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| Author | 半井 千晶 |
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Expression of *AKR1C3* and *CNN3* as markers for detection of lymph node metastases in colorectal cancer

（大腸癌のリンパ節転移における *AKR1C3* および *CNN3* 発現の検討）

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神戸大学大学院保健学研究科保健学専攻

半 井 千 晶
Expression of AKR1C3 and CNN3 as markers for detection of lymph node metastases in colorectal cancer

Abstract

The aim of the study was to identify a set of discriminating genes that could be used for the prediction of Lymph node (LN) metastasis in human colorectal cancer (CRC), and for this, we compared the whole genome profiles of two CRC cell lines (the primary cell line SW480 and its LN metastatic variant, SW620) and identified eight genes [S100 calcium-binding protein P; aldo-keto reductase family 1 (AKR1), member B1 (aldose reductase; AKR1B1); AKR1, member C3 (AKR1C3); calponin 3, acidic; metastasis associated in colon cancer 1; hemoglobin, epsilon 1; trefoil factor 3; and FGGY carbohydrate kinase domain containing]. These genes were examined by quantitative RT-PCR in tissues and LNs in 14 CRC patients and 11 control patients. The level of AKR1C3 mRNA expression was significantly different between the Dukes’ stage A, B, and C groups and the control group ($p < 0.05$, $p < 0.001$, and $p < 0.001$), and was also significantly different between Dukes’ stage C and A or B groups ($p < 0.05$ and $p < 0.001$ respectively). The expression of CNN3 was significantly different between the Dukes’ stage C and B or control groups ($p < 0.001$ and $p < 0.01$, respectively). There were significant correlations between the expression levels of AKR1C3 and CNN3. AKR1C3 and CNN3 expressions are more accurate and suitable markers for the diagnosis of LN metastasis than the other six genes examined in this study.

Keywords: AKR1C3; CNN3; lymph node metastasis; colorectal cancer; real-time quantitative PCR
Background

Lymph node (LN) evaluation is an important factor for determining prognosis in colorectal cancer (CRC). LN metastases cause recurrence of CRC and are related to prognosis and survival [1]. Carcinoembryonic antigen (CEA) was first described as a gastrointestinal oncofetal antigen and is now known to be overexpressed in most carcinomas [2]. CEA is generally used for the detection of LN metastases in CRC [3, 4]. Detection of cytokeratin-20 by RT-PCR in peritumoral, histopathologic, tumor-free LNs is an independent prognostic factor for overall survival in CRC [5]. A biomarker for identifying patients at high risk of metastasis could have extensively clinical applications. Recent evidence indicates that CXC Chemokine Ligand 10 (CXCL10), an interferon-inducible protein, is downregulated in recurrent CRC. Detection of CXCL10 as a prognostic marker for advanced stage CRC patients may help predict clinical outcomes [6]. Our recent study suggests that the expression of the E74-like factor 3 gene (ELF3) in LNs signals the possibility of metastases and that ELF3 may be more suitable than CEA as a gene marker for the detection of LN metastases from CRC [7].

In the present study, we compared the whole genome profiles of two isogenic CRC cell lines (the primary cell line SW480 and its LN metastatic variant, SW620) to identify a set of discriminating genes that could be used for the prediction of metastasis in human CRC. A total of 54,359 genes in SW480 and SW620 cells were analyzed using the Whole Genome Bioarray. As a result, we identified 8 genes that had a fivefold increase in the intensity ratio in SW620 cells as compared with SW480 cells and examined by quantitative RT-PCR (qRT-PCR) in tissues and LNs in 14 CRC patients and 11 control patients. The genes selected for examination were S100 calcium-binding protein P (S100P); aldo-keto reductase family 1 (AKR1), member B1 (aldose reductase; AKR1B1); AKR1, member C3 (AKR1C3); calponin 3, acidic (CNN3); metastasis associated in colon cancer 1 (MACC1); hemoglobin, epsilon 1 (HB E1); trefoil factor 3 (intestinal; TFF3); and FGGY carbohydrate kinase domain containing (FGGY). S100P is known to regulate the cellular processes, such as cell cycle progression and differentiation [8, 9]. The protein encoded by AKR1B1 catalyzes the reduction of a number of aldehydes, including the aldehyde form of glucose, and the protein encoded by AKR1C3 catalyzes the conversion of aldehydes and ketones [10, 11]. The protein encoded by CNN3 regulates actin cytoskeleton rearrangement, which is needed for the
plasma trophoblasts membranes to become fusion competent [12]. MACC1 is more frequently expressed in advanced CRC [13]. HBE1 is normally expressed in adult hemoglobin and the leading known cause of a β-thalassemia with gene mutation in Southeast Asia [14]. TFF3 is expressed in goblet cells in the intestines and the colon, and overexpression of TFF3 after chemoradiotherapy for rectal cancer is associated with a higher risk of relapse [15]. FGGY encodes a member of the FGGY kinase family that acts as a phosphotransferase [16]. In this study, we investigated whether these genes could be used as biomarkers for detecting LN metastases of CRC by qRT-PCR.

