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Comprehensive Genetic Search to Clarify the Molecular Mechanism of Drug Resistance Identifies *ASCL2-LEF1/TSPAN8* Axis in Colorectal Cancer

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ABSTRACT

Background. Treatment-resistance genes limiting anticancer therapy have not been well clarified in colorectal cancer (CRC). We explored gene expression profiles to identify biomarkers for predicting treatment resistance to an anticancer drug in CRC.

Methods. Six CRC cell lines were treated with phenylbutyrate (PB). The gene expression profiles were then compared using microarrays (harboring 54,675 genes), and genes associated with PB resistance were identified. Candidate genes were functionally examined in cell lines and clinically validated for treatment resistance in clinical samples.

Results. Both DLD1 and HCT15 cells were PB resistant, while HCT116 cells were identified as PB sensitive. On microarray analysis, among the PB resistance-related genes, the expression of the genes *ASCL2*, *LEF1*, and *TSPAN8* was clearly associated with PB resistance. PB-sensitive cells transfected with one of these three genes exhibited significant ($P < 0.001$) augmentation of PB resistance; *ASCL2* induced expression of both *LEF1* and *TSPAN8*, while neither *LEF1* nor *TSPAN8* induced *ASCL2*. RNA interference via *ASCL2* knockdown made PB-resistant cells sensitive to PB and inhibited both genes. *ASCL2* knockdown also played a critical role in sensitivity to treatment by 5-fluorouracil and radiotherapy in addition to PB. Finally, ASCL2 expression was significantly correlated with histological grade of rectal cancer with preoperative chemoradiation therapy.

Conclusions. *ASCL2* was identified as a causative gene involved in therapeutic resistance against anticancer treatments in CRC.

Colorectal cancer (CRC) has been treated with chemo(radio)therapy[1] and molecular target therapy.[2,3] However, some cases exhibit treatment failure and/or severe adverse effects associated with anticancer therapy. Hence, the mechanisms involved in drug resistance must be clarified to develop new therapeutic strategies and biomarkers for primary CRC.

We hypothesized that the resistance mechanism of anticancer treatments may be independent of the modalities or kinds of drugs applied. To identify genes uniquely involved in anticancer treatments in CRC, one must use individual anticancer drugs such as 5-FU, oxaliplatin, and radiation, respectively, because of their daily use in

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concurrent multimodality treatments for CRC such as neoadjuvant chemoradiation therapy (radiation and 5-FU)[4] or FOLFOX (5-FU and oxaliplatin).[5]

We recently constructed a model using phenylbutyrate (PB) to identify genes involved in PB resistance in breast cancer, and identified the *ZEB1* gene,[6] which has been confirmed to be related to resistance to chemoradiation therapy (multiplex chemotherapy drug and radiation) in breast cancer.[7,8] So, in the current study, we applied the same model to identify genes involved in drug resistance in CRC.

MATERIALS AND METHODS

Cell Lines

We used six CRC cell lines (DLD1, HCT15, COLO320, LOVO, COLO205, and HCT116) as described previously.[9] The cell lines excluding LOVO and HCT116 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Carlsbad, CA). The LOVO cells were maintained in 50:50 RPMI 1640:F-12 Ham's medium (Sigma-Aldrich N6658, St Louis, MO, USA), and the HCT116 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich D6429). All media contained 10% fetal bovine serum.

PB Treatments in CRC Cell Lines

One tablet of PB (1 g, triButyrate®; Fyrklövern Scandinavia AB, Sweden) was used as previously described.[6] Serum PB concentrations reached 0.5–3 mM in humans when administered at dosages of 27 or 36 g/day.[10] Hence, we added 1-, 2-, 4-, 10-, and 20-fold dosages of PB solution (onefold dosage = 0.5 mM, 0.5–10 mM) as previously described.[6]

Expression Microarrays

Messenger RNA (mRNA) was extracted from the PB-sensitive strain (HCT116) and the PB-resistant strains (DLD1 and HCT15) using an RNeasy Mini Kit (QIAGEN Sciences, MD, USA). The gene profiles were analyzed using Affymetrix 3′ IVT Express Kit microarrays (harboring 54,675 genes) according to the manufacturer's instructions.

Semiquantitative RT-PCR

Total RNA from cell lines was reverse-transcribed using a SuperScript III reverse transcriptase kit (Invitrogen) as described previously.[9] The thermal cycling conditions were as follows: an initial hold at 95 °C for 3 min and 30 cycles of 1 min at 95 °C, 1 min at 56 °C, 1 min at 72 °C, and 10 min at 72 °C. PCR primer sequences are presented in Supplementary Table S1.

5-Aza-dC and TSA Treatments in CRC Cell Lines

Cells from the PB-resistant strains (1×10^6 cells/T-75 flask) were treated with 1 or 5 μM of the demethylation agent 5-aza-dC (Sigma-Aldrich) dissolved in 50% acetic acid (Wako Pure Chemical Industries, Osaka, Japan) once every 24 h for 4 days followed by 300 nM of the HDAC inhibitor TSA (Sigma-Aldrich) for the final 24 h, as described previously.[6] On day 5, the cells were detached using Trypsin-ethylenediaminetetraacetic acid (EDTA), and mRNA was extracted using the RNeasy Mini Kit (QIAGEN).

Plasmid and Transfection

The full-length cDNA sequences of the *TSPAN8* and *LEF1* genes were isolated using PCR and subcloned into a pcDNA™3.1D/V5-His-TOPO vector (Invitrogen). A vector with self-ligation was used as control. The *ASCL2* plasmid was purchased from OriGene Technologies (Rockville, MD, USA).

The cells were transfected with 4 μg plasmid vector using Lipofectamine 2000 reagent (Invitrogen) in OPTI-MEM medium (GIBCO) according to the manufacturer's instructions. At 3 days after transfection, the viable cells were counted and used for RT-PCR.

siRNA and Transfection

siRNA targeting human *ASCL2* was prepared using the method described by Jubb et al.[11] A scramble siRNA was prepared according to the manufacturer's instructions as control: sense, 5′-agggucagacggauagcaa-3′; antisense, 5′-uugcuauccgucugacccu-3′ (Sigma-Aldrich).

