Histological differences between preoperative chemoradiotherapy and chemotherapy for rectal cancer: a clinicopathological study

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Pathological studies on the different histological effects between neoadjuvant chemotherapy (NAC) and preoperative chemoradiation therapy (preoperative CRT) have not been performed. The purpose of this study is to elucidate the histological differences in tissue received from NAC and preoperative CRT for rectal cancer to evaluate whether a pathological assessment method used after CRT can be applied for NAC. One hundred and thirty-eight patients were enrolled in this study; 88 patients underwent their operations after preoperative CRT or NAC, and 50 patients underwent surgery only. Residual tumor area was measured using morphometry software and we compared the stromal component of myofibroblasts, immune cells, and vasculature to elucidate the difference of therapeutic effect between them. The grade of reduction after preoperative CRT was more prominent than that seen in NAC. Also, ypT downstaging was more prominent in preoperative CRT than in NAC, and ypN downstaging was more frequent in NAC than in preoperative CRT. Preoperative CRT showed more marked myofibroblasts and fewer immune cells than did NAC, which indicates different effects on the cancer microenvironment. Our histological results suggest different effects between NAC and preoperative CRT on tumor tissue. The best assessment method available for a variable therapeutic protocol should be further investigated.

Key words: neoadjuvant chemotherapy, preoperative chemoradiotherapy, rectal cancer

Although preoperative CRT improves local tumor control, it is reported to induce postoperative anal dysfunction. Therefore, neoadjuvant chemotherapy (NAC) without radiation therapy can be another treatment that may result in better anal function.4–6 The tumor-reducing effect is found even with NAC and it may preserve better anal function.7,8 Currently, various pathological assessment methods have been reported for those receiving preoperative CRT, but they are not standardized.9 Furthermore, the utility of the assessment method after preoperative treatment has been evaluated only in those patients receiving preoperative CRT. In addition, so far there are no studies that compared the histopathological features of tissue from those who received NAC and those who received preoperative CRT. A histological comparison between NAC and CRT may allow us to estimate the validity of adopting for NAC the same pathological assessment method currently used after preoperative CRT. Biological differences in the therapeutic effect may also be elucidated.

In this study, we compared the histological differences of the cancer tissue that received either NAC or preoperative CRT to estimate the phenomenon due to the therapeutic differences. In addition to the histological features, the area of residual tumor (ART) and stromal features of the residual tumor tissue that received each treatment were compared to elucidate the different biological effects between NAC and preoperative CRT.10

MATERIAL AND METHODS

Patients, tumors, and treatment characteristics

From January 2001 to April 2014, a total of 2184 patients underwent surgery for rectal cancer at the National Cancer Center Hospital East, Chiba, Japan. Of these, 44 patients underwent preoperative CRT (5-fluorouracil and radiation with a total dose of 45 Gy in 25 fractions) before surgery and surgical resection was performed 4–6 weeks after the completion of the treatment.
Another 44 patients received NAC (FOLFOX was given in six courses) before undergoing surgery scheduled during the 4–8 weeks after the completion of treatment. Fifty age- and sex-matched patients who did not receive preoperative therapy were used as a control group. Preoperative CRT was used from 2001 to 2006, and the NAC and surgery only treatment was used from 2010 to 2014.

Histological assessment

The preoperative clinical staging was performed using the classification of UICC 7th edition.

All resected surgical specimens were fixed in 10% formalin. Tumor tissue was longitudinal sliced serially in 5mm intervals and embedded in paraffin. Sections of 4-μm thick were cut from paraffin blocks, stained with HE, and were evaluated independently by two authors (M.K. and N.S.) who were unaware of the clinical findings. Discrepancies between their findings were resolved by discussion. The residual tumor was pathologically staged according to the UICC 7th classification. In the present study, both reduction of pathological T stage (ypT) from clinical T stage, and that of pathological N stage from clinical N stage (ypN) were regarded as downstaging. Histological tumor regression grade was semiquantitatively evaluated according to the method described by Dworak et al., which is as follows: Grade 1, dominant tumor mass with obvious fibrosis and/or vasculopathy; Grade 2, dominantly fibrotic changes with few tumor cells or groups (easy to find); Grade 3, very few (difficult to find microscopically) tumor cells in fibrotic tissue with or without mucous substance; and Grade 4, no tumor cells, only fibrotic mass (total regression or response).11

All tumors were examined for vascular, lymphovascular, and perineural invasion. To assess the histological alteration after therapy, we first evaluated the presence or absence of mucus lakes in the tumor.12,13 Cases in which the mucus lake constituted less than 10% of the entire tumor area were assessed as Grade A. Grades B and C reflected mucus lakes of 10%–30% and >30%, respectively, of the tumor area. Tumor budding was defined as an isolated single cancer cell or a cluster composed of fewer than five cancer cells. After choosing one field where budding was the most intensive, a budding count was made in the field measuring 0.785 mm² using a ×20 objective lens. A field with five or more buds was viewed as positive.14

