to the Unio Internationalis Contra Cancrum. All tissue samples were collected at Kitasato University Hospital, and informed consent was obtained. This study was performed with approval of the Ethics Committee of the Kitasato University School of Medicine.

Cell Lines and Culture

We used eight CRC cell lines; HCT116, DLD1, COLO320, COLO205, and LOVO were kindly provided by the Cell Response Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan), SW480 was purchased from the American Type Culture Collection (Manassas, VA, USA), HCT15 was purchased from the RIKEN BioResource Center (Tsukuba, Japan), and LS174T was purchased from the European collection of authenticated cultures. LOVO and SW480 were grown in 50:50 RPMI 1640:F-12 HAMS medium (Sigma-Aldrich N6658, USA) and Leibovitz L15 medium, while all other cell lines were grown in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and penicillin–streptomycin (Gibco).

Small Interfering RNAs and Transfection

Two individual small interfering RNAs (siRNAs) were purchased from Sigma-Aldrich. Target sequences for WiNTRLINC1 siRNAs were as follows: siRNA1, 5′-GCAAGAAGAACGAGGCCAAUU-3′, and siRNA3, 5′-CAGGGAGGCUGAAGAGCAAUU-3′. SiRNA oligonucleotides in Opti-MEM (Invitrogen, Carlsbad, CA, USA) were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacture's protocol. After 48 h, WiNTRLINC1 expression levels were measured.

RNA Extraction

During surgery, all specimens were immediately collected and stored in RNAlater RNA Stabilization Reagent (Qiagen, Hilden, Germany) at 4 °C overnight. The next day, the specimens were sliced down and stored at − 80 °C. RNA extraction from homogenized specimens and cell lines was performed using the RNeasy Midi Kit (Qiagen) following the manufacture's protocol. The RNase-Free DNase Set (Qiagen) was used to digest DNA during RNA purification. Two micrograms of total RNA were reverse transcribed into complementary DNA (cDNA) using oligo-d(T) primer and the SuperScript III reverse transcriptase kit (Invitrogen).

Semi-quantitative Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

The pooled assay mix contained primers at a final concentration of 0.2 μM, 1 μL of dNTP mixture, 1.5 μL of $MgCl_2$, 5 μL of PCR buffer, and 0.2 μL of Platinum Taq DNA polymerase (Invitrogen) in a final volume of 50 μL. The thermal cycling conditions were an initial hold at 95 °C for 3 min and 25–35 cycles of 30 s at 95 °C, 30 s at annealing temperature, 30 s at 72 °C, and 10 min at 72 °C.

Quantitative Reverse Transcriptase–Polymerase Chain Reaction (Q-PCR)

Gene expression levels were validated by Q-PCR with iQ™ Supermix (Bio-Rad, Hercules, CA, USA) in triplicate on the iCycler iQ™ Real-Time PCR Detection system (Bio-Rad). PCR conditions were an initial hold at 95 °C for

3 min and 40 cycles of 20 s at 95 °C, 30 s at annealing temperature, 30 s at 72 °C in a 25 μL reaction volume containing 200 nmol/L fluorescent probe and 12.5 μL iQ™ Supermix. Target gene expression levels were normalized to β-actin and analyzed using the comparative cycle threshold method.

Chemosensitivity Assay

WiNTRLINC1 knocked-down cells were seeded onto a 96-well plate at a density of 1×10^4 cells per well and incubated for 24 h. The cells were then exposed to different concentrations of 5-fluorouracil and CPT-11 for 48 h. Cell viability was measured using the CytoSelect™ WST-1 Cell Proliferation Assay Reagent (Cell Biolabs, Inc., San Diego, CA, USA) according to the manufacture's protocol.

Statistical Analysis

Continuous variables were evaluated using the Student's *t* test, and categorical variables were evaluated using the Fisher's exact test or Chi-square test, as appropriate. A p value < 0.05 was considered to indicate statistical significance. All calculations were performed using JMP® version 14 software (SAS Institute Japan, Tokyo, Japan).

