Expression pattern of placenta specific 8 and keratin 20 in different types of gastrointestinal cancer

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Abstract. The aim of the present study was to investigate the expression of keratin 20 (KRT20) and placenta specific 8 (PLAC8) in gastrointestinal (GI) cancer with various differentiation phenotypes. The present study retrospectively investigated archived formalin-fixed paraffin-embedded tissue samples from 12 patients at different stages of GI cancer [four with gastric cancer, four with pancreatic cancer and four with colorectal cancer (CRC)]. The stages were pre-determined, according to differentiation phenotypes, by a pathologist of the Department of Pathology at Sijhih Cathay General Hospital. KRT20 and PLAC8 expression levels were assessed using immunohistochemistry. The CRC cell lines SW620 and Caco-2 were used to assess interactions between KRT20 and PLAC8 via reverse transcription-quantitative PCR. PLAC8 and KRT20 expression was observed consistently only in the well-differentiated CRC tissue samples. Low KRT20 expression levels were observed in the PLAC8 knockdown SW620 cells. In addition, there was a positive association between PLAC8 and KRT20 expression in the differentiated Caco-2 cells. According to the results of the present study, the differentiation status of GI cancer influenced KRT20 expression, particularly in CRC, which may explain why patients with well-differentiated CRC display better clinical outcomes. Therefore, the prognostic significance of KRT20 and PLAC8 may be particularly crucial for patients with CRC displaying a well-differentiated phenotype.

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Introduction

Gastrointestinal (GI) cancer develops in the organs of the alimentary canal, including the esophagus, liver and bile ducts, gallbladder, pancreas, stomach and small and large intestines (1). Although some distinct mutations have been reported in different GI organs, GI tumors display several key molecular alterations (2,3).

Keratins (KRTs) are a family of fibrous structural proteins that are present in normal epithelia, however, some are upregulated in neoplasms (4). The differential expression of KRTs facilitates the diagnosis of several tumors, including GI tumors, using molecular techniques and allows KRTs to be used as biomarkers to discriminate primary from metastatic adenocarcinoma (5-8). In addition, KRTs have long been considered epithelial differentiation markers (9) and the differentiation of cells within the GI tract is associated with an increased susceptibility to GI cancer (10,11).

In addition to being involved in conventional tumorigenesis, epithelial-mesenchymal transition (EMT) plays key roles in cellular differentiation and cancer progression (12,13). KRTs have been reported to be aberrant in cells undergoing EMT (14,15). For example, the detection of KRT20-positive circulating tumor cells is associated with worse prognosis in patients with colorectal cancer (CRC) (16). In addition to structural KRTs, other genes often exhibit dysregulated expression during EMT. Previously, placenta specific 8 (PLAC8), a gene expressed under physiological conditions, was reported to play

a key role in the tumorigenic and EMT pathways in a number of types of human cancer, such as CRC or pancreatic cancer (CaP) (17,18). PLAC8 was expressed at high levels in the GI tract and KRT20 expression patterns were highly specific (19-21). In addition, elevated PLAC8 levels are positively associated with tumor metastasis and recurrence in CRC (18,22). Understanding the interaction between KRT20 and PLAC8 could have clinical implications for the treatment of GI cancer. In the present study, immunostaining was employed to detect the expression of KRT20 and PLAC8 in the tissues of patients with GI cancer [gastric cancer (GC), CaP and CRC]. Furthermore, the mRNA levels of KRT20 in CRC cells displaying differential PLAC8 expression were quantified.

Materials and methods

Tissues and cell lines. Archived 4 μm formalin-fixed paraffin-embedded (FFPE) tissue sections from four GC, four CaP and four CRC patients who had undergone surgery at the Department of Surgery of Cathay General Hospital before December, 2000 were obtained and used in the present study. All procedures were approved by the Cathay General Hospital Institutional Ethics Committee and a waiver of consent was approved by the same committee. Patient information was anonymized.