Tumor differentiation in the initial biopsy specimen before preoperative treatment was reviewed and classified as low-grade (low differentiated) or high-grade (well to moderately differentiated) adenocarcinomas, or no grade if prominent tumor regression disturbed accurate histological evaluation (ie, prominent colloid formation).12

The fibrosis degree of the primary tumor was evaluated with a 4-point scale. Grade 0, 1, 2, and 3 reflected <10%, 10%–<25%, 25%–50% and >50% replacement of tumor tissue by fibrosis, respectively. Other histological features of acidophilic degeneration of cytoplasm and calcification were also evaluated.12,13,15

Measurement of the area of residual tumor (ART)

Hematoxylin and eosin (HE) stained slides from the maximum slice of the tumor were photographed using a NanoZoomer Digital Pathology Virtual Slide Viewer (Hamamatsu Photonics, Hamamatsu, Japan) and were used for morphometric analysis. The depth of tumor invasion beyond the muscular layer was measured between the inferior margin of the muscular layer and the outermost portion of the tumor. In those cases where the muscular layer had been destroyed or replaced by fibrosis, the shortest line between the residual muscular layers was drawn on the picture and the distance between the line and outermost portion of the tumor was measured.

We performed morphometric measurements of the area of residual tumor (ART) within the muscular layer (WM-ART) and beyond the muscular layer with perirectal adipose tissue (BM-ART), and calculated a total (T-ART) using tumor slices of the largest residual tumor. ART was measured using viewer software, and mucus lakes were excluded from the ART. All tumor nests >0.1 mm² were measured for ART. Inside the inferior margin of the muscular layer of ART was defined as WM-ART, and outside the inferior margin was defined as BM-ART. If the muscular layer was broken by inflammation, necrotic tissue, or fibrosis, a connecting line between the residual tumor muscular layers was drawn on the picture to discriminate WM-ART and BM-ART (Figure 1).10 Mucosa showing ulceration, inflammation, necrosis, or adenoma components was excluded from ART.

Histochemical and immunohistochemical study of the stromal component

Representative formalin-fixed, paraffin-embedded specimens obtained from a rectal cancer were cut into 3-μm-thick serial sections. The sections were stained with HE, azan-mallory (azan), and for immunohistochemical analysis, with α-smooth muscle actin (α-SMA), CD3, CD20, CD31, and CD68. Automated immunohistochemical staining was performed by using a Ventana Benchmark ULTRA (Ventana Medical Systems, Tucson, AZ, USA). Monoclonal anti-human α-SMA antibody (Dako, Glostrup, Denmark) was used at a dilution of 1:100, and the conditions for antigen retrieval and primary antibody incubation were set at 91°C for 8 minutes and 35°C for 60 minutes, respectively. Anti-human CD31 antibody (Dako) was used at a dilution of 1:200. Antigen retrieval and primary antibody incubation were performed at 95°C for 8 minutes and 35°C for 60 minutes, respectively. Monoclonal anti-rabbit CD3, anti-mouse CD20, and anti-mouse CD68 antibody (Dako) were used and the conditions for antigen
retrieval and primary antibody incubation were set at 95°C for 8 minutes and 35°C for 64 minutes, respectively. The slides were photographed by using a NanoZoomer Digital Pathology Virtual Slide Viewer system and were subjected to morphometric analysis.

We chose three hot spots from the WM-tumor-area and BM-tumor-area and six points in total were used for the evaluation of the immunohistochemical slides. The azan-positive areas and α-SMA-positive areas were calculated using the tracing algorithm of the WinROOF version 6.5 software (Mitani Corporation, Tokyo, Japan). The azan-positive areas and α-SMA-positive areas in ×40 pictures were taken and calculated, using each color-detecting algorithm of the software. The numbers of CD31-positive vessels in ×20 pictures were counted manually. The numbers of CD3 (T cell), CD20 (B cell), and CD68 (macrophage) positive cells were counted manually at a magnification of ×40.

The azan-positive ratios and the α-SMA-positive ratios in ×40 pictures were also calculated. The histological analyses of the morphometric analysis of α-SMA and azan-positive areas are shown in Figure 2. One investigator (N.S.) carried out all histological analyses under the supervision of an experienced pathologist (M.K.).

Statistical analysis

The associations between ART and the histopathological and immunohistochemical features were evaluated using the t-test. All calculated P values were 2-sided, and P < 0.05 was considered statistically significant. All statistical analyses were performed using the SPSS Statistics version 22.0 software (IBM SPSS Statistics, Armonk, NY, USA).