RESULTS

Clinical Significance of WiNTRLINC1 Expression in 40 Primary Colon Cancers

WiNTRLINC1 expression was initially investigated by semi-quantitative PCR in the three consecutive colon cancers, and all cases clearly overexpressed it in primary tumors compared with the corresponding non-cancerous mucosas (Fig. 1a). We then investigated WiNTRLINC1 expression in the eight CRC cell lines by semi-quantitative (Fig. 1b) and TaqMan quantitative PCR (Q-PCR) (Fig. 1c; Colo320 cells as the positive control).

In Q-PCR, WiNTRLINC1 expression was significantly higher in primary tumors than in the corresponding non-cancerous mucosas ($p = 0.0005$) (Fig. 2a). There was no significant difference of WiNTRLINC1 expression in pathological T (pT), pN, pM, and pStage, although it was slightly higher in patients with LNM and pStage III (Fig. 2b). WiNTRLLINC1 expression was rather low in cases with distant metastasis. All other clinicopathological factors did not significantly correlate with WiNTRLINC1 expression in primary colon cancer (electronic supplementary Table S1).

Clinical Significance of ASCL2 Expression in 40 Primary Colon Cancers

ASCL2 expression was then investigated in eight CRC cell lines by semi-quantitative PCR (Fig. 1b) and Q-PCR (Fig. 1c; DLD1 cells as the positive control). Interestingly, ASCL2 expression was significantly correlated with WiNTRLINC1 expression in CRC cell lines, as previously reported[16] (Fig. 1b). In Q-PCR, ASCL2 expression was significantly higher in primary tumors than the corresponding non-cancerous mucosas ($p < 0.0001$) (electronic supplementary Fig. S1a). ASCL2 expression was faint at messenger RNA (mRNA) in the non-cancerous mucosas, however fluorescent staining confirmed ASCL2 expression at the base of the large intestinal crypts, as previously reported[17] (data not shown).

There was no significant difference of ASCL2 expression in clinicopathological factors (electronic supplementary Fig. S1b). ASCL2 expression was strongly correlated with WiNTRLINC1 expression in both tumor and mucosa tissues ($r = 0.72$, $p < 0.0001$) (Fig. 3a, Table 1). Moreover, ASCL2 expression was significantly correlated with young onset ($p = 0.0381$) (Fig. 3b) and histological differentiation ($p = 0.0307$) in tumors (Fig. 3c). WiNTRLINC1 expression was not significantly correlated with these two factors, however the tendencies were similar with ASCL2 (Fig. 3b, c). Nevertheless, the best cut-off value of WiNTRLINC1 expression level (3.8) clarified that there were significantly different rates of high expression (75% vs. 15.6%) between young and elderly patients (defined as age $\geq$ 55 years, $p = 0.0071$).

Clinical Significance of Wnt-Related Gene Expression in 40 Primary Colon Cancers

The WiNTRLINC1/ASCL2 axis is involved in Wnt pathway activation in CRC,[16] and c-Myc is a representative and essential Wnt-targeted oncogene.[18] PRL-3 also activates the Wnt signal through the non-canonical pathway,[19] which is considered necessary for CRC metastasis.[20] We therefore investigated both genes at mRNA level in eight CRC cell lines, by semi-quantitative (Fig. 1b) and quantitative PCR (Fig. 1c; HCT116 for c-Myc and Colo320 for PRL-3 as the positive controls).

There was no significant difference of c-Myc expression in clinicopathological factors (electronic supplementary Fig. S1c). c-Myc expression was significantly correlated with young onset ($p = 0.0003$) (Fig. 3b), as in WiNTRLINC1/ASCL2 expression. Furthermore, c-Myc expression was not significantly correlated with histological differentiation, however the tendencies were similar with ASCL2 ($p = 0.101$) (Fig. 3c). c-Myc expression was strongly correlated with either ASCL2 ($r = 0.72$, $p < 0.0001$) (Fig. 3a)