Materials and Methods

Microarray analyses
A total of 54,359 genes in two isogenic CRC cell lines (the primary cell line SW480 and its LN metastatic variant, SW620) were analyzed using a CodeLink™ Human Whole Genome Bioarray (Applied Microarrays, Inc. Tempe, AZ, USA). We entrusted microarray analyses to Filgen, Inc. (Nagoya, Japan). The procedure was identical to that of a previous study [17]. Thirty-five genes with a fivefold increase in the intensity ratio in SW620 cells compared with SW480 were arbitrarily defined as being overexpressed in SW620 cells (data not shown). Of the 35 genes that were overexpressed, we selected eight genes (S100P, AKR1C3, CNN3, AKR1B1, MACC1, HBE1, TFF3, and FGGY) that were extremely overexpressed and were unlikely to be related to inflammation in Table 1.

Patients
Twenty-seven tissue specimens (14 tumor specimens and 13 non-tumor specimens) and 125 LNs were dissected from 14 patients with CRC. Non-tumor specimens were located far from primary cancer and confirmed not including tumor cells pathologically. Eleven inflammatory tissue specimens and 35 LNs were dissected as controls from 11 patients who were undergoing surgery for

| Accession number | Gene symbol | Gene name | Intensity ratio | qRT-PCR |
|------------------|-------------|-----------|----------------|---------|
| NM_005980        | S100P       | S100 calcium binding protein P | 48.41 | 311.22 |
| NM_003739        | AKR1C3      | aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II) | 18.19 | 37.83 |
| NM_001839        | CNN3        | calponin 3, acidic calmodulin-binding troponin-like protein | 16.39 | 98.34 |
| NM_001628        | AKR1B1      | aldo-keto reductase family 1, member B1 (aldosoc reductase) | 15.24 | 153.11 |
| NM_187262        | MACC1       | metastasis associated in colon cancer | 11.29 | 27.32 |
| NM_005330        | HBE1        | hemoglobin, epsilon 1 | 9.79 | 886.31 |
| NM_003226        | TFF3        | trefoil factor 3 (intestinal) | 5.90 | 336.22 |
| NM_018291        | FGGY        | FGGY carbohydrate kinase domain containing | 5.02 | 23.46 |
ulcerative colitis (UC). LNs and tissue specimens were obtained from surgical resections performed in the Department of Surgery, Hyogo College of Medicine, Nishinomiya, Japan between September, 2009 and March, 2010. The study design was approved by the Ethics Review Committee on Genetic and Genomic Research, Kobe University Graduate School of Health Sciences, Kobe, Japan. Sections of formalin-fixed, paraffin-embedded LNs were examined using hematoxylin-eosin staining (HES) in the Department of Surgical Pathology, Hyogo College of Medicine. All LNs from CRC patients were categorized according to Dukes’ staging system [18] (Table 2). The patients were categorized into three groups: A (n=2), B (n= 4) and C (n=8). Almost all cases had lymphatic invasion and/or venous invasion regardless of LN metastasis. In almost all cases, invasion reached the subserosa. Routine HES diagnosis of LNs detected metastasis in 4 (28.6%) out of 14 patients, lymphatic invasion in 10 (71.4%), and venous invasion in 13 (92.9%). Extramural cancer deposits (EX) were detected in three cases. EX were defined as cancer foci that were not adjacent to the primary tumor and not associated with LN [19]. Case 10 was EX-positive diagnosed with metastasis-negative LNs on conventional pathologic of staging.