RESULTS

Clinicopathological characteristics

The clinicopathological characteristics of the 138 patients are shown in Table 1. There were no significant differences in age or sex among those in the NAC, preoperative CRT, and control groups. The numbers of clinical/pathological stage IV tumors in the NAC group were higher than that seen in the other two groups. All patients in the CRT group underwent intersphincteric resection (ISR). The NAC and control groups included cases with other operative procedures, including abdominoperineal resection (APR) and low anterior resection (LAR).

Downstaging

Forty-four patients who received NAC were administered FOLFOX for six cycles and the rate of downstaging was 59.1%. The ypT and ypN downstaging rates were 25% and 59.1%, respectively, and four lesions (9.1%) in the NAC group were diagnosed as having a complete response for grade of regression.11 However, in the 44 patients that received preoperative CRT, the downstaging rate was 52.3%. The ypT and ypN downstaging rates were 47.7% and 20.5%, respectively. Dworak regression Grade 3 and 4 in the CRT group was more significant than that seen in the NAC group (NAC, 8 of 44 cases (18.2%); CRT, 24 of 44 cases (54.5%); P < 0.05), and the regression grade of primary tumors was also different between NAC and CRT.

Nine lesions (20.5%) in the CRT group were diagnosed as having a complete response for Dworak grade of regression. The ypT downstaging was less and ypN downstaging was more...
frequent in the NAC group than that of the preoperative CRT group, and the pattern of downstaging was found to be different between the NAC and preoperative CRT groups (P < 0.05) (Figure 3).

**Table 1** Patient characteristics.

|                     | NAC group (n=44) | CRT group (n=44) | control (n=50) |
|---------------------|------------------|------------------|----------------|
| Male                | 30               | 32               | 30             |
| Female              | 14               | 12               | 20             |
| Median age (range)  | 57.4 (28-76)     | 56 (27-77)       | 61 (35-86)     |
| Median AV (cm) (range) | 4.0 (0.0-6.0)   | 3.3 (0.0-5.0)   | 2.5 (0.0-5.0)  |
| Operative procedure (%) | 34 (77.2)       | 44 (100)         | 37 (74)        |
| ISR                 | 10 (22.8)        | 0 (0)            | 13 (26)        |
| cT (0/1/2/3/4)      | 0/0/0/3/1/6      | 0/0/9/35/0       | 0/0/63/11      |
| cN (0/1/2/3/4)      | 5/16/7/11/6/0    | 27/10/6/1/0      | 26/20/2/0      |
| pT (0/1/2/3/4)      | 4/21/0/23/5      | 9/1/12/2/0       | 0/0/4/15/5     |
| pN (0/1/2/3/4)      | 23/10/3/8/0      | 29/8/7/0/0       | 29/13/2/4/3    |
| Clinical stage (0/I/IIA/IIIB/IV) | 0/0/5/1/4/21/4 | 0/6/19/9/10/0  | 0/0/21/12/8/0  |
| Pathology stage (0/I/IIA/IIIB/IV) | 4/8/10/8/11/3   | 6/13/11/5/9/0   | 0/2/25/14/9/0  |
| Tumor down staging (UICC)(%) | Present | 26 (59.1) | 23 (52.3) | - |
|                      | Absent           | 18 (40.9)       | 21 (47.7)      | - |
| Dworak grade of regression (0/1/2/3/4) | 0/1/25/3/5 | 0/3/17/15/9 | - |
| 3/4(%)               | 8 (18.2)         | 24 (54.5)       | -              |

ISR, intersphincteric resection; cT, clinical T stage; cN, clinical lymph node metastasis; ypT, pathological T stage; ypN, pathological lymph node metastasis.

**Histopathological features**

The histopathological features of preoperative CRT, NAC, and the control group are shown in Table 2. Tumor differentiation...
was not different in each group. Budding grade tended to be higher in the CRT group than that seen in the NAC group, but was not statistically significant.

Fibrosis Grade 3 was observed in 26/44 (59.1%) of cases in the preoperative CRT group, whereas that accounted for 3/50 (6.0%) of cases in the control group, and 3/44 (6.8%) of cases in the NAC group. There was a significantly higher fibrosis rate in the preoperative CRT group, compared with results for the NAC and control groups (Table 2) \( (P < 0.05) \). The NAC group also showed a significantly higher fibrosis rate than that seen in the control group \( (P < 0.05) \). Next, the NAC group had a higher lymphovascular invasion rate than that seen in the preoperative CRT group.