For each cancer (GC, CaP and CRC), two patients had well-differentiated cancer [one at American Joint Committee on Cancer (AJCC) stage II and one at stage III] and two had poorly differentiated cancer (one at AJCC stage II and one at stage III) (23). Cancer diagnoses were performed by a pathologist. The human CRC cell lines SW620 [cat. no. CRL-1831; AJCC stage III) and Caco-2 (cat. no. HTB-37) were obtained from the American Type Culture Collection (ATCC) and the medium suggested by the ATCC for each cell line was used for culture; Leibovitz's L-15 medium for SW620 cells and the Eagle's Minimum Essential medium for Caco-2 cells. The two CRC cell lines were selected due to their high PLAC8 expression levels (SW620 cells) (24) and their differentiation capacity (Caco-2 cells) (25). SW620 cells were incubated at 37˚C and 100% air (with very low CO_2) in a humidified incubator and subcultured 2 to 3 times per week (25). To induce intestinal differentiation, Caco-2 cells were cultured to confluence in a humidified incubator at 37˚C with 5% CO_2 for 21 days, as described in a previous study (25).

PLAC8 knockdown and detection of PLAC8 mRNA level in CRC cells. PLAC8 mRNA levels were knocked down in SW620 cells using a lentivirus-mediated small hairpin (sh) RNA targeting PLAC8 to obtain shPLAC8-SW620 cells. Control (shLUC-SW620) cells were obtained using a lentivirus-mediated shRNA targeting luciferase (25). The lentiviruses and the protocol for lentivirus infection were acquired from the National RNAi Core Facility of Academia Sinica. Briefly, $1x10^6$ SW620 cells were grown in a 10 cm plate for 24 h, and then infected with lentivirus at a multiplicity of infection of 3. Stable infected cells were selected and maintained in medium containing 2 μg/ml puromycin for 48 h (Thermo Fisher Scientific, Inc.). The total RNA of the transfected cells was then extracted using RNAzol® RT (Molecular Research Center) and reverse transcribed into cDNA using a high-capacity cDNA Reverse Transcription kit (Catalog No. 4368813; Thermo Fisher Scientific, Inc.) in the presence of oligo(dT) primers, according to the manufacturer's instructions. The level of mRNA was considered as the gene expression level and was measured by PCR in the presence of specific amplification primers (Table I), a TaqMan probe and TaqMan master mix (Roche Diagnostics GmbH), according to the manufacturer's instructions. Cycling conditions were: 2 min at 50˚C and 10 min at 95˚C, followed by 50 cycles each consisting of 15 sec at 95˚C and 1 min at 60˚C. mRNA levels were adjusted relative to the level of GAPDH to estimate the relative levels of gene expression with the method of the $2^{-\Delta\Delta Cq}$ method (26). LightCycler (version 4.05; Roche Diagnostics GmbH) was used to analyze the PCR kinetics.

Immunohistochemical staining of PLAC8 and KRT20 in the archived FFPE tissue sections. For the PLAC8 immunohistochemical staining, a VECTASTAIN® Elite ABC kit (Vector Laboratories, Inc.) was used according to the manufacturer's instructions. Briefly, cancer tissue sections were incubated in oven at 65˚C for 30 min to deparaffinize, and then each tissue was rehydrated with 100, 90 and 70% ethanol, sequentially. The rehydrated slides were immersed in citrate buffer (10 mM; pH 6.0), boiled (95-99˚C) for 20 min and then cooled to room temperature for 20 min. To inactivate endogenous peroxidases within the tissues, the slides were incubated for 30 min at room temperature in 3% methanolic hydrogen peroxide. The tissue sections were then blocked for 30 min at room temperature using a blocking solution with 5% rabbit serum (Vector Laboratories, Inc.) and incubated overnight at 4˚C with anti-PLAC8 antibody (1:200; cat. no. ab122652; Abcam) or anti-KRT20 antibody (1:200; cat. no. ab76126; Abcam). After washing the tissues several times with Tris-buffered saline, the samples were incubated with a secondary biotinylated goat anti-rabbit IgG antibody (1:200; cat. no. BA-1000; Vector Laboratories) for 1 h at room temperature. Subsequently, the slides were stained with 3-amino-9-ethylcarbazole (DAKO; Agilent Technologies, Inc.) for 1 min at room temperature to visualize PLAC8 and KRT20. Tissues were stained with hematoxylin for 4 min and then with eosin for another 1.5 min at room temperature for identification of normal and tumor areas. The stained sections were subsequently diagnosed by a pathologist via a light microscope Olympus BX41 with magnification at x200 (Olympus Corp.). Then, the GI cancer tissue sections were divided into two differentiation states (well or moderately differentiated, >50% glandular formation and poorly differentiated, 0-49% glandular formation) (27).