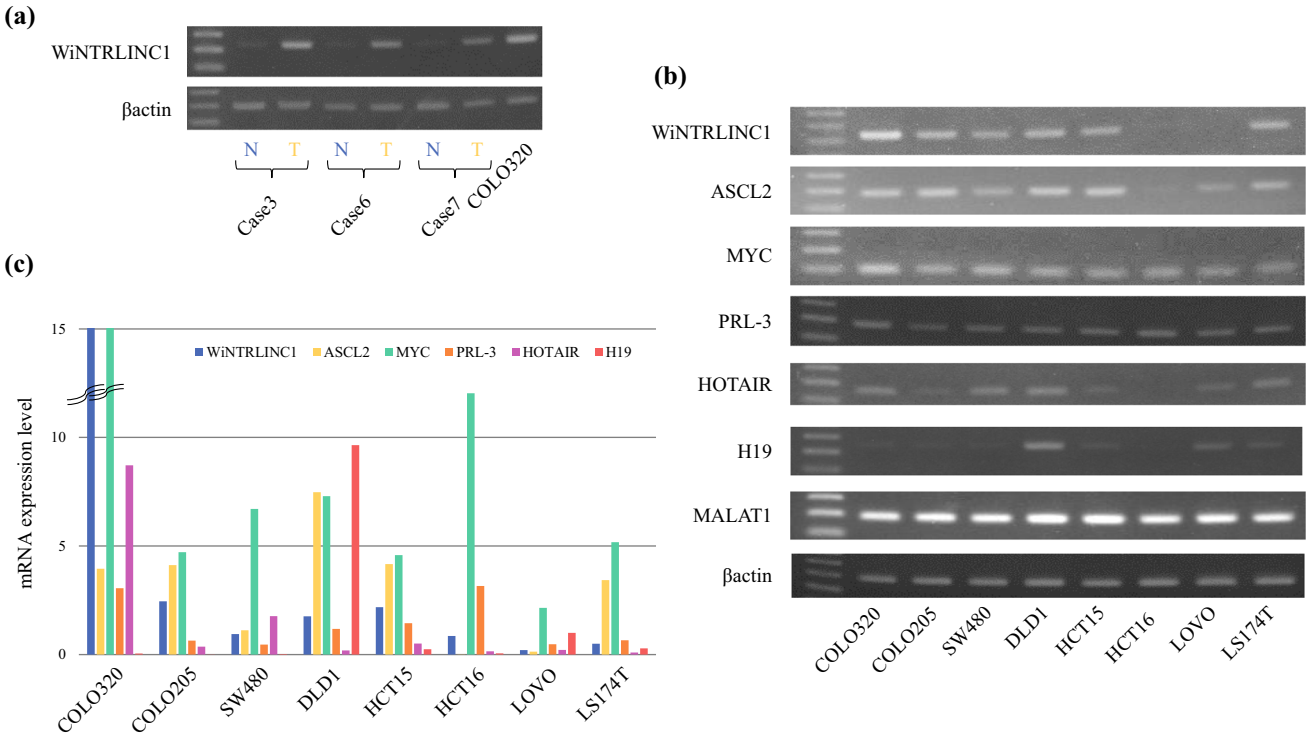

FIG. 1 Wnt pathway-related genes and major lncRNA expression status in primary colon cancer and CRC cell lines. **a** RT-PCR analysis of mRNA expression of WiNTRLINC1 in clinical samples. **b** RT-PCR analysis of mRNA expression of seven genes (WiNTRLINC1, ASCL2, MYC, PRL-3, HOTAIR, H19, and MALAT1) in eight CRC cell lines. **c** Q-PCR analysis of mRNA expression of six genes (WiNTRLINC1, ASCL2, MYC, PRL-3, HOTAIR, and H19) in eight CRC cell lines. *lncRNA* long non-coding RNA, *RT-PCR* reverse transcriptase–polymerase chain reaction, *mRNA* messenger RNA, *CRC* colorectal cancer, *Q-PCR* quantitative reverse transcriptase–polymerase chain reaction, *N* corresponding normal mucosa, *T* primary tumor

or WiNTRLINC1 expression in both tumor and mucosa tissues ($r = 0.50$, $p < 0.0001$) (Fig. 3a). c-Myc expression was also significantly correlated with aggressive lymphatic permeation ($p = 0.0199$). Thus, the WiNTRLINC1/ASCL2/c-Myc axis activated by WiNTRLINC1 is commonly found in early tumorigenesis of differentiated colon cancer at young onset (Fig. 4c, red box).

There was no significant difference of PRL-3 expression in pT, pN, pM, and pStage (electronic supplementary Fig. S1d), but there was close association of PRL-3 expression with the WiNTRLINC1/ASCL2/c-Myc axis (Table 1, Fig. 3); however, PRL-3 expression did not significantly correlate with both histological differentiation and age.

