INTRODUCTION

Colorectal cancer (CRC) is the third-most commonly diagnosed cancer in males and the second-most commonly diagnosed in females, with an estimated 1.4 million new cases and 693,900 deaths occurring in 2012. Complete resection is essential for the cure of CRC. Although the resection rate has gradually been increasing, some patients recur even after curative surgery. Once recurrence has developed, the prognosis is poor. Therefore, it is important to identify reliable predictive factors for patients at high risk of recurrence.

Recently, the loss of cell polarity and disruption of intracellular adhesion have been shown to have important roles in cancer progression. Tight junctions form the apical junctional complex in epithelial and endothelial cellular sheets, and these junctions are essential for controlling the paracellular ion flux and barrier function, thereby maintaining tissue homeostasis. Claudins are a 24-member family of proteins that are major components of tight junctions, the epithelial cell-cell contacts that play crucial roles in cell polarity maintenance. Members of this family are membrane proteins composed of four transmembrane domains and two extracellular loops. Claudin-4 is a particularly suitable representative member of the claudin multigene family given its association with cancer. Several reports have confirmed the high expression of claudin-4 in ovarian, breast, and pancreatic cancers. In ovarian cancer, due to the consistent overexpression of claudin-4 and its association with a poor prognosis, claudin-4 is being investigated as a diagnostic or prognostic biomarker. In CRC, however, the relationship between claudin-4 expression level and outcomes remains no reports.

In this study, we measured the expression of the claudin-4 gene in 202 pairs of cancer tissue and adjacent normal mucosa obtained from patients with CRC. To evaluate the clinical significance of claudin-4, we examined the correlation between the relative expression of this gene and the outcomes in patients with CRC.
MATERIALS AND METHODS

Patients

This was a retrospective multi-institutional study. Patients’ records were retrieved from the collected database of Yokohama City University, Department of Surgery, between 2003 and 2006. The inclusion criteria were as follows: (i) histologically proven CRC, (ii) age over 20 years, and (iii) curative surgery with lymph node dissection received as primary treatment for CRC.

Surgical procedure

The appropriate length of resection and the levels of lymph node dissection were generally determined by the Japanese Society for Cancer of the Colon and Rectum (JSCCR) Guidelines 2010. Pathological staging was carried out according to the UICC classification.

Follow-up

Patients were followed up in accordance with the protocol of the present study. In brief, during protocol treatment, the clinical findings and laboratory data were evaluated every two weeks. After completion of the protocol treatment, patients were followed up in accordance with a predefined surveillance schedule until recurrence or death was confirmed for five years after surgery. Recurrence was assessed based on computed tomography (CT). These tests were carried out every four months during the first two years after surgery and once every six months from the third year onward.

Quantitative real-time, reverse-transcription polymerase chain reaction (PCR)

Each tissue sample was embedded in O.C.T. compound (Sakura Finetechical Co., Ltd., Tokyo) and immediately stored at -80 °C until use. No patient had any other malignancies. The histopathological features of specimens stained with hematoxylin and eosin were examined, and sections that consisted of >80% carcinoma cells had their total RNA prepared. Total RNA isolated from CRC and adjacent normal mucosa was prepared using Trizol (Gibco, Life Tech, Gaithersburg, MD, USA). Complementary DNA (cDNA) was synthesized from 2 μg of total RNA with an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, USA). After synthesis, the cDNA was diluted 1:4 with water and stored at -20 °C until use. Quantitative real-time PCR was performed with an iQ SYBR-Green Supermix (Bio-Rad Laboratories). PCR was carried out in a total volume of 15 μl, containing cDNA derived from 75 μg of RNA; 0.27 μM of each primer; 7.5 μl of iQ SYBR-Green Supermix containing dATP, dCTP, dGTP, and dTTP at a concentrations of 400 μM each; and 50 units/ml of iTag DNA polymerase. The PCR protocol was 10 min at 94 °C followed by 50 cycles of denaturation of the cDNA for 30 sec at 94 °C, annealing for 30 sec at an appropriate temperature (Table 1), and primer extension for 1 min at 72 °C followed by 72 °C for 10 min. The PCR primer sequences of claudin-4 and β-actin, which was used as an internal control, are shown in Table 1. The expression of the claudin-4 gene was categorized as low or high according to the median value.