Statistical analysis. Data were presented as mean ± standard deviation. The relative expression levels of PLAC8 and KRT20 in cells were compared between samples using the Student's t-test. All statistical analyses were performed using SPSS (version 19; IBM Corp.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

PLAC8 and KRT20 expression in well-differentiated GI stage II and III cancer cells. Cellular KRT20 and PLAC8 proteins were detected in each of the cancer tissues using immunohistochemistry. In the well-differentiated

Table I. Quantitative PCR primers.

Gene	Primers (5' → 3')	
	Forward	Reverse
PLAC8	CGTCGCAATGAGGACTCTCT	CTCTTGATTTGGCAAAGAGTACAA
KRT20	CAGTCCCATCTCAGCATGAA	ACAGCGACTGGAGGTTGG
GAPDH	CTCTGCTCCTCCTGTTCGAC	ACGACCAAATCCGTTGACTC

PLAC8, placenta specific 8; KRT20, keratin 20.

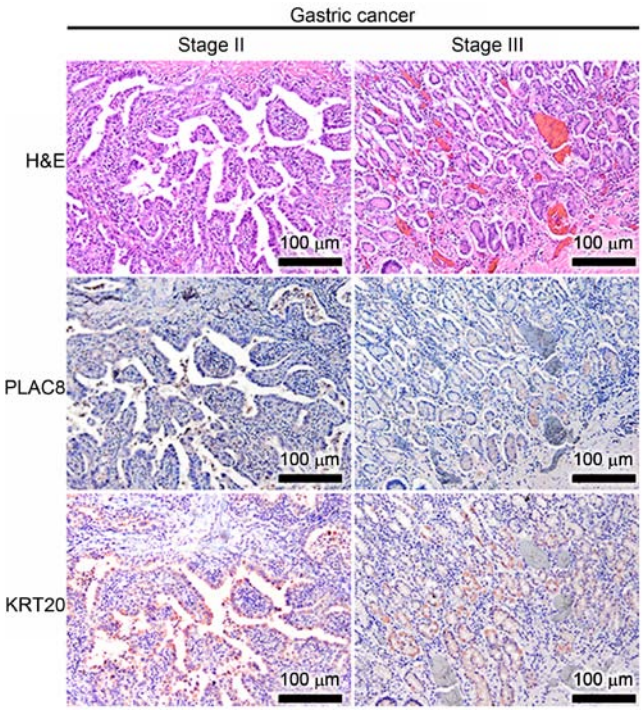

Figure 1. Representative immunohistochemical staining for PLAC8 and KRT20 expression in well differentiated stage II and III gastric cancer tissue. Scale bar displayed in bottom right corner, magnification, x200. H&E, hematoxylin and eosin staining; PLAC8, placenta specific 8; KRT20, keratin 20.