Clinical Significance of Long Non-coding RNA Expression in 40 Primary Colon Cancers

We investigated three lncRNAs at mRNA in eight CRC cell lines. Strong H19 expression was only seen in DLD1 cells (positive control) (Fig. 1b, c), while MALAT1 expression was ubiquitously recognized in all eight cells (Fig. 1b). HOTAIR expression was the highest in Colo320 cells (positive control) among the eight CRC cells (Fig. 1c). In Q-PCR, HOTAIR expression was faint ($\sim$ 0.3 at most) in colon cancer tissues in contrast to the positive control Colo320 ($\sim$ 8), and its overexpression in tumor tissues was marginal ($p = 0.0641$) (electronic supplementary Fig. S2a). In addition, HOTAIR had no association with any clinicopathological factors (data not shown).

On the other hand, H19 expression was significantly higher (mean 38.0-fold) in colon cancer tissues than the corresponding non-cancerous tissues ($p = 0.0233$) (electronic supplementary Fig. S2a). The case with its highest expression was unique (giant undifferentiated tumor). Putatively due to this case, H19 expression was significantly correlated with histological undifferentiation ($p = 0.0158$) (electronic supplementary Fig. S2b) and tumor size ($p = 0.0048$) (electronic supplementary Fig. S2b). The top three highest cases were all right-sided colon in female.

WiNTRLINC1 Knockdown in Colo320 Cells

Knockdown of WiNTRLINC1 expression was performed using RNA interference, as previously described.[16] Q-PCR confirmed significant inhibition of WiNTRLINC1 mRNA expression in Colo320 cells by two types of RNA interference (Fig. 4a). Interestingly, expression change was