| Case | Location | Stage | Histology | Depth | LN metastasis | EX |
|------|----------|-------|-----------|-------|---------------|----|
| 1    | R        | A     | tub1      | sm    | -             | -  |
| 2    | R        | A     | tub1      | mp    | -             | -  |
| 3    | R        | B     | tub1      | ss    | -             | -  |
| 4    | D        | B     | tub2      | ss    | -             | -  |
| 5    | A        | B     | por1      | ss    | -             | -  |
| 6    | A        | B     | tub1      | ss    | -             | -  |
| 7    | A        | B     | por1      | ss    | -             | -  |
| 8    | S        | B     | tub2      | ss    | -             | -  |
| 9    | A        | B     | tub2      | ss    | -             | -  |
| 10   | A        | B     | muc      | se    | +             | +  |
| 11   | D        | C     | por       | ss    | +             | +  |
| 12   | D        | C     | tub2      | ss    | +             | -  |
| 13   | Rb       | C     | tub2      | mp    | +             | -  |
| 14   | R        | C     | tub2      | ss    | +             | +  |

*D, descending colon; A, ascending colon; R, rectum; Rb, rectum below peritoneal reflection; S, sigmoid colon
T, transverse colon
*tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por1, poorly differentiated solid adenocarcinoma; muc, mucinous adenocarcinoma. sm, submucosa; mp, muscularis propria; ss, subserosa; se, serosa-exposed
EX, extramural cancer deposits without lymph node structure
**Tissue preparation / RNA extraction and cDNA synthesis**

Tissue preparation, RNA extraction, and cDNA synthesis performed in the same way as described in the previous report [7]. Each RNA from the tissues and LNs was standardized equal concentration.

**Real-time qRT-PCR**

One microliter of cDNA was used as the template in the reaction mixture for real-time qRT-PCR. For determination of specific gene expression, each primer was designed with *Perfect real time primer* (Takara, Ohtsu, Japan). The primers for *S100P* (GenBank Acc. No. NM_005980), *AKR1C3* (GenBank Acc. No. NM_003739), *CNN3* (GenBank Acc. No. NM_001839), *AKR1B1* (GenBank Acc. No. NM_001628), *MACC1* (GenBank Acc. No. NM_182762), *HBE1* (GenBank Acc. No. NM_005330), *TFF3* (GenBank Acc. No. NM_003226), *FGGY* (GenBank Acc. No. NM_018291), and β-actin (*ACTB*; GenBank Acc. No. NM_001101) are listed in Tables 1 and 3. The parameter threshold cycle (Ct) was used as the cycle number to detect the fluorescence increasing. The housekeeping gene *ACTB* was used to calculate the relative level of expression for each gene and data normalization to correct RNA quality and quantity using the 2-ΔΔCt method. qRT-PCR was performed on a MyiQ Real-time PCR System (Bio-Rad, Hercules, CA, USA) using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA, USA) according to the manufacturer’s recommendations. The protocol was as follows: initial denaturation at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s, annealing at the temperature suitable for each gene marker for 10 or 20 s, and extension at 72°C for 10 s (Table 3). Each sample was assayed in duplicate. A control and two references were included in every run to confirm each examination.

**Statistical analysis**

Statistical analysis was performed using PASW for Windows version 17.0 (SPSS Japan Inc., Tokyo, Japan). To set cutoff values for each gene marker, receiver operating characteristic (ROC) curve analysis was performed by plotting the true-positive fraction (sensitivity) and false-positive fraction (specificity) pairs with area under the curve (AUC) values for LNs dichotomized according to LN metastasis diagnosed by HES [20,21]. Data were evaluated using the Kruskal–Wallis test, followed by the Mann–Whitney U test with a Bonferroni correction. Analyses of correlations between levels of different mRNA species were performed using a two-tailed Spearman’s rank correlation test. Differences were considered as statistically significant at \( p < 0.05 \).
Results

Genes with a fivefold increase in the intensity ratio in SW620 cells as compared with SW480 cells were arbitrarily defined as being overexpressed. Eight candidate genes, S100P, AKR1C3, CNN3, AKR1B1, MACC1, HBE1, TFF3, and FGGY, were selected on the basis of their remarkable overexpression in SW620 cells and unlikable to be related to inflammation (Table 1). We were examined these genes in tissues and LNs of 14 colorectal cancer patients and 11 controls by real-time qRT-PCR.