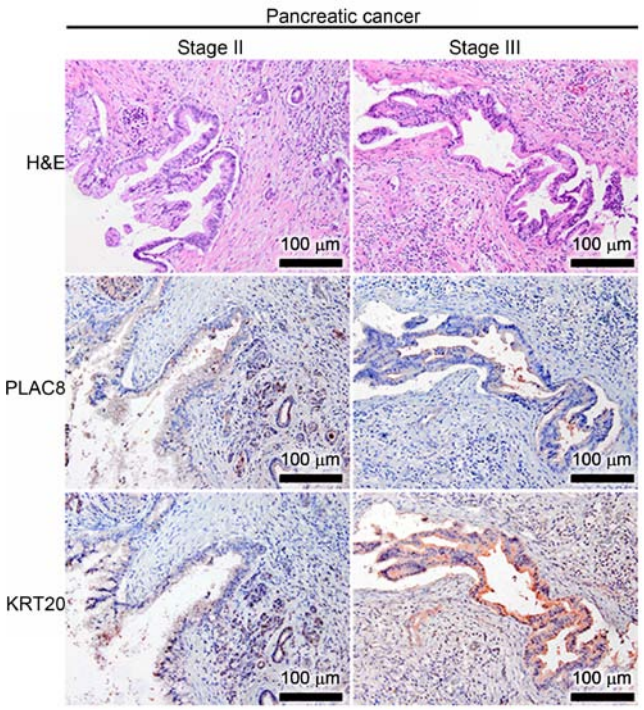

Figure 2. Representative immunohistochemical staining for PLAC8 and KRT20 expression in well differentiated stage II and III pancreatic cancer tissue. Scale bar displayed in bottom right corner, magnification, x200. H&E, hematoxylin and eosin staining; PLAC8, placenta specific 8; KRT20, keratin 20.

GC cancer tissues, PLAC8 levels were low and tissues were KRT20-positive regardless of tumor stage (Fig. 1). Conversely, tissues were positive for both PLAC8 and KRT20 in the well-differentiated CaP tissues at stages II and III, but PLAC8 displayed a luminal staining pattern (Fig. 2). In addition to the expression patterns in the well-differentiated GC and CaP cases, the expression patterns of PLAC8 and KRT20 were also immunodetected in well-differentiated CRC cases (Fig. 3). PLAC8 signals were consistently low in the well-differentiated CRC at stage II (the left panel of Fig. 3), but the small number of PLAC8-positive CRC cells appeared to also express KRT20. Furthermore, a co-expression of PLAC8 and KRT20 was observed in the well-differentiated CRC cells at stage III (the right panel of Fig. 3).

PLAC8 and KRT20 expression in the poorly differentiated GI cancer cells at stages II and III. Immunohistochemical staining revealed that although poorly differentiated GC tissues expressed similar levels of PLAC8 at stage II and III (the middle panel of Fig. 4), these tissues expressed higher levels of KRT20 at stage III compared to stage II (the bottom panel of Fig. 4). Conversely, the poorly differentiated CaP at stage II displayed higher levels of PLAC8 compared with the CaP tissues at stage III, and KRT20 levels were low in the poorly differentiated CaP at both stage II and III (Fig. 5). The PLAC8 and KRT20 expression patterns in poorly differentiated CRC were different from those in well-differentiated CRC (Fig. 6). PLAC8 expression was higher in the poorly differentiated CRC tissue at stage III than at stage II (the middle panel of Fig. 6). However, the late tumor stage (stage III) did not appear to increase the expression of KRT20 in the poorly differentiated CRC tissue.

PLAC8 and KRT20 expression levels in transfected and differentiated CRC cell lines. PLAC8 expression was knocked