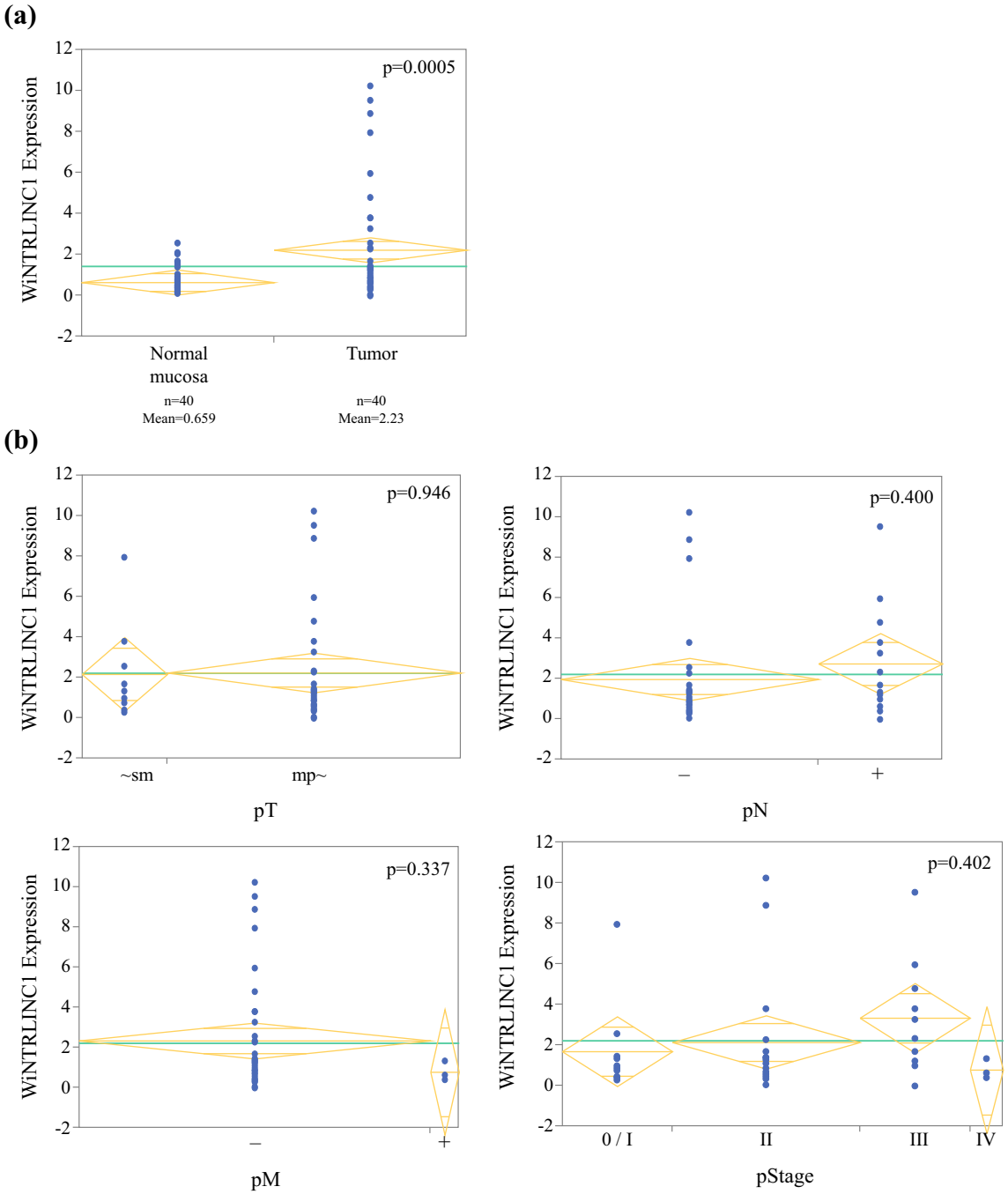

FIG. 2 Clinicopathological analysis of WiNTRLINC1 gene expression in primary colon cancer tissue samples. **a** WiNTRLINC1 mRNA expressions are quantified by Q-PCR in 40 colon cancer tissues in contrast to the 40 corresponding non-cancerous mucosa tissues (p = 0.0005). **b** WiNTRLINC1 mRNA expressions are shown according to pT, pN, pM, and pStage. There was no significant difference of WiNTRLINC1 mRNA expression in pT, pN, pM, and pStage. *mRNA* messenger RNA, *Q-PCR* quantitative reverse transcriptase–polymerase chain reaction

accompanied by reduced expression of ASCL2/c-Myc (Fig. 4a), suggesting that WiNTRLINC1 is on the upstream of ASCL2/c-Myc overexpression in colon cancer cells, while the degree of reduction was modest in c-Myc. These findings suggested that ASCL2 is a direct target of WiNTRLINC1[16] whereas c-Myc is indirect (Fig. 4c). On the other hand, WiNTRLINC1 knockdown rather significantly augmented PRL-3 expression (Fig. 4a). PRL-3 is considered an alternate activator of ASCL2 in primary tumors (Fig. 4c).