Evaluations and statistical analyses

The significance of the correlation between the claudin-4 expression and the clinicopathological parameters was determined using Fisher’s exact test or the χ² test. The overall survival (OS) was defined as the period between surgery and death, and the recurrence-free survival (RFS) was defined as the period between surgery and recurrence or death, whichever came first. The data of the patients who had not experienced an event were censored at the date of the final observation. The OS and RFS were evaluated by univariate and multivariate analyses. OS and RES curves were calculated using the Kaplan-Meier method and compared using the log-rank test. The univariate and multivariate survival analyses were performed using Cox’s proportional hazards model. P values of <0.05 were considered to be statistically significant.

The survival data were obtained from hospital records or from the city registry system. The SPSS software program (v11.0 J Win; SPSS, Chicago, IL, USA) was used for all of the statistical analyses. The Ethics Committees of Yokohama City University Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study.

### Table 1  PCR primers and conditions

| Gene      | Primer 1                     | Primer 2                     | Annealing Temperature (℃) | Product size (bp) |
|-----------|------------------------------|------------------------------|---------------------------|-------------------|
| Claudin-4 | 5'-TGCCCTTGCTCCACCAACCC-3`  | 5'-CCTCTAAACCCGTCATCCACTC-3` | 55.1                      | 91                |
| β-actin   | 5'-AGTTGCGTTACACCACTTGTGAC-3`| 5'-GCTCGCTCCAACCGACTGC-3`   | 60.0                      | 171               |
RESULTS

Patients

A total of 202 patients were eligible for inclusion in the present study between 2003 and 2006. The patients’ ages ranged from 32-90 years (median: 68 years); 110 patients were male, and 92 were female. Forty-five patients underwent right side colectomy, 65 underwent left side colectomy, and 92 underwent rectal cancer resection. The median follow-up period was 65 months (12-180 months).

The relationship between the clinicopathological factors and claudin-4 expression

The clinicopathological factors were compared between the patients with a high and low claudin-4 expression. In total, eight clinicopathological factors were evaluated. Although a significant difference was observed in the tumor location between the two expression groups, there were no marked differences between the two groups in any of the other clinicopathological parameters (Table 2).

Survival analyses

The OS rates at 3 and 5 years after surgery were 78.2% and 62.5%, respectively, in the patients with a high claudin-4 expression and 89.0% and 83.5%, respectively, in the patients with a low claudin-4 expression (Fig. 2). This result was also statistically significant (p=0.0149). Multivariate analyses showed that the claudin-4 expression status was a significant risk factor for the OS (Table 3 and 4).

DISCUSSION

The aim of the present study was to evaluate the clinical impact of the claudin-4 status in the CRC patients who underwent curative resection. The major of the finding was that the OS of the patients differed significantly based on their claudin-4 status. Our results suggest that the claudin-4 status had a clinical impact on the survival in CRC patients who underwent curative resection. Novel treatments are needed in order to improve the survival of patients with a high claudin-4 expression.

First, we will discuss the differences in the mRNA expression of the claudin-4 gene between CRC tissue and adjacent normal mucosa. The loss of claudins has been associated with tumor genesis. Tight junctions, together with adherens junctions (AJs) and desmosomes, form the apical junctional complex in epithelial and endothelial cellular sheets. They also act to maintain cell polarity and control paracellular permeability as well as create a
Fig. 1  A comparison of the claudin-4 expression between colorectal cancer tissue and adjacent normal mucosa. The claudin-4 expression was higher in the cancer tissue than in the adjacent normal mucosa (P=0.001).