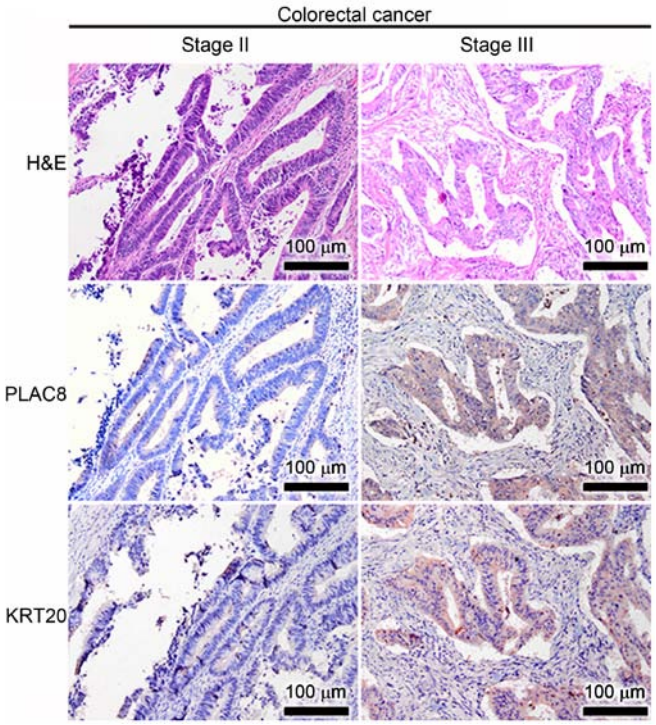

Figure 3. Representative immunohistochemical staining for PLAC8 and KRT20 expression in well differentiated stage II and III colorectal cancer tissue. Scale bar displayed in bottom right corner, magnification, x200. H&E, hematoxylin and eosin staining; PLAC8, placenta specific 8; KRT20, keratin 20.

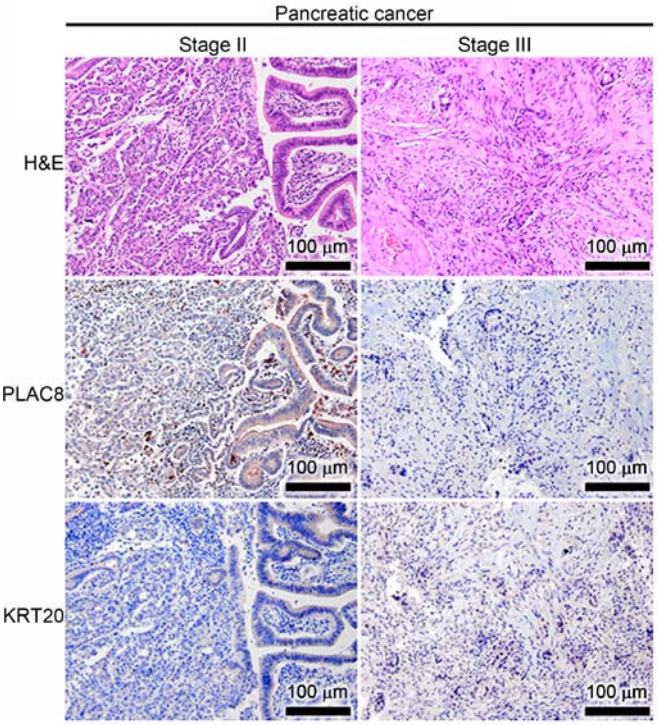

Figure 5. Representative immunohistochemical staining for PLAC8 and KRT20 expression in poorly differentiated stage II and III pancreatic cancer tissue. Scale bar displayed in bottom right corner, magnification, x200. H&E, hematoxylin and eosin staining; PLAC8, placenta specific 8; KRT20, keratin 20.