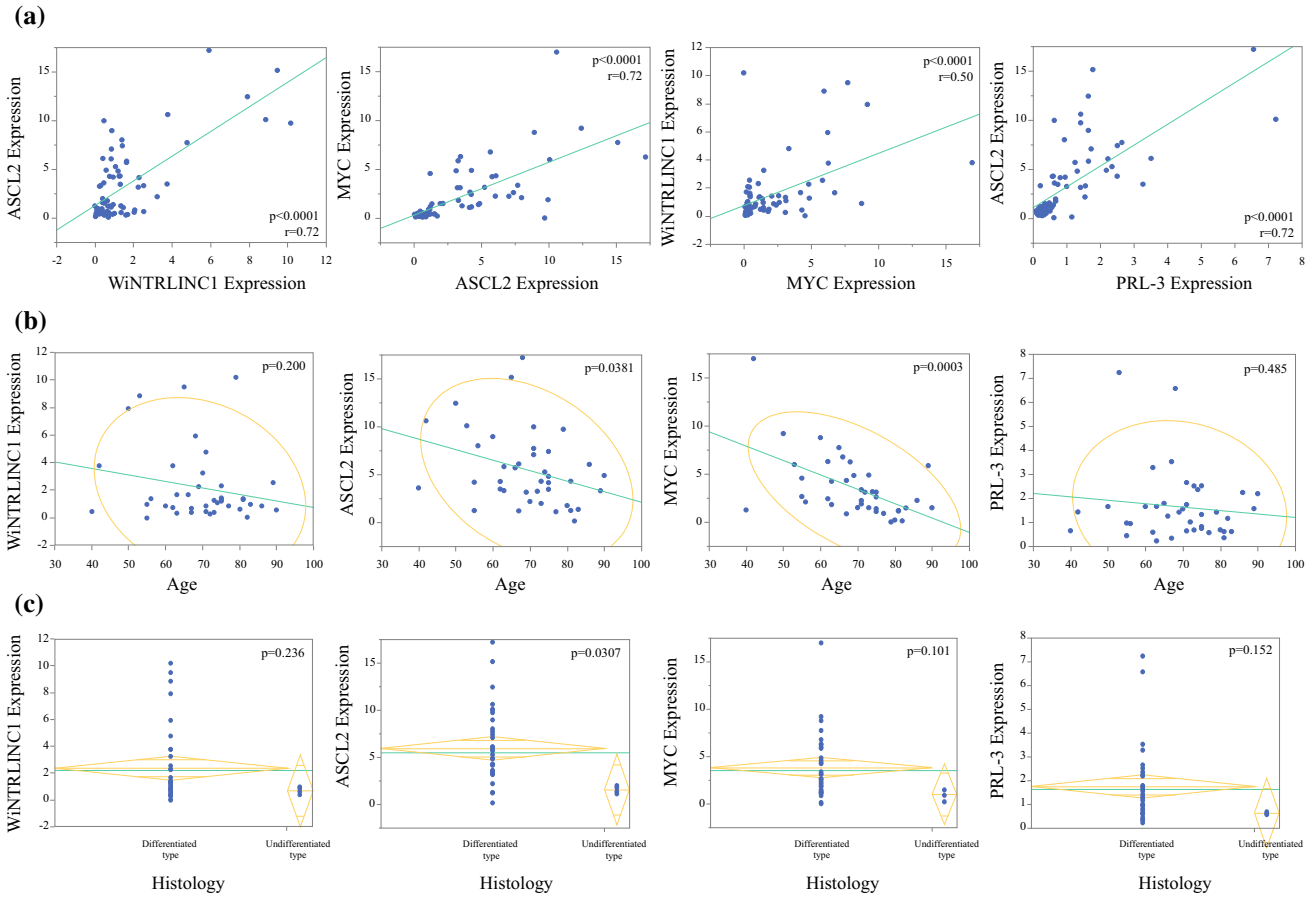

FIG. 3 Associated expressions between Wnt-related genes (WiNTRLINC1, ASCL2, MYC, and PRL-3) in clinical samples of primary colon cancer patients. **a** Significant associations ($p < 0.0001$) are seen between WiNTRLINC1 and ASCL2 ($r = 0.72$), ASCL2 and MYC ($r = 0.72$), MYC and WiNTRLINC1 ($r = 0.50$), and PRL-3 and ASCL2 ($p = 0.72$) in 40 tumor tissues and 40 corresponding non-cancerous mucosa tissues. **b** Age is inversely correlated with expressions of WiNTRLINC1 ($p = 0.20$), ASCL2 ($p = 0.0381$), MYC ($p = 0.0003$), and PRL-3 ($p = 0.485$) in 40 tumor tissues. **c** Differentiated histology showed higher expression than undifferentiated histology in WiNTRLINC1 ($p = 0.236$), ASCL2 ($p = 0.0307$), MYC ($p = 0.101$), and PRL-3 ($p = 0.152$) in 40 tumor tissues

TABLE 1 Correlation analysis of the 6 genes expression in 40 primary colon cancer patients

	WiNTRLINC1	ASCL2	MYC	PRL-3	H19	HOTAIR
WiNTRLINC1		0.72	0.5	0.59	0.12	0.31
ASCL2			0.72	0.72	0.16	0.25
MYC				0.54	0.084	0.24
PRL-3					0.2	0.078
H19						0.053
HOTAIR						

WiNTRLINC1 Knockdown Robustly Decreases Cell Viability in CRC Cell Lines

We investigated the effect of WiNTRLINC1 knockdown on the chemosensitivity of Colo320 cells using anticancer drugs such as 5-fluorouracil and CPT-11. Cells were exposed to 0–100 μg/ml of 5-fluorouracil and CPT-11 for 48 h. Inhibition of WiNTRLINC1 expression potently suppressed cell viability even without drug treatment (0 concentration) and in almost all concentrations compared with both the scr of #1 and #3, respectively (Fig. 4b).