The cells were seeded in six wells overnight to reach 30–40% confluence, then transfected with 100 pmol siRNA using Lipofectamine 2000 reagent in OPTI-MEM medium according to the manufacturer's instructions. After 24 h, the cells were treated with a single administration of tenfold PB solution, 1 μg/mL of 5-FU (Wako), 4 μg/mL of L-OHP (Sigma-Aldrich), or 3 Gy/day of radiation for 2 days. At 3 days after transfection, the cells were counted and used for RT-PCR.

ASCL2 Genomic Gain Status in Primary CRC and Liver Metastasis

The genomic gain status of *ASCL2* gene was investigated in a total of 33 primary CRC patients with liver metastasis who underwent surgical resection for their primary tumors and liver metastases at Kitasato University Hospital in 2000 as previously described for *PRL3* genomic amplification[12] (Supplementary Table S2). This study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Kitasato University School of Medicine (number B17-355). All patients agreed to use of their pathological specimens for research purposes. Q-PCR with a TaqMan probe was performed in triplicate samples using iQ^{TM} Supermix (Bio-Rad Laboratories, Hercules, CA) and the iCycler iQ^{TM} Real-Time PCR Detection system (Bio-Rad). To normalize each gene copy number per cell, β-actin was used as endogenous reference, as described previously.[13] The copy number of the tumor tissues relative to corresponding normal tissues was determined as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta C_t = \Delta C_t(\text{tumor}) - \Delta C_t(\text{corresponding normal})$. The *ASCL2* primer and probe were prepared using Primer 3 software (Supplementary Table S1). A DNA ratio in the tumor (primary CRC or liver metastasis) tissues (T) relative to the corresponding normal tissues (N) (*T*/*N* ratio) that was equal to or greater than twofold was defined as positive genomic gain.

Immunohistochemistry

Immunohistochemical staining for ASCL2 was investigated initially for tumor tissues (primary CRC and liver metastasis of CRC) and noncancerous mucosa tissues from two CRC patients with positive *ASCL2* genomic gain, then for before neoadjuvant chemoradiation therapy (NCRT) biopsy samples from 57 primary rectal cancer patients who underwent NCRT followed by surgical resection at Kitasato University Hospital between 2004 and 2014 (Supplementary Table S3). This study was conducted in accordance with the Declaration of Helsinki and was approved by the Research Ethics Committee of Kitasato University School of Medicine (number B17-355).

Tissues from the patients with CRC were immunohistochemically stained for ASCL2 using Anti-Achaete Scute homolog 2, clone 8F1 (mouse monoclonal IgG1 $_k$; Merck Millipore Corp., Darmstadt, Germany) at antibody concentration of 10 μg/mL. This antibody has been demonstrated to provide strong contrast between positive and negative controls.[14]

Statistical Analysis

Continuous variables were evaluated using the Student *t* test, and categorical variables were evaluated using the Fisher exact test or the Chi square test, as appropriate. $P < 0.05$ was considered to indicate statistical significance. All calculations were performed using JMP® 11 software (SAS Institute Inc., Cary, NC, USA).

RESULTS

Classification of PB Resistance in CRC Cell Lines

Cell proliferation was assessed at various concentrations, ranging from 0.5 to 10 mM, of PB in the six CRC cell lines. Viable cells were counted on day 7 and compared with the control cells (Fig. 1a). The numbers of HCT116, COLO205, COLO320, and LOVO cells were lower after treatment with 0.5 mM PB than those of corresponding control cells (reduction rates of 45%, 35%, 26%, and 26%, respectively). The numbers of DLD1 and HCT15 cells, however, were similar after treatment with 0.5 mM or 1 mM PB, while they were reduced in number by more than 80% after treatment with 5 mM or 10 mM PB. In 0.5 mM PB treatments, both DLD1 and HCT15 cells (PB-resistant CRC cells) did not show reduced numbers, while HCT116 cells (PB-sensitive CRC cells) showed significantly ($P = 0.005$) reduced numbers (Fig. 1b). The increase of DLD1 cells in the 0.5 mM treatment was not statistically significant, but epigenetic treatments increase both oncogenic and tumor suppressor genes,[15] thus specific conditions of PB may affect oncogenic genes more than tumor suppressor genes. Based on the results of these experiments, it was considered that differential PB sensitivity was seen in the six CRC cell lines, whereas cell proliferation could always be suppressed by high concentration of PB in all six cell lines, consistent with results in breast cancer.[6] Hence, the HCT116 cells were designated as a PB-sensitive strain, while both the DLD1 and HCT15 cells were designated as PB-resistant strains throughout the remainder of the study.

Identification of Genes Whose Expression Is Significantly Associated with PB Sensitivity and PB Resistance Using Expression Microarrays

To clarify the differential gene expression profiles that significantly differed between PB sensitivity and PB residence, Affymetrix expression microarrays harboring 54,675 genes were used. “High” expression in CRC cell lines by microarrays was initially defined as a raw signal value of 100 or beyond, because RT-PCR can steadily amplify such expression levels, as previously experienced.[6]