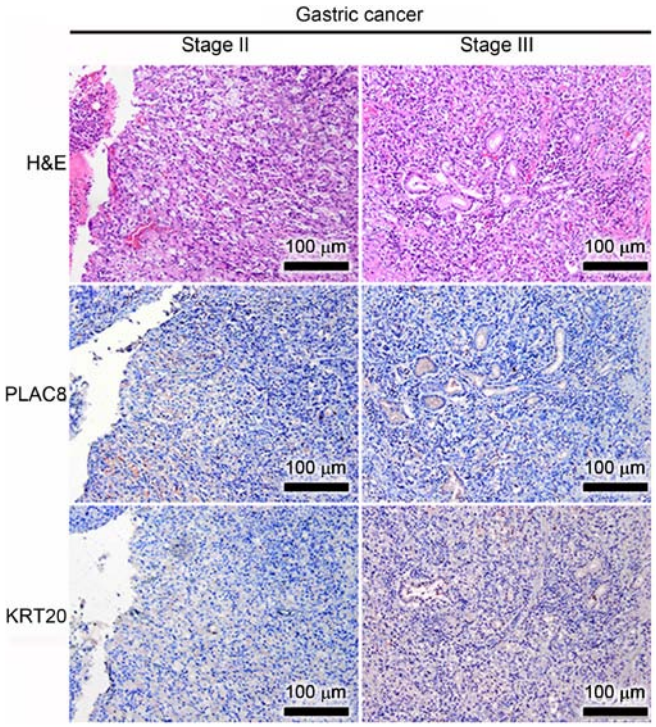

Figure 4. Representative immunohistochemical staining for PLAC8 and KRT20 expression in poorly differentiated stage II and III gastric cancer tissue. Scale bar displayed in bottom right corner, magnification, x200. H&E, hematoxylin and eosin staining; PLAC8, placenta specific 8; KRT20, keratin 20.

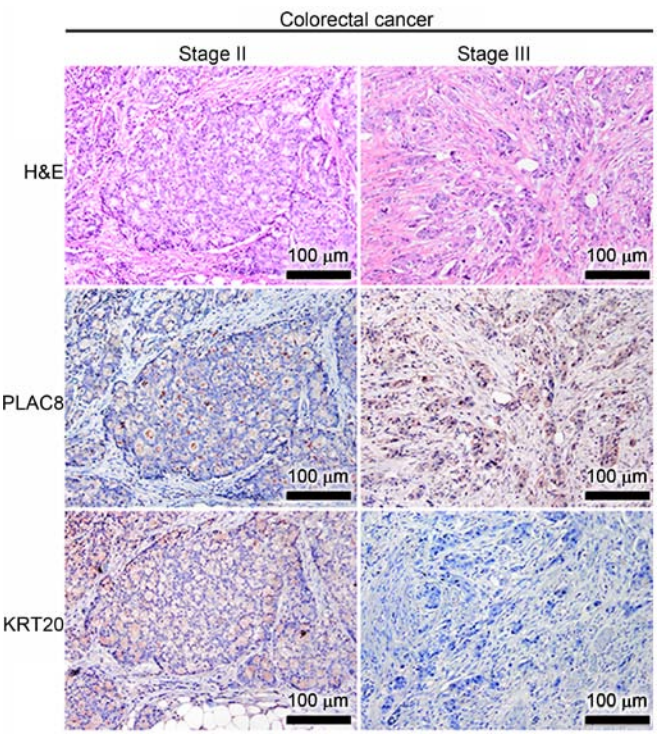

Figure 6. Representative immunohistochemical staining for PLAC8 and KRT20 expression in poorly differentiated stage II and III colorectal cancer tissue. Scale bar displayed in bottom right corner, magnification, x200. H&E, hematoxylin and eosin staining; PLAC8, placenta specific 8; KRT20, keratin 20.

down in SW620 cells, displayed by an 80% decrease in PLAC8 mRNA levels (shPLAC8-SW620) compared with the levels in the control cells (shLUC-SW620; Fig. 7A). The KRT20 mRNA levels in the shPLAC8-SW620 cells also decreased by 76%