qRT-PCR was performed to quantify these genes in tumor tissues (n = 14), non-tumor tissues from CRC patients (n = 13), and inflammatory tissues from patients with UC; the latter tissues served as the controls (n = 11). The results are shown in Fig. 1. There were no significant differences in the relative levels of mRNA expression for S100P, AKR1C3, CNN3, AKR1B1, HBE1, TFF3, and FGGY among tumor tissues, non-tumor tissues, and inflammatory tissues. For MACC1, there were significant differences in levels of expression between tumor tissues (Mean±SD, 145.37±289.74), non-tumor tissues

| Table 3. Primer sequences and PCR conditions used for real-time quantitative RT-PCR. |
|----------------|-----------------|-----------------|----------------|
| primer         | Sequence        | length | annealing |
| S100P          | F 5'-TAGCACTTGGACCTAGAGCA-G-3'  | 182        | 53°C 10sec |
|                | R 5'-TGACCAATTTTCACTGCCATC-3'   |            |            |
| AKR1C3         | F 5'-GGATTGCGACCTAGACCT-3'      | 91         | 52°C 10sec |
|                | R 5'-CTATATGGCGAACCACCTTCA-3'   |            |            |
| CNN3           | F 5'-TTCATAACCAACATGATGGAG-3'   | 127        | 52°C 20sec |
|                | R 5'-GCTGACTGACATTTTAGTGGTTC-3' |            |            |
| AKR1B1         | F 5'-TATGACTGGCGAGCTGGCTTTTA-3' | 71         | 60°C 10sec |
|                | R 5'-GAACCACTGGCCGACTCA-3'      |            |            |
| MACC1          | F 5'-AGGTACACATGGTTTACCTAGAGG-3' | 65         | 52°C 20sec |
|                | R 5'-CAATAGAGAAGTGGCATTTGG-3'   |            |            |
| HBE1           | F 5'-CTGAGTGACTGTCGTAGCAAG-3'   | 75         | 60°C 10sec |
|                | R 5'-AATCACCACATCACTACCCAGGA-3' |            |            |
| TFF3           | F 5'-CTGGTGTCTTGAGCTCTCAAGAT-3' | 90         | 63°C 10sec |
|                | R 5'-CGCTGAGGTCCTGCTCAGAA-3'    |            |            |
| FGGY           | F 5'-AGGACCCTTTGATCTTGGGCCATTC-3' | 93         | 52°C 20sec |
|                | R 5'-CTCTGCTCTGATCCAGCCTTCTA-3' |            |            |
| ACTB           | F 5'-TGCCACCCAGCACAATGAA-3'     | 186        | 52°C 10sec |
|                | R 5'-CTAAGTCATAGTCTGGCTAGAA-3'  |            |            |
Figure 1. Relative mRNA expression of *S100P*, *AKR1C3*, *CNN3*, *AKR1B1*, *MACC1*, *HBE1*, *TFF3*, and *FGGY* in tissues from colorectal cancer (CRC) patients determined by real-time quantitative RT-PCR. Dots showed mRNA levels in 13 non-tumor tissues and 14 tumor tissues from CRC patients compared with 11 inflammatory tissues from ulcerative colitis patients as controls. Bars showed means. A: The relative quantity values (Means±SD) of *S100P* are 28.68±24.20, 14.43±12.90 and 199.51±549.78 (control, non-tumor and tumor). B: Those of *AKR1C3* are 2.15±3.55 and 0.96±1.63. C: Those of *CNN3* are 2.86±1.53, 10.44±16.20 and 6.93±10.30. D: Those of *AKR1B1* are 1.32±1.57, E: Those of *MACC1* are 10.04±6.12, 69.13±158.41 and 145.37±289.74. F: Those of *HBE* are 0.89±1.43, 0.55±1.15 and 0.67±1.61. G: Those of *TFF3* are 154.79±216.76, 1116.46±1610.72 and 908.91±1158.59. H: Those of *FGGY* are 1.02±0.50, 13.30±25.12 and 30.65±49.14, respectively. The *p*-values are based on Kruskal-Wallis test. *+* *p*<0.05 and *p*<0.01 are based on Mann-Whitney *U* test with Bonferroni correction.