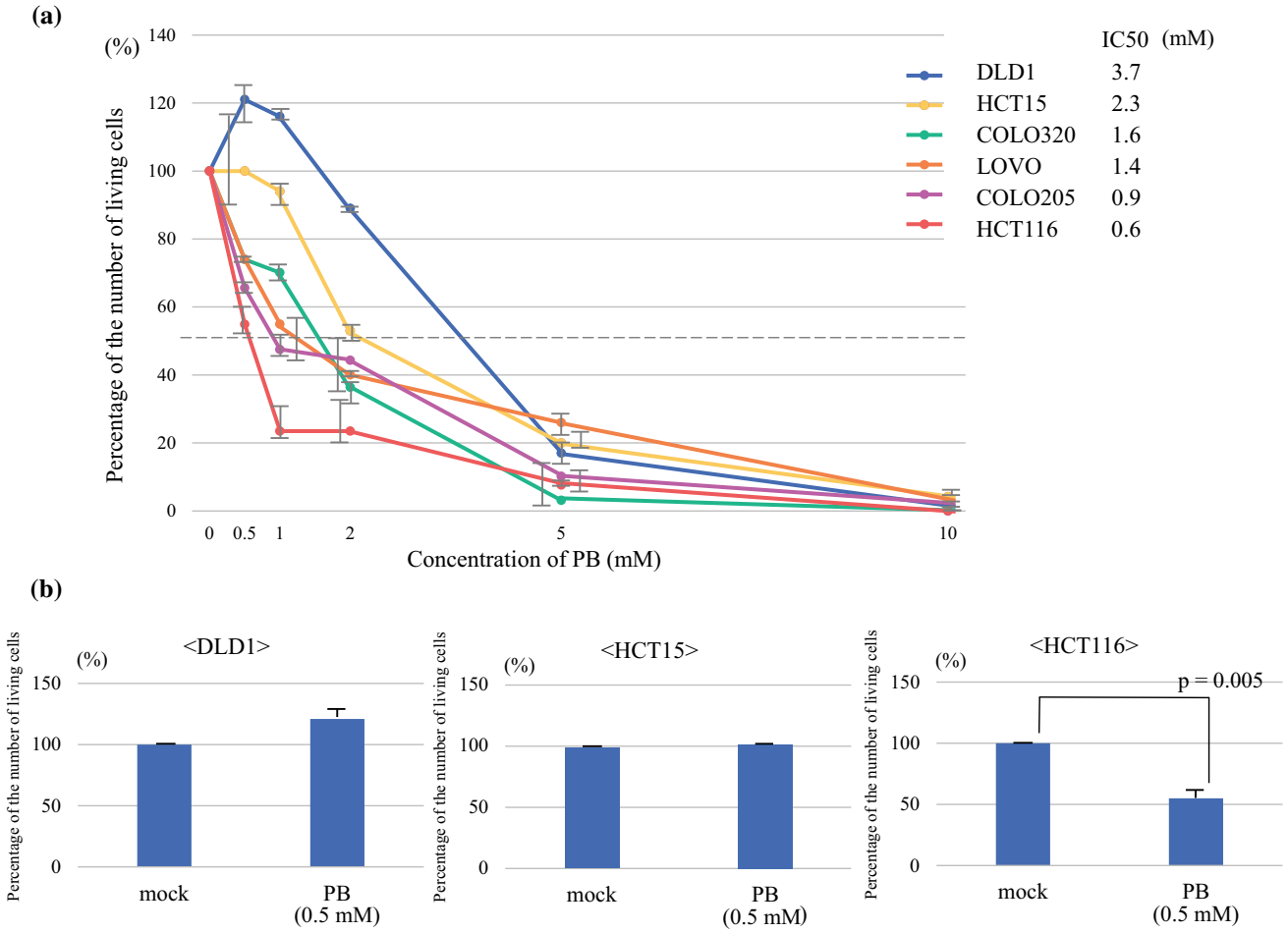

FIG. 1 Classification of PB sensitivity in CRC cell lines. **a** Percentage of number of living cells in CRC cell lines according to concentration of PB treatment. The number of HCT116 cells was reduced by a onefold PB dose, while the numbers of DLD1 and HCT15 cells were barely reduced by either a onefold or even twofold PB dosage. **b** Reduction rate of viable cells in three cell lines. The number of HCT116 cells was significantly reduced by onefold PB treatment. Error bars indicate respective standard deviation

Genes with the top 40 highest expression ratios between sensitive and resistant strain were categorized as PB sensitivity related, and those with the top 26 highest expression ratios between resistant and sensitive strain as PB resistance related. Heat maps of such genes according to their expression ratio between PB-resistant strains (DLD1 and HCT15) and PB-sensitive strain (HCT116) are shown in Fig. 2a. Semiquantitative RT-PCR confirmed that the microarray results were almost accurate (representative genes are shown in Fig. 2b). The top 40 genes that were highly expressed in the PB-sensitive strains included *BCL2 interacting protein 3* (*BNIP3*), *cysteine-rich intestinal protein 1* (*CRIP1*), and *retinol binding protein 1* (*RBP1*), which have been previously reported as being associated with tumor suppressor activity.[15,16] On the other hand, the 26 genes that were highly expressed in the PB-resistant strains included *achaete-scute like 2* (*ASCL2*), *lymphoid enhancer-binding factor 1* (*LEF1*), and *Tetraspanin8* (*TSPAN8*), which are known to be involved in Wnt pathway activation.[11,17]

Demethylation Treatment Results in Differential Expression in Either PB Sensitivity- or Resistance-Related Genes

Simultaneous treatment with 5-aza-2′-deoxycytidine (5-aza-dC) and trichostatin A (TSA), a highly effective demethylation method, was used to evaluate whether epigenetic factors affect the differential expression of the genes identified above.[15] Almost all the PB sensitivity-related genes were reactivated after demethylation treatment, while the gene expressions of almost all the PB resistance-related genes showed no change after the same treatment (Fig. 2c). These findings suggest that the expression of the PB-resistant genes is regulated in a manner different from those of the PB-sensitive genes, and the molecular mechanism of PB resistance is likely to be mediated genetically,