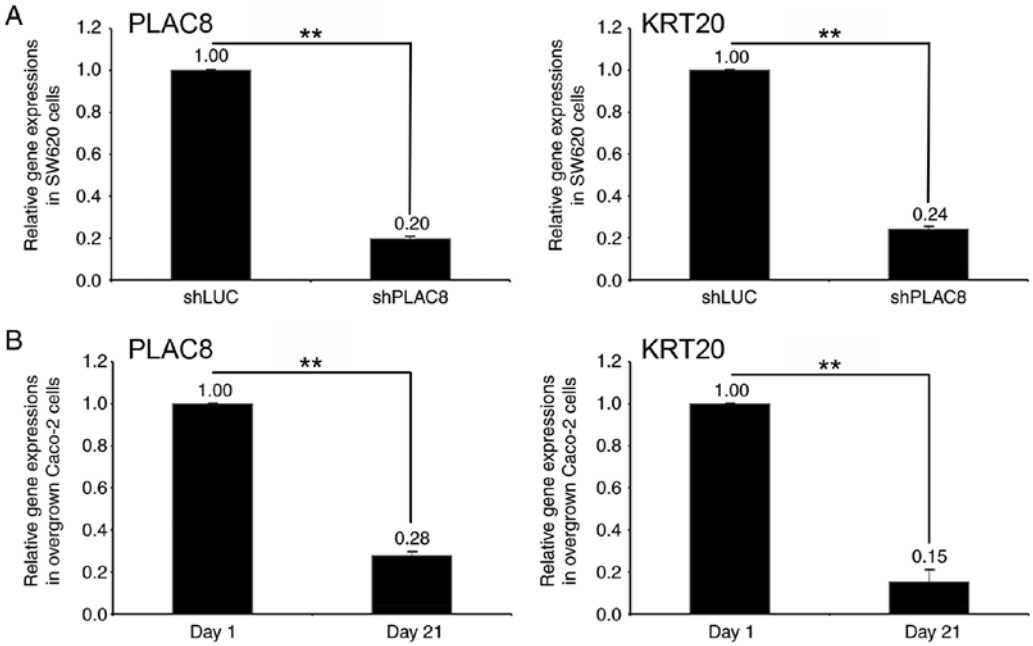

Figure 7. Expression patterns of PLAC8 and KRT20 in CRC cell lines. (A) Relative gene expression of PLAC8 and KRT20 in transfected SW620 cells. SW620 cells were cultured in a humidified and CO_2-free incubator. (B) Relative gene expression of PLAC8 and KRT20 in Caco-2 cells. Caco-2 cells were differentiated in a humidified incubator with 5% CO_2 for 21 days. Data were presented as mean ± standard deviation and compared between samples using the Student's *t*-test, **P<0.01. PLAC8, placenta specific 8; KRT20, keratin 20. CRC, colorectal cancer; sh, short hairpin.

compared with the levels in the control cells (Fig. 7A). The mRNA levels of both PLAC8 and KRT20 in the differentiated Caco-2 cells (day 21) decreased by 72 and 85%, respectively, compared with the levels in the day 1 Caco-2 cells (Fig. 7B).

Discussion

In clinical settings, patients with different types of GI cancer should be diagnosed using appropriate biomarkers, even though gut-derived adenocarcinomas display similar genetic alterations (28-32). Lukyanchuk *et al* (33) reported that KRT20 had clinical significance in GI cancer, including GC, CaP and CRC. Thus, the present study focused on investigating KRT20 and PLAC8 expression in these types of GI cancer. In the present study, the aberrant co-expression of the cytoplasmic protein PLAC8 and the cytokeratin KRT20 were found in the well-differentiated CRC at stage III, but this expression pattern was not observed in poorly differentiated CRC. No such co-expression was observed in the GC and CaP tissue sections, regardless of tumor stage and differentiation state. CRC tissues at stages II and III have been frequently studied to improve prognosis and to avoid the incorrect use of chemotherapeutic agents (34,35).

Cytoskeletal rearrangement is required for cell migration and invasion, which are key steps in cancer metastasis (36,37). Highly dynamic biological processes of cytoskeletal organization in cancer have been extensively explored (38-42). Among the different cytoskeletal molecules, KRTs might be the most examined based on clinical significance (43,44), and several KRTs have been previously studied from a tumor progression perspective (45-47). For example, previous studies have reported that upregulation of KRT17 and KRT19 may be involved in tumor metastasis (5,48) and that KRT18 and KRT19 are associated with colorectal malignancy (49-52). In addition, aberrant KRT20 expression has been observed in generalized GI cancer (16,19,53) and is recognized as a marker of circulating CRC cells (54). Therefore, KRT20 could be a suitable marker for the evaluation of the primary origin of GI cancer, including CRC (19,55).