| marker  | cut-off value | AUC value | SE  | 95% CI       | *p*-value |
|---------|---------------|-----------|-----|--------------|-----------|
| *S100P* | 0.42          | 0.887     | 0.036 | 0.817-0.957 | 0.00026   |
| *AKR1C3* | 56.74        | 0.919     | 0.043 | 0.829-1.000 | 0.00008   |
| *CNN3*  | 18.31         | 0.951     | 0.037 | 0.900-1.000 | 0.00002   |
| *AKR1B1* | 1.35          | 0.755     | 0.054 | 0.649-0.862 | 0.01592   |
| *MACC1* | 4.13          | 0.774     | 0.095 | 0.587-0.960 | 0.00981   |
| *HBE*   | 0.18          | 0.833     | 0.052 | 0.731-0.936 | 0.00165   |
| *TFF3*  | 1.97          | 0.713     | 0.105 | 0.507-0.918 | 0.04471   |
| *FGGY*  | 5.90          | 0.626     | 0.102 | 0.425-0.827 | 0.23394   |
(Mean±SD, 69.13±158.41), and inflammatory tissues (Mean±SD, 10.04±6.12) (Kruskal-Wallis test; p < 0.05). MACC1 mRNA expression was significantly different between tumor and inflammatory tissues (Mann–Whitney U test with a Bonferroni correction; p < 0.05). For FGGY, there were significant differences in levels of expression between tumor tissues (Mean±SD, 30.65±49.14), non-tumor tissues (Mean±SD, 13.30±25.12), and inflammatory tissues (Mean±SD, 1.02±0.50) (Kruskal-Wallis test; p < 0.001). A subsequent Mann–Whitney U test with a Bonferroni correction showed that the mean values for FGGY expression were significantly different between non-tumor and inflammatory tissues (p < 0.05) and between tumor and inflammatory tissues (p < 0.01).

To determine the cutoff values for use in qRT-PCR, ROC curve analysis was performed using relative gene expression values from LNs from CRC patients categorized according to the degree of LN metastasis as evaluated by HES. The cutoff values are shown in Table 4. The AUC values were as follows: CNN3 = 0.951, SE = 0.037, 95% confidence interval (CI) = 0.000–1.000, p = 0.00002; and AKR1C3 = 0.919, SE = 0.043, 95% CI = 0.829–1.000, p = 0.00008. The cutoff values for CNN3 and AKR1C3 were set at 18.31 with 87.5% sensitivity and 96.6% specificity rates, and 56.74 with 87.5% sensitivity and 93.2% specificity rates, respectively. The AUC values of the other six genes were below 0.9.