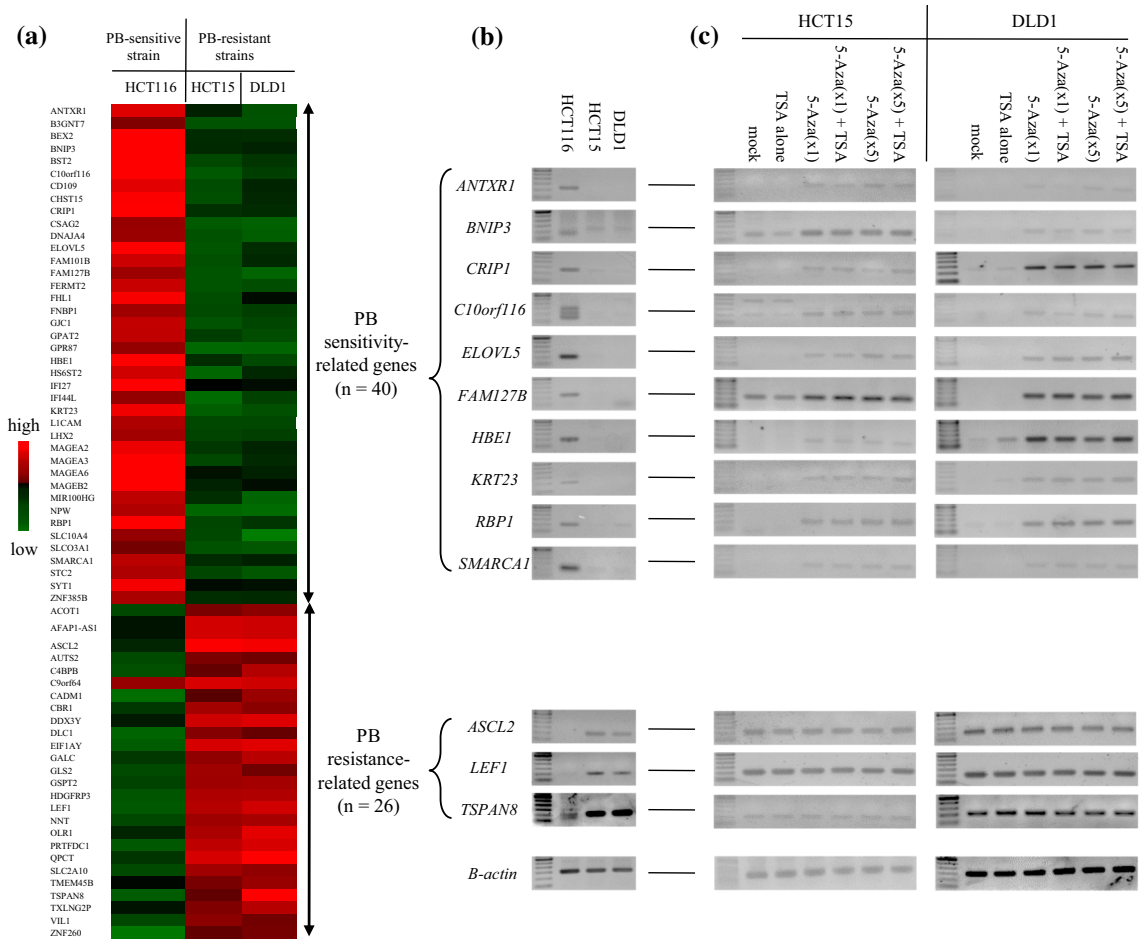

FIG. 2 Gene profiles of PB-resistant and PB-sensitive strains using an expression microarray. **a** Heat maps of gene expression. A red bar shows a high level of expression, while a green bar shows a low level. **b** mRNA expression profiles of PB sensitivity- and resistance-related genes using semiquantitative RT-PCR. The expression patterns were almost the same as the microarray results. **c** Changes in expression after demethylation treatment. Some PB sensitivity-related genes were recognized after demethylation treatment, but none of the PB resistance-related genes showed any change after the same treatment. Representative genes are shown

which is different from the epigenetic regulation of *ZEB1* in breast cancer.[6] Actually, *ZEB1*, which is associated with PB resistance in breast cancer, was silenced in all three CRC cell lines, and no association between *ZEB1* expression and PB resistance was observed in CRC. Considering these unexpected results, we conclude that PB resistance-related genes are regulated by genetic aberrations unique to CRC. Importantly, CRC is well known to be generated from adenomatous polyposis coli (APC)/β-catenin Wnt genetic pathway activation differently from breast cancer,[18] and *ASCL2*, *LEF1*, and *TSPAN8* genes are well known to be involved in Wnt pathway activation, as described above.

Transfection of ASCL2, LEF1, and TSPAN8 Genes into PB-Sensitive CRC Cells

We next made expression plasmids of *ASCL2*, *LEF1*, and *TSPAN8* genes from CRC cell lines. Transient transfection of a plasmid vector with the full-length *ASCL2* gene into HCT116 cells (PB-sensitive cells) induced mRNA expression of both *LEF1* and *TSPAN8* genes (Fig. 3a). This transient transfection of an *ASCL2*-expressing vector significantly (*t* test, $P < 0.001$) augmented PB resistance to the same level as observed in PB-resistant cells (DLD1 mock cells) after 5 mM PB treatment.

On the other hand, transient transfection of a plasmid vector with the full-length *LEF1* or *TSPAN8* gene into HCT116 cells (PB-sensitive cells) did not induce expression of the two other genes (Fig. 3b, c). The transient transfection of either an *LEF1*- or *TSPAN8*-expressing vector significantly (*t* test, $P < 0.001$ for both vectors)