Fig. 2  A comparison of the overall survival in the high and low claudin-4 expression groups.
barrier between the apical and basolateral compartments of the plasma membrane\(^5\)-\(^7\). The suppression of cell-to-cell adhesiveness may trigger the release of cancer cells from primary cancer nets and increase tumor invasiveness. Claudin-4 has been shown to be overexpressed in several cancers. For example, Kominsky et al. reported that the claudin-4 gene expression was higher in breast cancer tissue than in normal mammary epithelial cells\(^12\). Sato et al. reported that the claudin-4 gene was overexpressed in the neoplastic cells of pancreatic cancers and not expressed in normal pancreatic duct showing a high prevalence of hypomethylation in pancreatic cancer cell lines and primary pancreatic carcinomas\(^13\). Mees et al. reported that the tight junction protein claudin-4 was more often overexpressed in CRC tissues than in normal colorectal tissue\(^14\). They evaluated the expression of the tight junctions proteins claudin-1-4, occludin, and ZO-1 and the AJ protein beta-catenin in 16 colectomy specimens and found that the mucosa of the crypts and surfaces of CRC exhibited significantly elevated expression.

### Table 3  Univariate analysis of clinicopathological factors for 5-year overall survival

| Variables/Categories | n   | Hazard ratio | 95% CI   | P-value |
|----------------------|-----|--------------|----------|---------|
| Age                  |     |              |          | 0.582   |
| <65                  | 84  | 1.000        |          |         |
| ≥65                  | 118 | 1.182        | 0.651-2.148 | 0.500   |
| Gender               |     |              |          |         |
| Male                 | 110 | 1.000        |          |         |
| Female               | 92  | 0.815        | 0.451-1.475 | 0.418   |
| Size                 |     |              |          | <0.001  |
| <50mm                | 112 | 1.000        |          |         |
| ≥50mm                | 90  | 3.502        | 1.837-6.675 | 0.011   |
| Histological type    |     |              |          |         |
| Well, Mod            | 174 | 1.000        |          |         |
| Por, Mac             | 28  | 2.410        | 1.220-4.761 | 0.005   |
| Depth of invasion    |     |              |          |         |
| T1, T2               | 50  | 1.000        |          |         |
| T3, T4               | 152 | 17.410       | 2.398-126.396 | 0.159   |
| Location             |     |              |          |         |
| Colon                | 110 | 1.000        |          |         |
| Rectum               | 92  | 1.526        | 0.847-2.747 | 0.418   |
| Lymph node metastasis|     |              |          |         |
| N0-N1                | 151 | 1.000        |          |         |
| N2-N3                | 51  | 1.298        | 0.690-2.444 | 0.037   |
| Lymph vascular invasion| |  | | |
| Absent               | 56  | 1.000        |          |         |
| Present              | 146 | 2.495        | 1.055-5.901 | 0.017   |
| Claudin-4            |     |              |          |         |
| Low                  | 101 | 1.000        |          |         |
| High                 | 101 | 2.122        | 1.141-3.945 |         |

CI: confidence interval, Well: well differentiated, Mod: moderately differentiated, Por: Poorly differentiated, Muc: mucinous

### Table 4  Multivariate analysis of clinicopathological factors for 5-year overall survival

| Variables/Categories | n   | Hazard ratio | 95% CI   | P-value |
|----------------------|-----|--------------|----------|---------|
| Size                 |     |              |          | 0.021   |
| <50mm                | 112 | 1.000        |          |         |
| ≥50mm                | 90  | 2.181        | 1.126-4.225 | 0.079   |
| Histological type    |     |              |          |         |
| Well, Mod            | 174 | 1.000        |          |         |
| Por, Mac             | 28  | 1.862        | 0.930-3.728 | 0.022   |
| Depth of invasion    |     |              |          |         |
| T1, T2               | 50  | 1.000        |          |         |
| T3, T4               | 152 | 10.531       | 1.399-19.267 | 0.080   |
| Lymph vascular invasion| |  | | |
| Absent               | 56  | 1.000        |          |         |
| Present              | 146 | 2.169        | 0.911-5.168 | 0.032   |
| Claudin-4            |     |              |          |         |
| Low                  | 101 | 1.000        |          |         |
| High                 | 101 | 1.984        | 1.060-3.716 |         |

CI: confidence interval, Well: well differentiated, Mod: moderately differentiated, Por: Poorly differentiated, Muc: mucinous
of claudin-1, claudin-3, claudin-4, and beta-catenin compared to intraepithelial neoplasia and normal mucosa. These results were similar to the present study findings. On the other hands, upregulation of some claudin family members has also been reported in other human cancers. It is possible that claudin upregulation could come from two signaling pathways: EGFR and Wnt, both are able to increase claudins expression and are permanently activated in many cancer types, including colorectal cancer\(^\text{18}\).