To investigate whether each gene was overexpressed in metastatic LNs from CRC, we measured mRNA expression in 12 LNs from patients categorized into Dukes’ stage A, 97 LNs from patients categorized into Dukes’ stage B, and 16 LNs from Dukes’ stage C. As a control, we also measured mRNA expression in 35 LNs dissected from UC patients. As shown in Fig. 2, each level of S100P, AKR1C3, CNN3, AKR1B1, MACC1 and HBE1 mRNA expression was significantly different among the Dukes’ stage A, B, and C groups and the control group (S100P, AKR1C3, CNN3: p < 0.001, ; AKR1B1, MACC1, HBE1: p < 0.01, respectively; Mann–Whitney U test with a Bonferroni correction). The level of AKR1C3 mRNA expression was significantly different between the Dukes’ stage A, B, and C groups and the control group (p < 0.05, p < 0.001, and p < 0.001, respectively; Mann–Whitney U test with a Bonferroni correction), and was also significantly different between Dukes’ stage C and Dukes’ stage A or Dukes’ stage B groups (p < 0.05 and p < 0.001 respectively). A subsequent Mann–Whitney U test with a Bonferroni correction showed that expression of CNN3 and MACC1 was significantly
Figure 2. Relative mRNA expression of S100P, AKR1C3, CNN3, AKR1B1, MACC1, HBE1, TFF3, and FGGY in lymph nodes (LNs) from colorectal cancer (CRC) patients categorized by Duke’s classification. Dots showed mRNA levels in 125 LNs from CRC patients with Dukes’ stage A, B and C, compared with 35 LNs from ulcerative colitis patients as controls. Black dots indicate LNs with tumor cells, and grey dots indicate LNs without tumor cells identified by hematoxylin–eosin staining. Broken lines show cut-off values of eight genes in Table 4. Bars showed means. A: The relative quantity values (Means±SD) of S100P are 53.17±208.51, 1.30±2.86, 1.10±6.44 and 6.24±16.00 (control, Dukes’ A, Dukes’ B and Dukes’ C). B: Those of AKR1C3 are 1.97±3.14, 13.99±17.82, 34.03±201.50 and 87.72±127.72. C: Those of CNN3 are 5.46±6.73, 5.73±5.28, 4.06±6.39 and 22.97±26.78. D: Those of AKR1B1 are 5.49±7.29, 3.44±2.72, 5.15±15.99 and 3.64±4.01. E: Those of MACC1 are 4.53±6.06, 10.87±10.81, 6.43±18.75 and 34.17±95.53. F: Those of HBE are 8.57±22.92, 1.61±4.06, 0.48±1.14 and 5.52±9.51. G: Those of TFF3 are 7.75±13.53, 13.54±25.01, 5.48±16.74 and 16.70±43.65. H: Those of FGGY are 6.45±14.38, 3.67±2.87, 3.20±5.24 and 4.41±4.33, respectively. The p-values are based on Kruskal-Wallis test. *p<0.05, **p<0.01 and ***p<0.001 are based on Mann-Whitney U test with Bonferroni correction.
different between the Dukes’ stage B and C groups ($p < 0.001$, $p < 0.01$, respectively), and between the control and the Dukes’ stage C groups ($p < 0.01$, $p < 0.05$, respectively). The $S100P$ and $HBE1$ was significantly different between the Dukes’ stage B and C groups ($p < 0.001$, $p < 0.001$, respectively), and the $S100P$ and $AKR1B1$ was significantly different between the control and the Dukes’ stage B groups ($p < 0.001$, $p < 0.05$, respectively). On the other hand, there were no significant differences in $TFF3$ and $FGGY$ mRNA expression among those four groups. The mRNA expression of $AKR1C3$, $CNN3$, and $MACC1$ was significantly higher in the Dukes’ stage C group than in the control group.

Furthermore, to investigate the correlation between the mRNA levels for the eight biomarkers, we compared mRNA expression in the LNs of CRC...
patients and controls. LNs from the controls and each staging group were analyzed separately. The results worthy of special mention are shown in Table 5. There were significant correlations between the levels of AKR1C3 and CNN3 mRNA expression overall ($r = 0.635; p < 0.001$), in Dukes’ A ($r = 0.888; p < 0.001$), Dukes’ B ($r = 0.712; p < 0.001$) and Dukes’ C ($r = 0.844; p < 0.001$) groups, and in the control group ($r = 0.475; p < 0.01$).

The relationships between the qRT-PCR results and histological examination are shown in Table 6. The results can be summarized as follows: there were 7 of 7 true-positives for $S100P$, $AKR1C3$, $CNN3$, $AKR1B1$, and $HBE1$; 6 of 7 for $MACC1$; 5 of 7 for $TFF3$; and 3 of 7 for $FGGY$ (statistical analysis was omitted due to low case numbers).

## Discussion

Recently, there have been many reports regarding the use of novel gene markers to detect colon tumors in early stages and diagnose the status of the disease appropriately. The purpose of this study was to find new markers for the detection of LN metastases in CRC by qRT-PCR. On the basis of comparative microarray analyses, we identified eight candidate genes ($S100P$, $AKR1C3$, $CNN3$, $AKR1B1$, $MACC1$, $HBE1$, $TFF3$, and $FGGY$).

In this study, $S100P$ was remarkably overexpressed in SW620 cells as compared with SW480 (intensity ratio = 48.41). It has been reported that expression of the $S100P$ mRNA and protein is significantly higher in cancerous regions than in non-cancerous tissues [22]. It has been known to express in cancer cells in adult specifically and to mediate tumor growth, drug resistance, and metastasis [23-25]. However, our study revealed that there are no significant differences in levels of $S100P$ expression between non-tumor tissues and tumor tissues. Our findings conflict with those reported by others.