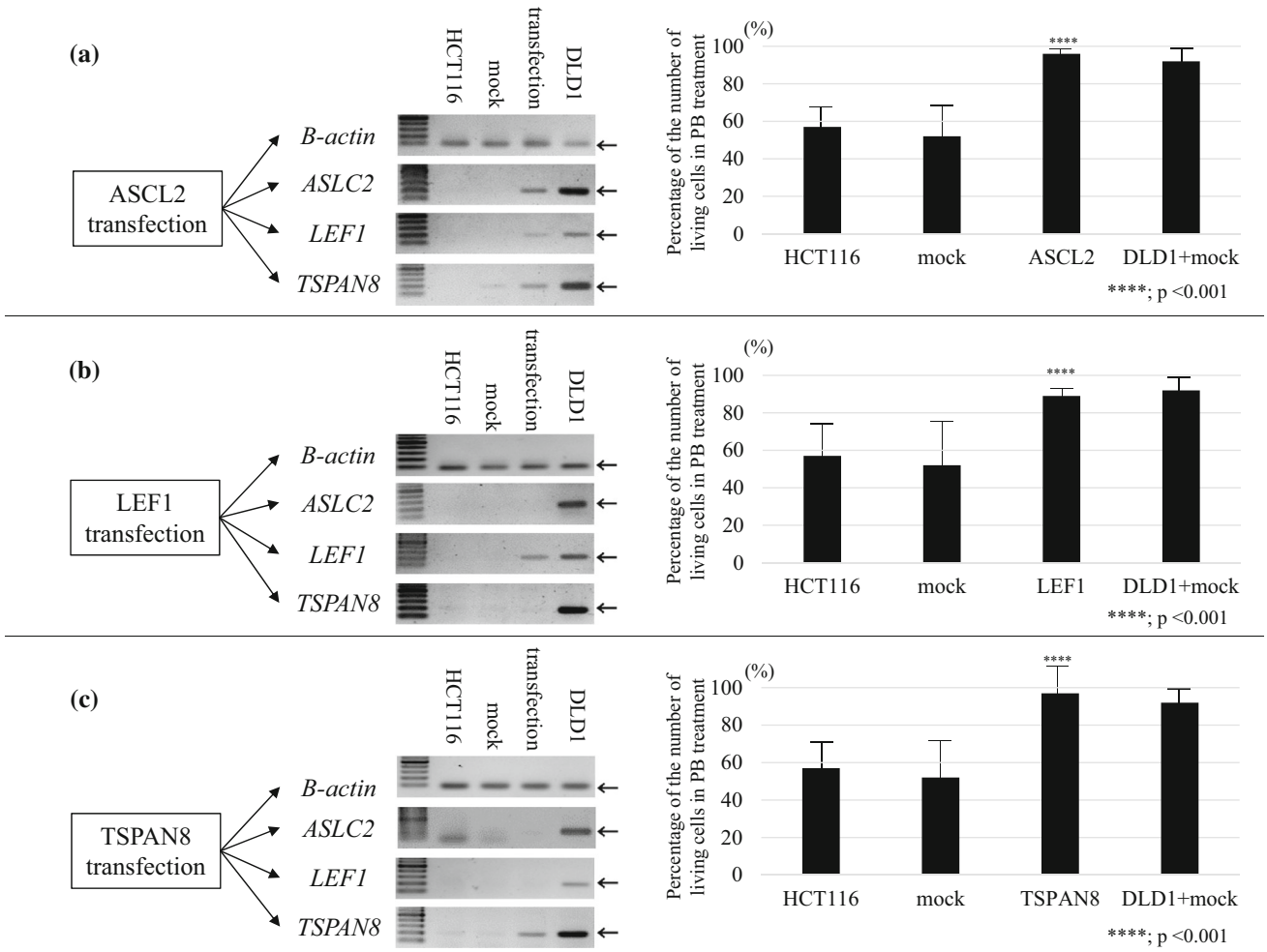

FIG. 3 EMT-related gene transfection experiments in PB-sensitive CRC cells. **a** *ASCL2* transfected into HCT116. Transfection of *ASCL2* induced expression of *LEF1* and *TSPAN8* and significantly augmented PB resistance. **b** *LEF1* transfected into HCT116. Transfection of *LEF1* significantly augmented PB resistance. **c** *TSPAN8* transfected into HCT116. Transfection of *TSPAN8* significantly augmented PB resistance

augmented PB resistance to a level similar to that observed in DLD1 mock cells after 5 mM PB treatment.

These findings suggest that *ASCL2* is an upstream activator of either *LEF1* or *TSPAN8*, and we proceeded to perform detailed experiments focusing on *ASCL2* among the three PB-resistance-related genes.

ASCL2 Gene Knockdown by Transfection of Small Interfering RNA (siRNA) into PB-Resistant CRC Cells

We then performed knockdown of *ASCL2* gene in the PB-resistant cell lines (DLD1 and HCT15 cells). Transient transfection of *ASCL2* siRNA into HCT15 cells reduced the expression of *LEF1* and *TSPAN8* genes as expected (Fig. 4a). Under PB treatment, the transient transfection of *ASCL2* siRNA significantly (*t* test, $P = 0.002$) increased PB sensitivity in HCT15 cells. On the other hand, the transient transfection of *ASCL2* siRNA into DLD1 cells also reduced the expression of *TSPAN8* gene, whereas *LEF1* gene was little changed (Fig. 4b). Under PB treatment, the transient transfection of *ASCL2* siRNA remarkably (*t* test, $P = 0.006$) increased PB sensitivity in DLD1 cells.

As PB is not a drug used in daily clinical practice to treat CRC, we further investigated the associations between the gene expression of *ASCL2* and anticancer treatments other than PB treatment, viz. 5-fluorouracil (5-FU), oxaliplatin (L-OHP), and radiation. Under 5-FU treatment, transient transfection of *ASCL2* siRNA into HCT15 and DLD1 significantly (*t* test, $P < 0.0001$ and $P = 0.008$, respectively) increased 5-FU sensitivity. Under L-OHP treatment, on the other hand, transient transfection of *ASCL2* siRNA into HCT15 and DLD1 did not cause any changes in L-OHP sensitivity. Under radiation treatment, transient transfection of *ASCL2* siRNA into HCT15 and DLD1 significantly (*t* test, $P < 0.0001$ and $P = 0.0002$,