PLAC8, a novel oncogenic marker that mediates tumor progression, has also been reported to play a key role in the EMT of CRC (18,22). In the present study, an association between KRT20 and PLAC8 expression was observed in CRC cells. The KRT20 mRNA levels decreased in the PLAC8-knockdown SW620 CRC cells, which were diagnosed as AJCC stage III. In addition, the intestinal differentiation of Caco-2 cells was used to evaluate the well-differentiated state of GI cancer (56,57). Such spontaneously differentiated Caco-2 cells displayed decreasing levels of KRT20 and PLAC8 expression upon differentiation. The Caco-2 cell line, which is applied extensively as an intestinal epithelial barrier model, displays favorable differentiation in a continuous culture (58,59). In addition, the positive association between KRT20 and PLAC8 expression levels in the well-differentiated CRC was confirmed by immunostaining of archived FFPE tissue sections. The FFPE tissue sections of other well-differentiated GI cancer (GC and CaP) at stages II and III did not display patterns similar to those of CRC and no association between PLAC8 and KRT20 expression levels were observed in the three poorly differentiated GI cancer tissues (GC, CaP and CRC). The results from the present study suggested that understanding the expression of PLAC8 and KRT20 could be critical for predicting the prognosis of patients with CRC.

Experiments exploring the molecular heterogeneity of CRC could facilitate the formulation of effective therapies (60,61).

CRC development and progression is a complex process involving multiple genetic changes (62-64). The genes involved in CRC tumorigenesis should therefore be identified for clinical applications (65). Chemotherapy, target molecule therapy (with vascular endothelial growth factor or epidermal growth factor receptor) and immunotherapy (anti-programmed death-1) lead to increased survival rates and decreased recurrence rates in CRC (66-68). Imai *et al* (69) revealed that the KRT20 expression was closely associated with the invasive histological phenotype in poorly differentiated colorectal adenocarcinoma. However, in the present study, it was suggested that the differentiation status of GI cancer may influence KRT20 expression, particularly in CRC.

A recent animal study reported that PLAC8 expression might be associated with the gut microbiota (70) and others detected that aberrant KRT20 expression is induced by altering the gut microbiota (71). Taken together, these results implied that KRT20 and PLAC8 might work cooperatively in different types of GI cancer. The present study suggested that PLAC8 expression could influence KRT20 expression. Therefore, it could be hypothesized that a well-differentiated CRC may have poor prognosis if KRT20 is induced via the upregulation of PLAC8. Conversely, the downregulation of PLAC8 may reduce the expression levels of KRT20. These suggested molecular dynamics imply that RNA interference of PLAC8 expression could be used as a therapeutic technique for the treatment of GI cancer at stages II and III. A similar concept that uses small interfering RNA as a cancer therapeutic agent has been explored extensively (72-74). In addition, PLAC8 promotes tumor growth, invasion and metastasis in other tumors, which could explain why patients with well-differentiated CRC display different clinical outcomes, in comparison with the patients with poorly-differentiated CRC (17,22,75,76). Therefore, the prognostic significance of KRT20 and PLAC8 could be particularly essential for patients with CRC displaying well-differentiated phenotypes.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

CSH, YCW and CYL designed the study. CSH and CYL wrote the initial version of the manuscript. JWG and CCH performed the cell studies. JWG, JYY and CYL performed the immunostaining and pathologic diagnosis. RNY, CLL, MHS and CCH interpreted the patient data regarding the well-differentiated and poorly-differentiated CRC. CCH and CJH performed the statistical analyses. CYL provided supervision throughout the study. All authors discussed, modified and approved of the final version.

Ethics approval and consent to participate

All procedures were approved by The Cathay General Hospital Institutional Ethics Committee and a waiver of consent was approved by the same committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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