As a result of our ROC curve analysis, we conclude that $AKR1C3$ and $CNN3$ expression are more accurate and suitable for the diagnosis of LN metastasis than the other 6 genes. The AUC values of $AKR1C3$ and $CNN3$ were 0.919 and 0.951, respectively, whereas our previous data showed AUC values for $ELF3$ and $CEA$ of 0.955 and 0.903, respectively [7]. From this point of view, $AKR1C3$ and $CNN3$ are more accurate markers than $CEA$ and may be considered to be as accurate as $ELF3$.

The mRNA expressions of $AKR1C3$
and CNN3 were found to be significantly higher in the Dukes’ stage C group than in the control groups. The AKR1C3 mRNA expression was also found to be significantly different between the Dukes’ stage C group and the other Dukes’ groups. To our knowledge, this report is the first study on LN metastasis in CRC that has focused on AKR1C3 and CNN3. We found that the mRNA expression of both genes in primary tumor tissues was different from that in non-tumor or inflammatory tissues. In addition, there was a significant correlation between AKR1C3 and CNN3 mRNA expression (Table 5). AKR1C3 expression has been demonstrated in sex hormone-dependent tissues, including breast [11], endometrial [26], testis [27], and prostate tissues [11] as well as in sex hormone-independent tissues, including kidney, bladder, and urothelial tissues [28]. Elevated expression of AKR1C3 has been identified in prostate and breast cancer and is correlated with the aggressiveness of the disease [11, 29, 30]. Positive immunoreactivity AKR1C3 was widely present in both adenocarcinoma and squamous cell carcinoma of the lung and gastroesophageal junction [31]. A previous study showed that AKR1C3 mRNA and protein were overexpressed in castration-resistant prostate cancer tissue as compared to benign prostate and primary prostate cancer tissue [32]. CNN3 was identified the gene in tumorigenic parameter as ovarian cancer and mucosa-associated lymphoid tissue lymphoma [33, 34].

MACC1 is a key regulator of the hepatocyte growth factor receptor pathway, including in cellular growth, invasiveness, and metastasis, and is useful to identify the poor prognosis in CRC patients [35]. We found that the level of MACC1 mRNA expression differs between primary tumor tissues and inflammatory tissues (p < 0.05, Kruskal–Wallis test). As MACC1 overexpression was not found in all the histologically positive LNs examined in this study, we conclude that MACC1 may be inferior to AKR1C3 and CNN3 in detecting LN metastases.

However, AKR1B1, HBE1, TFF3, and FGGY were not suitable for detecting LN metastases in view of the fact that there were no significant differences in the expression levels of these genes between the control group and the Dukes’ stage C group. AKR1B1 is overexpressed in human tumors, such as those found in liver, breast, and lung cancer, and may play an important role in the development and progression of cancer [36]. There is no previous report of HBE1’s expression
in cancer cells. *TFF3* expression may play a role in promoting LN metastases in CRC [37]. *FGGY* expression has been recently associated with an increased susceptibility to sporadic amyotrophic lateral sclerosis [38]. We would like to emphasize our study limitations, especially the number of patients were not enough for definitive conclusion. Thus, it may be biased by the relatively small number of patients.

In conclusion, *AKR1C3* and *CNN3* expression are more accurate and suitable markers for the diagnosis of LN metastasis than the other six genes examined in this study. We found that the difference in *AKR1C3* expression between all Dukes’ stage groups and the control group was statistically significant. In addition, there were significant correlations between the expression levels of *AKR1C3* and *CNN3*. *AKR1C3* and *CNN3* might be more suitable than the other six genes as gene markers for the detection of LN metastases from CRC and requires further verification as biomarkers in a larger population study.

**Abbreviations**

LN Lymph node
CRC colorectal cancer
UC ulcerative colitis
CEA Carcinoembryonic antigen

CXCL10 CXC Chemokine Ligand 10
ELF3 E74-like factor 3
S100P S100 calcium-binding protein P
AKR1 aldo-keto reductase family 1
AKR1B1 AKR1, member B1
AKR1C3 AKR1, member C3
CNN3 calponin 3, acidic
MACC1 metastasis associated in colon cancer 1
HBE1 hemoglobin, epsilon 1
TFF3 trefoil factor 3
FGGY FGGY carbohydrate kinase domain containing.
HES hematoxylin–eosin staining

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**Competing interests**

No conflicts of interest exist in the submission of this manuscript.
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