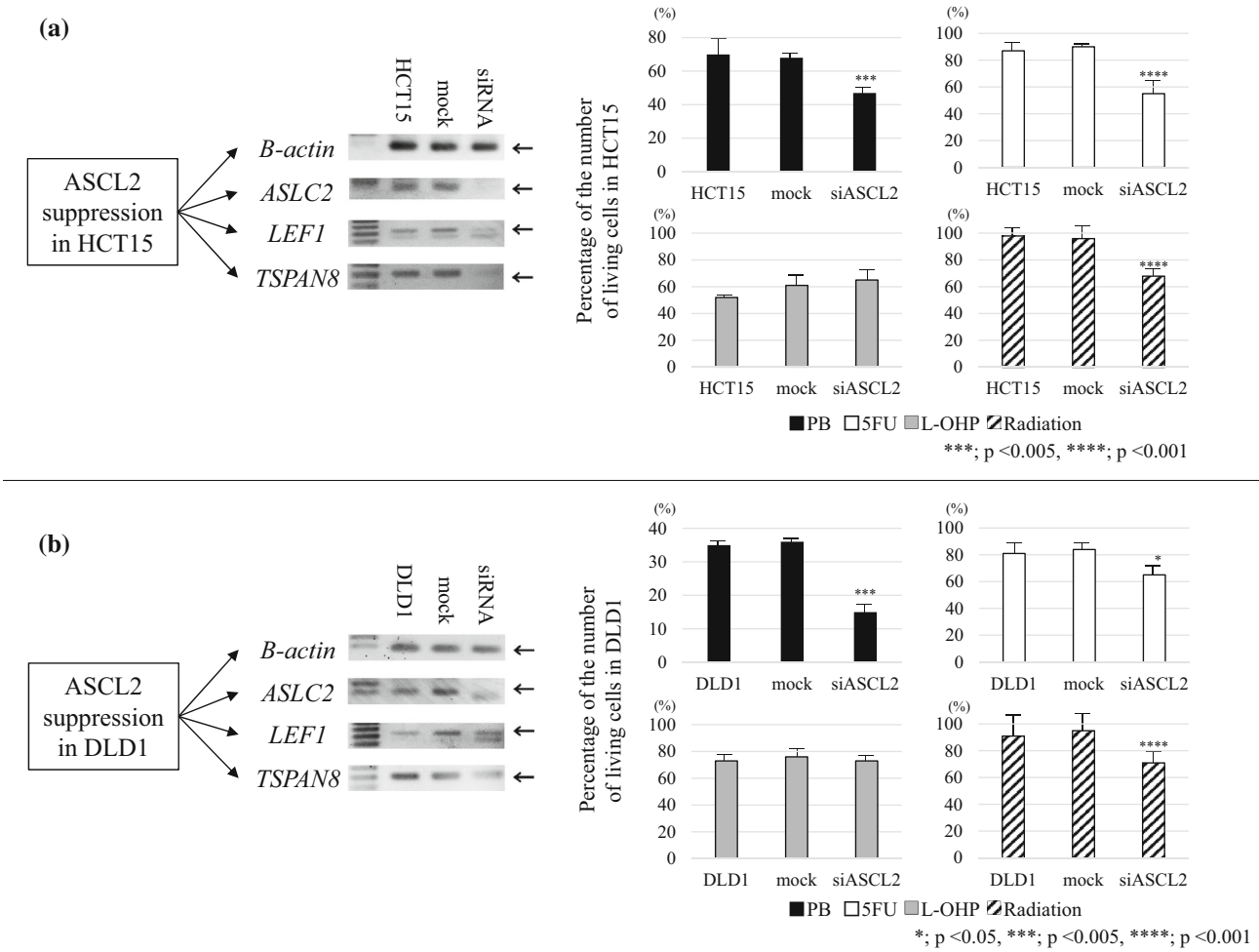

FIG. 4 Knockdown experiments in PB-resistant CRC cells. Transfection of *ASCL2* siRNA into HCT15 (**a**) and DLD1 (**b**). Suppression of *ASCL2* gene expression reduced expression of *LEF1* and *TSPAN8* and significantly increased the sensitivities of both HCT15 and DLD1 cells to PB, 5-FU, and radiation treatment

respectively) increased radiation sensitivity. 5-FU and radiation treatments are main components of NCRT in advanced rectal cancer treatment at present,[4] raising interest in the clinical association between *ASCL2* expression and NCRT response in rectal cancer.

Genomic Amplification of ASCL2 and Immunostaining of ASCL2 in CRC Clinical Materials

The above results suggest that genomic changes in *ASCL2* may be involved in the sensitivity of cells to a variety of anticancer treatments for CRC. Gene copy number gain of *ASCL2* gene has been reported in liver metastasis of CRC.[19] We thus investigated the genomic gain status of *ASCL2* gene in primary CRC tumor tissues and corresponding liver metastases with respect to corresponding noncancerous mucosa tissues.

Quantitative PCR (Q-PCR) for *ASCL2* DNA was initially performed to assess the gene copy number. The T (CRC tumor or liver metastasis tissue)/N (noncancerous mucosa tissue) ratios of the *ASCL2* DNA are shown in Fig. 5a. All 33 cases had both primary CRC and liver metastasis. An *ASCL2* genomic gain (defined as *T*/*N* ratio of 2 or above) was seen in three cases, composed of one liver metastasis (case 1) and two primary CRC (cases 2 and 5), which is much less than in a previous report.[19] In case 1, *ASCL2* genomic gain was only found in liver metastasis but not in primary CRC tumor. In cases 2 and 5, *ASCL2* genomic gain was seen only in primary CRC tumors. A representative genomic quantification (case 5) is shown in Fig. 5b. A positive correlation of *ASCL2* genomic gain was seen between the primary CRCs and corresponding liver metastases (Fig. 5c, $P = 0.02$). The expression of ASCL2 protein was finally investigated in two cases with genomic gain (cases 1 and 2) in immunohistochemistry (Fig. 5d). ASCL2-positive cells were observed in the intestinal crypts of normal colon mucosa as previously described,[11] while