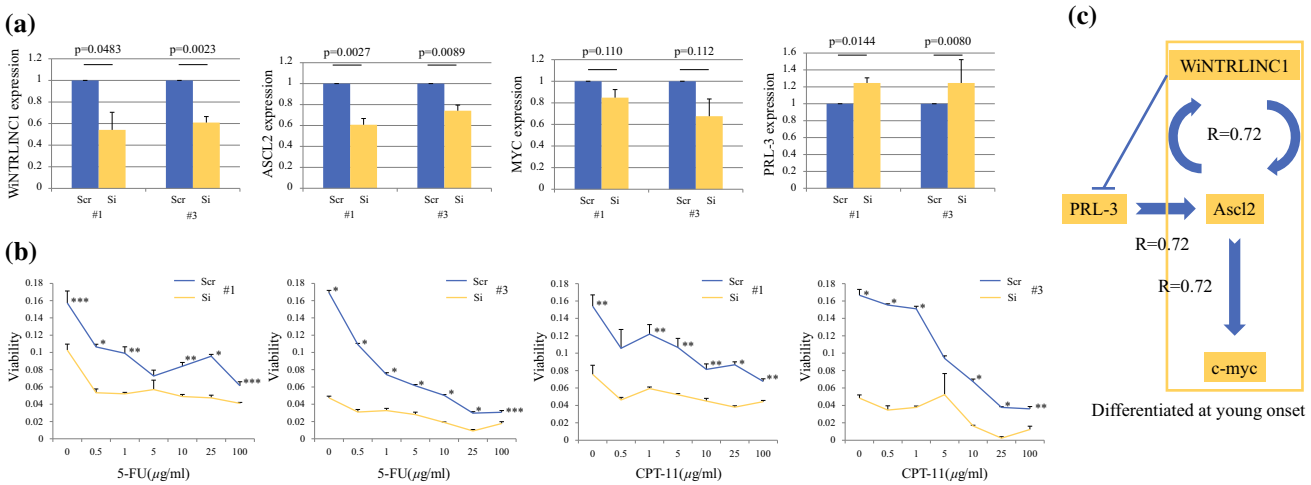

FIG. 4 WiNTRLINC1 knockdown experiments describing its relation to cell viability in the context of the WiNTRLINC1/ASCL2/c-Myc axis. **a** Inhibition of WiNTRLINC1 expression using #1 and #3 RNA interference (Si) resulted in the reduction of ASCL2/Myc expression, while expression of PRL-3 was inversely augmented compared with its negative control (scr representing scrambled sequences) of #1 and #3, respectively. **b** Inhibition of WiNTRLINC1 expression significantly reduced cell viability in almost all concentrations of anticancer drugs, compared with the scr of #1 and #3. Data were representative of three independent experiments. **c** Correlation coefficient of the clinical samples and knockdown experiments proposed model of molecular interactions of the WiNTRLINC1/ASCL2/c-Myc axis. $*p < 0.0001$, $**p < 0.001$, $***p < 0.01$

DISCUSSION

The lncRNA WiNTRLINC1 induced its genomic neighbor ASCL2, a transcription factor that controls intestinal stem cell fate. WiNTRLINC1 interacts with TCF4/β-catenin to mediate the juxtaposition of the promoter with the regulatory regions of the ASCL2 gene.[16] WiNTRLINC1-induced ASCL2 results in closing a feed-forward regulatory loop through ASCL2-induced WiNTRLINC1 (Fig. 4c).[16] Furthermore, ASCL2 was previously proved to be overexpressed in both adenoma and primary CRC.[17] This regulatory circuitry is highly amplified during CRC tumorigenesis, while there have been no reports describing the clinical relevance of WiNTRLINC1 in human cancers. Reflected by these early reports, our study of colon cancer demonstrated, for the first time, WiNTRLINC1 overexpression and the association of WiNTRLINC1/ASCL2/c-Myc in clinical samples, which was unique to differentiated histology at young onset. This feature may be largely derived from the close links of this pathway in tumor tissues.