Second, we will discuss the relationship between the claudin-4 gene expression and the clinicopathological features. We found that the expression of claudin-4 correlated with only the tumor location. However, there have been no reports on the relationship between the claudin-4 gene expression and the clinicopathological features in CRC, although a few reports on other types of malignancies have been published. For example, Matsuda et al. reported that the heterogeneous expression of claudin-4 was detected in advanced gastric cancer, but there was no significant association between the claudin expression and the clinicopathological parameters\(^\text{15}\). Sheehan et al. reported that the expression of claudin-4 was correlated with advanced-stage tumors\(^\text{19}\). Pan et al. found a slight though insignificant trend towards positive associations of claudin-4 levels with the tumor grade and disease stage in patients with endometrial carcinoma\(^\text{17}\). Further studies should focus on this issue.

Third, we will discuss the relationship between the claudin-4 gene expression and the outcomes of CRC. In our study, a high claudin-4 gene expression was associated with a poorer five-year OS than a low claudin-4 gene expression in patients with CRC. In a univariate Cox regression analysis, a higher claudin-4 gene expression was a significant predictor of the five-year OS in these patients. Although there have been no reports about the relationship between the claudin-4 gene expression and the survival in CRC, a few reports on other types of malignancies have been published. Lanigan et al. reported that high levels of claudin-4 protein were associated with adverse outcomes in breast cancer patients\(^\text{16}\). Lechpammer et al. reported that a moderate to strong claudin-4 expression was also associated with a decreased survival in patients with renal clear cell carcinoma\(^\text{17}\). Szász et al. reported that the expression of claudin-4 could be used to distinguish between prostate cancer patients who had metastases and those who did not\(^\text{20}\). Although the exact mechanism underlying this finding is unclear, the following is speculated: Claudin-4 is a receptor for Clostridium perfringens enterotoxin (CPE), and studies in other cancer types have shown that CPE may be effective against tumor cells that are highly resistant to chemotherapy. Furthermore, Santin et al. reported that CPE may have potential as a novel treatment for either chemotherapy-resistant or recurrent ovarian cancer\(^\text{21}\), and in uterine cancer, they reported that claudin-4 receptors may offer promising targets for the use of CPE as a novel type-specific therapy against this highly aggressive and chemotherapy-resistant variant of endometrial cancer\(^\text{22}\). If claudin-4 does prove to be a useful CRC biomarker, then the existence of a ready-made target treatment would be invaluable for future chemotherapy.

Special attention is required when interpreting the current results, as the present study is associated with several potential limitations. First, the present study was a retrospective analysis performed with a small sample size. We cannot deny the possibility that our findings were observed by chance. Second, there was some selection bias in the patients in this series. Third, the evaluation method of the claudin-4 expression was not standardized. The optimal claudin-4 cut-off value has not yet been elucidated. Therefore, future studies should be carried out to determine the optimal cut-off value of claudin-4 in CRC. In addition, we did not investigate the expression using western blot or immunohistochemistry in the present study. Because the clinical impacts of the claudin-4 was unclear in CRC when we conducted the present study. Further study should be focus and use the expression using western blot or immunohistochemistry of claudin-4 in CRC. Given these limitations, the results must be confirmed in another cohort or in a prospective multicenter-study.

In conclusion, the claudin-4 gene expression was higher in cancer tissue than in normal adjacent mucosa in patients with CRC, and a high expression of this gene was significantly related to a poor outcome. Our findings suggest that the overexpression of the claudin-4 gene is a useful predictor of the outcome in patients with CRC.

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Availability of data and materials
The datasets analyzed during the current study are not publicly available due to including patients’ personal information.

Authors’ Contributions
HT, OT and TA: collected all references and wrote the draft. HT, TO, TA, KY, MN, MS, CK, MM, YR: collected all data of the clinical. HT and TO: offered the conception and design, revised and discussed the meaning of the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate
The Ethics Committees of Yokohama City University Medical Center and Kanagawa Cancer Center approved the protocol before initiation of the study.

Patient consent for publication
Voluntary written consent was obtained from all patients.
Competing interests
The authors declare no competing interests in association with the present study.

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