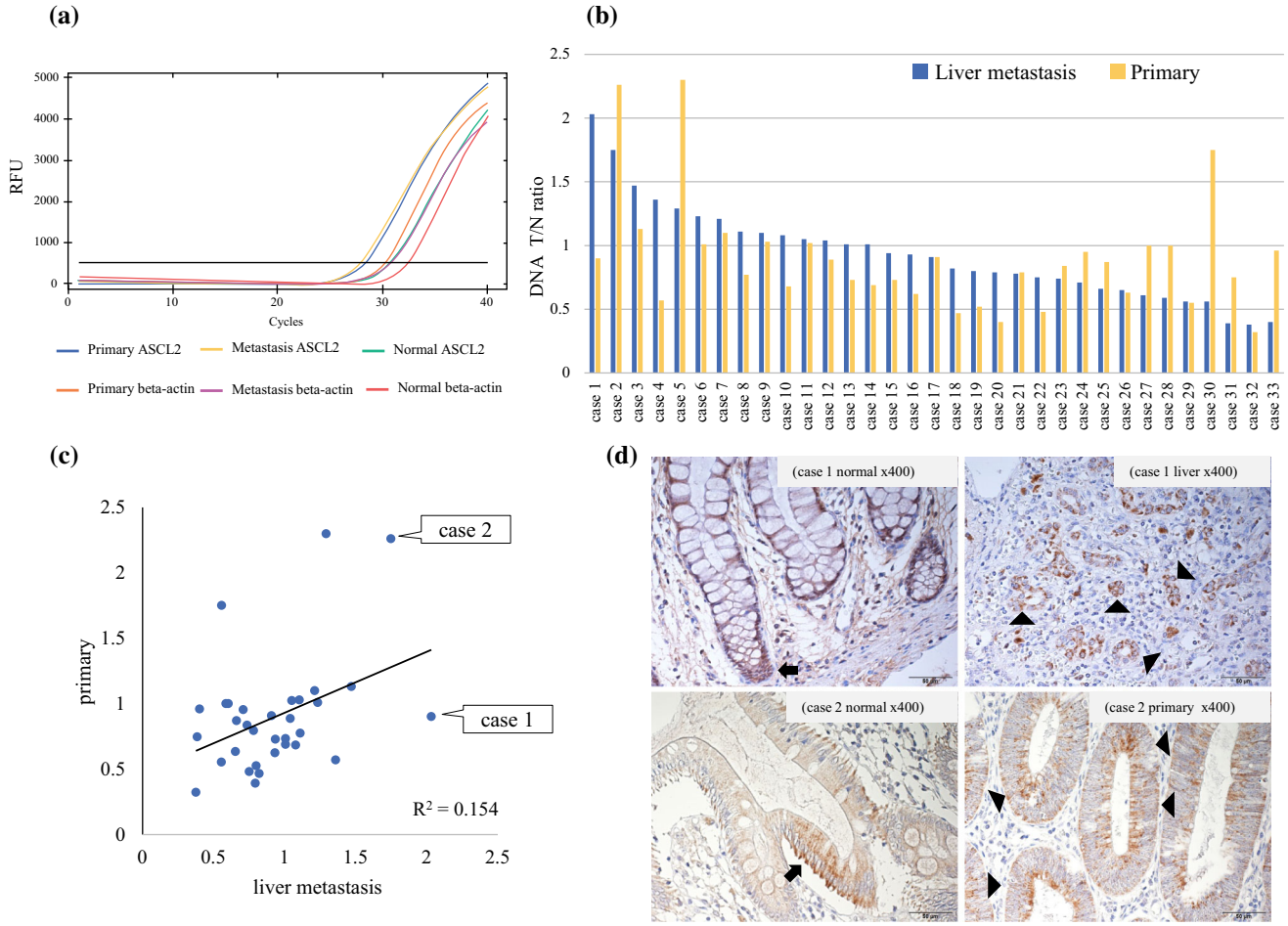

FIG. 5 Genomic amplification and immunostaining of *ASCL2* in primary CRC and corresponding liver metastasis. **a** Representative genomic quantification (case 5). **b** *T/N* ratio of *ASCL2* gene between primary CRC and corresponding liver metastasis. **c** *T/N* ratio of *ASCL2* gene. **d** Immunostaining of ASCL2 in case 1 and case 2. ASCL2-positive cells are clearly localized in the crypts of normal colon tissue and are increased in primary CRC and liver metastasis

higher numbers of ASCL2-positive cells were seen in the corresponding primary CRC and liver metastasis.

Prediction of Histological Grade by Immunohistochemistry of ASCL2 in Rectal Cancer Biopsy Samples

Finally, preoperative biopsy samples from 57 rectal cancer patients who underwent NCRT were immunostained for ASCL2. The 57 rectal cancers showed histological grade 1 (n = 23), 2 (n = 21), and 3 (n = 13) after NCRT, and the complete remission (CR) rate was 13/57 (22.8%), consistent with our previous report.[20] Immunohistochemistry of ASCL2 was classified into IHC0 (n = 7), IHC1+ (n = 19), and IHC2+ n = 31) (Fig. 6a). Interestingly, ASCL2 immunostaining was significantly (P = 0.006) correlated with pathological histology grade (Fig. 6b). Among rectal cancer patients with NCRT showing histological grade 1, 78% were immunostained for ASCL2 IHC2+, compared with only 15% for those with histological grade 3 (complete remission).

DISCUSSION

We recently identified *ZEB1-RAB25/ESRP1* as PB-resistance genes in breast cancer,[6] and *ZEB1* has received wide attention with regard to chemoresistance in breast cancer.[7,8] Utilizing the same search strategy, we again used the same model for molecules involved in PB chemoresistance in this work for CRC. Interestingly, *ZEB1* expression was robustly suppressed by epigenetic (demethylating) treatments,[6] and miRNA, e.g., the Mir200 family, which is epigenetically regulated in human cancer, might play a causative role in *ZEB1* overexpression in human cancers.[21] In our CRC cell lines, on the other hand, *ZEB1* was not expressed in either PB-sensitive or PB-

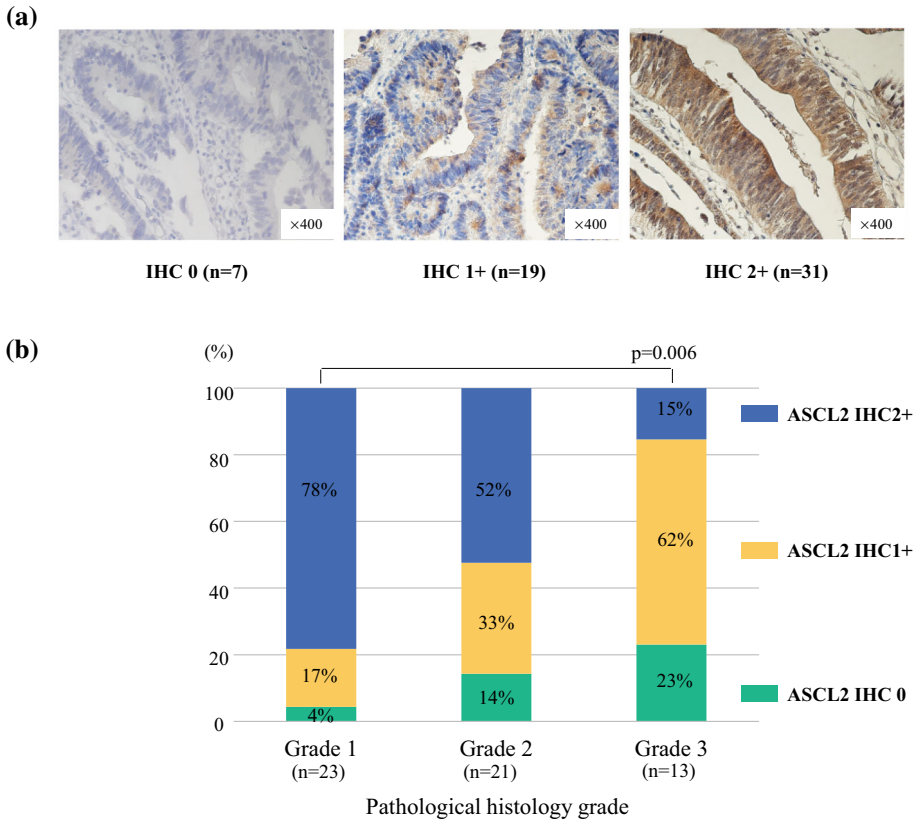

FIG. 6 Immunohistochemistry for ASCL2 in CRC tissue with NCRT.
a Immunohistochemistry of ASCL2 was classified as IHC0 for no staining, IHC1+ for moderate staining, and IHC2+ for diffuse staining.
b Distribution of ASCL2 staining score in each histological grade

resistant cells (data not shown). This finding suggests that *ZEB1* is not associated with PB resistance in CRC, unlike the situation in breast cancer.