In this study, testing was restricted to colon cancer only because the current mainstay of treatments for advanced rectal cancer include preoperative chemoradiation therapy,[4] where molecular information from the tumor tissues is considered to be highly modified. In our study, ASCL2 expression was strongly associated with both WiNTRLINC1 ($r = 0.72$) and c-Myc ($r = 0.72$), suggesting that both genes were directly associated with ASCL2 because they were proved to be both upstream and downstream of ASCL2 expression (Fig. 4c), respectively.[16] However, WiNTRLINC1 knockdown robustly attenuated ASCL2 expression, while c-Myc reduction was modest in Colo320 cells (Fig. 4a). We did not use LS174T cells as previously reported[16] because they did not express WiNTRLINC1 mRNA as much as Colo320 cells in our study (Fig. 1c). We also included knockdown of WiNTRLINC1 expression in both DLD1 and HCT116 cells because they showed the second and third largest expression of WiNTRLINC1 in Q-PCR; the results were almost similar to Colo320 cells (electronic supplementary Fig. S3). More intriguingly, WiNTRLINC1 knockdown clarified that PRL-3 expression was unexpectedly increased, which is consistent with modest attenuation of c-Myc expression in Colo320 cells.

There have been many reports describing the significant correlation of LNM, the most important prognostic factor of colon cancer, to expressions of ASCL2[21] c-Myc,[22,23] PRL-3[24–26] H19[27,28] and HOTAIR;[29,30] however, our data did not demonstrate that they were correlated with LNM. With regard to the ASCL2 gene,[21] previous reports used metastatic lymph node tissues, while our study examined primary tumors. While WiNTRLINC1/ASCL2/c-Myc expression was higher in cases with positive LNM than in those with negative LNM, the differences were not significant in 40 cases (this number may be too small to show statistical significance between each gene expression and LNM), and increased patient numbers might recapitulate the previous results. As WiNTRLINC1 is a newly emerging lncRNA, public databases such as The Cancer Genome

Atlas (TCGA) have not yet included the clinicopathological factors of WiNTRLINC1, and it is not available for clinicopathological factors.

Using comprehensive genetic searches, we recently identified the ASCL2 gene as a candidate gene, and demonstrated drug resistance against phenyl butyrate and 5-fluorouracil in CRC cell lines.[9] Therefore, in this study, we added CPT-11 to 5-fluorouracil, which are the most prevalent anticancer drugs for CRC. As ASCL2 expression was not affected by epigenetic (5-aza-2′-deoxycytidine, trichostatin A) treatments, genetic events such as APC mutations and ASCL2 genomic gains may play a central role in ASCL2 overexpression in CRC. Genomic gains of ASCL2 actually contribute to its overexpression,[31] but was not proved so frequently in Japanese patients with CRC.[9] Exploration of the upstream signal events has thus been highly demanding for molecular targets to regulate drug resistance, and we believe that WiNTRLINC1 is one of the important candidates. Our study clarified that the WiNTRLINC1/ASCL2/c-Myc axis is low in elderly differentiated colon cancer, which may indicate that elderly colon cancer patients can be actively treated with anticancer treatments, including chemoradiotherapy.

Including WiNTRLINC1, the lncRNAs were recently focused on their association with tumor aggressiveness, and HOTAIR,[29,30,32] MALAT1[1,33,34] and H19[27,28] were reported to be involved in LNM of CRC. Among these three genes, in our study the H19 gene was of particular interest. H19 gene expression was significantly (mean 38.0-fold) increased in colon cancer compared with that in noncancerous mucosa tissues ($p = 0.0233$). H19 overexpression was unique to undifferentiated colon cancer, which is consistent with a previous report,[35] and different from the WiNTRLINC1/ASCL2/c-Myc axis. More intriguingly, H19-expressed cancer cells were proved to be resistant to anticancer drugs.[36]

Our earlier study demonstrated that ASCL2 expression was significantly associated with histological response in biopsy samples, prior to neoadjuvant chemoradiotherapy (NCRT) in rectal cancer by immunohistochemistry, however the samples could not be used for mRNA analysis.[9] Prospective collection of mRNA from biopsy samples prior to NCRT would be required for quantitative analysis of lncRNAs in the next stem but not available at present.

CONCLUSIONS

The WiNTRLINC1/ASCL2/c-Myc axis, which could be augmented with PRL-3, plays a critical role in differentiated colon tumorigenesis with young onset, and is essential for cell viability of colon cancer. These findings may propose an active indication of anticancer drug treatments in a lower dose for elderly colon cancer patients than for young colon cancer patients.

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