In the current search, on the other hand, we discovered that *ASCL2* plays a critical role in PB resistance in CRC. *ASCL2* was significantly associated with resistance against various anticancer treatments, including PB, 5-FU, and radiation, but not L-OHP. *ASCL2* was not altered by epigenetic treatments, unlike the situation of *ZEB1* gene in breast cancer,[6] but we focused on *ASCL2* gene because it is critically involved in the Wnt pathway, together with *LEF1* and *TSPAN8*, which have well-known genetic aberrations in CRC. In a previous report, *LEF1* gene was shown to act upstream of *ASCL2*,[11] while the results for *TSPAN8* were not informative with regard to its relation to *ASCL2*. However, the results of this study reveal, for the first time, that both *LEF1* and *TSPAN8* are regulated by *ASCL2*. *ASCL2* and *LEF1* are involved in the Wnt pathway, suggesting that genomic alterations play causative roles in the Wnt activation phenotype linked to chemoresistance in CRC, unlike the situation in breast cancer.

Dominant negative *LEF1* was reported to inhibit *ASCL2* expression, suggesting that *LEF1* acts upstream of *ASCL2*.[11] However, in this study, this finding could not be confirmed. *ASCL2* induced expression of both *LEF1* and *TSPAN8* genes, whereas *ASCL2* gene inhibition suppressed either or both genes. These findings suggest that *ASCL2* is an upstream regulator of the critical Wnt pathway genes *LEF1* and *TSPAN8*. Experiments based on transfection of both *LEF1* and *TSPAN8* genes indicated that both genes augmented PB resistance, while neither *LEF1* nor *TSPAN8* could induce vice versa. Thus, we speculated that *LEF1* and *TSPAN8* genes were also involved in chemoresistance, and those actions are dependent on *ASCL2* overexpression in CRC.

LEF1 is a well-established molecule that converges with the Wnt pathway, with downstream genes of *LEF1* reportedly including *cyclin D1*, *c-myc*, and *Bcl-xl*.[22] These genes could be individually involved in chemoresistance.[23,24] On the other hand, to the best of the authors' knowledge, *TSPAN8* involvement in chemoresistance has not been reported, while *CD44* involved in the cancer-initiating process induces *TSPAN8*, which plays a critical role in cancer invasion.[25] Although *TSPAN8* was induced by *ASCL2* transfection in this study, the molecular mechanism has not yet been elucidated. Drug resistance capacity of cancer cells is often accompanied by augmented oncogenic capacity, because the molecular mechanisms that explain these two different phenotypes are redundant.[26] Although there are no reports describing the molecular mechanism of *TSPAN8* in anticancer drug resistance in human cancer, it has been reported that *TSPAN8* is actually

correlated with drug resistance in various human cancers,[17,27] and *TSPAN8* may be involved in cancer stemness[28–30] which has been considered to be involved in drug resistance in cancer cells. Based on these reports, the precise molecular mechanism is likely to be at least partially explained by cancer-derived exosomes including *TSPAN8* to yield cancer-initiating cells.

The *ASCL2* gene, which is for a basic helix–loop–helix transcription factor, has been reported to be a major transcription factor active in intestinal stem cells that can trigger formation of liver metastases if overexpressed.[14,31] In a previous study, *ASCL2*, which is located on chromosome 11p15.5, was reported to be amplified by 25% in liver metastases of CRC.[19] In this study, however, *ASCL2* genomic amplification was less than expected (Fig. 5a). The Cancer Genome Network has also focused specifically on *ASCL2* genomic amplification, which was also less than expected relative to the previous report,[19] and that *ASCL2* overexpression was unlikely to be associated with the frequent genomic gain of the 11p15 locus in CRC.[32] Among the genes at this locus, gene overexpression accompanied by genomic gain was recognized for *IGF2*, and *IGF2* overexpression was exclusive for expression of related genes, such as *IRS2*. These findings suggest that *ASCL2* is not dominantly responsible for the genomic gain of 11p15.

ASCL2 has been proposed to be a marker of dynamic cancer-initiating cells, and it was ubiquitously expressed in Lgr5-positive colon stem cells, but not in +4 stem cells, which are alternative stem cells which are positive for homeodomain-only protein homeobox (HOPX).[14,33] Immunohistochemistry studies clearly showed the localization of ASCL2 in the crypts of normal colon tissue as previously shown (Fig. 5d). Simultaneously, *ASCL2* is overexpressed in primary CRC tumors and liver metastasis, suggesting that such cancer cells were derived from progenitor dynamic stem cells overexpressing *ASCL2*.

Inhibition of *ASCL2* results in arrest at the G2/M checkpoint of the cell cycle.[11,14] Several recent reports describe that sensitivity to several anticancer drugs such as CPT11 or Cdk inhibitors is accompanied by G2/M cell cycle arrest.[34,35] In this study, *ASCL2* was for the first time confirmed to be involved in 5-FU or radiotherapy resistance, in addition to PB resistance, while L-OHP sensitivity was not affected by the expression level of *ASCL2*. A direct effect of L-OBP is cross-linking of double-stranded DNA, and DNA replication is inhibited, resulting in arrest at the G1/S rather than G2/M phase of the cell cycle.[36] Such a differential acting mechanism may be reflected in the types of anticancer drug resistance observed in this study.

The results of this study also demonstrate that *ASCL2* could be useful as a chemoresistance biomarker for actual anticancer treatments targeting the cell cycle or damaging DNA, such as PB, 5-FU, and radiation, even when using biopsy samples prior to NCRT for locally advanced rectal cancer. Recently, it was reported that *ASCL2* expression was mainly regulated by long noncoding RNA of WiNTRLINC1 in CRC, which is a direct target of Wnt pathways.[37] These findings, together with our data (showing that *ASCL2* genomic amplification is an infrequent event), suggest that the WiNTRLINC1/ASCL2 axis could contain key targets to control the Wnt pathway and CRC disease.

CONCLUSIONS

A comprehensive search for PB-resistance genes identified *ASCL2*, which was genetically regulated possibly through upstream Wnt pathway activation in CRC. The *ASCL2* gene was also involved in resistance to PB treatment as well as NCRT. The regulation of *ASCL2* may provide a clue for the molecular understanding of CRC treatment failure.

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