**Table 2** Histological features.

|                | NAC (n=44) | CRT (n=44) | Control (n=50) | \( P \) NAC vs CRT | \( P \) NAC vs control | \( P \) CRT vs control |
|----------------|-----------|------------|----------------|--------------------|------------------------|------------------------|
| Ly             | 22 (50%)  | 5 (11.4%)  | 29 (58%)       | \(<0.05^{*}\)       | 0.57                   | \(<0.05^{*}\)          |
| V              | 23 (52.3%)| 19 (43.2%) | 39 (78%)       | 0.4                | \(<0.05^{*}\)          | 0.29                   |
| PN             | 15 (34.1%)| 13 (29.5%) | 20 (40%)       | 0.65               | 0.56                   | 0.16                   |
| Acidophilic degeneration of cytoplasm | 1 (2.3%) | 5 (11.4%) | 0 (0%)         | 0.56 | 0.32 | 0.16 |
| Calcification  | 0 (0%)    | 1 (2.3%)   | 2 (4%)         | 0.32               | \( p=0.40\)            | 0.32                   |
| Mucus lake     |           |            |                |                    |                        |                        |
| (Grade)        | A: 5 (11.4%) | A: 5 (11.4%) | A: 0 (0%) | 0.28 | 0.25 | 0.15 |
|                | B: 1 (2.3%) | B: 3 (6.8%) | B: 3 (6%)     |                    |                        |                        |
|                | C: 2 (4.5%) |            |                |                    |                        |                        |
| Present        | 8          | 8          | 3              | 0.28               | 0.25                   | 0.15                   |
| Absent         | 36         | 36         | 47             |                    |                        |                        |
| Tumor differentiation (initial histological) | | | | | | |
| Low-grade      | 2          | 2          | 3              |                    |                        |                        |
| High-grade     | 40         | 42         | 45             |                    |                        |                        |
| Not grade      | 2          | 0          | 2              |                    |                        |                        |
| Budding grade  |           |            |                |                    |                        |                        |
| -              | 34         | 37         | 30             | 0.43               | \(<0.05^{*}\)          | 0.07                   |
| +              | 10         | 7          | 20             |                    |                        |                        |
| Fibrosis grade | 012 (0–50%)| 41         | 18             | 47                 | \(<0.05^{*}\)          | 0.76                   |
| 3 (>50%)       | 3          | 26         | 3              |                    |                        | \(<0.05^{*}\)          |

\( ^{*}P < 0.05 \). Ly, lymphovascular invasion; V, vein invasion; PN, perineural invasion.

**ART and depth**

The ART and depth of the tumor in the preoperative CRT, NAC, and control group are shown in Figure 4. The NAC group and preoperative CRT group showed smaller ARTs (T, WM, and BM-ART) than those seen in the controls \( (P < 0.05) \). The NAC group and preoperative CRT group showed more shallow tumor depths than those seen in the control group \( (P < 0.05) \). Although there was no statistical difference in WM-ART between the NAC and preoperative CRT groups, the preoperative CRT group showed the smallest T-ART and BM-ART, and shortest depth of tumor invasion (Fig. 4a). These results suggested that preoperative CRT has a more robust...
effect on total tumor regression than does NAC, and that preoperative CRT seemed to effect predominantly the tumor area beyond the muscular layer (Fig. 4b).

**Histochemical and immunohistochemical features**

Immunohistochemical features are shown in Table 3. CD3 positive T lymphocytes and CD20 positive B lymphocytes distributed more predominantly in the order of the control, NAC, and preoperative CRT group. All differences among them were statistically significant (P < 0.001). The azan-positive area was prominent in the order of the preoperative CRT, NAC, and control group. All differences among them were also statistically significant (P < 0.001). The preoperative CRT group showed significantly (P < 0.001) fewer CD31 positive vessels than those seen in the NAC and control group. These results were not affected by the tumor location of the WM-tumor area and BM-tumor area. The α-SMA expression in the WM-tumor area was more prominent in the order of the control, NAC, and preoperative CRT groups. However, the α-SMA expression in the BM-tumor area was more predominant in the order of the CRT, control, and NAC group. The difference between the NAC and preoperative CRT group was statistically significant. This result suggested that not only the amount of expression, but also the distribution of the α-SMA was different between the NAC and preoperative CRT groups. Similarly, CD68 positive cells in the WM-tumor area were more prominent in the order of the preoperative CRT, control, and NAC group. The differences between the NAC and preoperative CRT group (P < 0.001), and between the NAC and control group were statistically significant (P = 0.006). However, CD68 positive cells in the BM-tumor area were more predominant in the order of the control, NAC, and preoperative CRT group. All differences among them were statistically significant. Not only the number of CD68 positive cells, but also their distribution were different between the NAC and preoperative CRT groups. The cancer microenvironment was thought to be heterogeneous within one tumor, but our result revealed that NAC and preoperative CRT altered the quality and distribution of cancer microenvironment.

**DISCUSSION**

In this study, we compared the clinicopathological characteristics of tumors with the effect of preoperative CRT or NAC in rectal cancer. Detailed analysis using morphometry...
and immunostaining area was also performed. Our study revealed marked clinicopathological differences between preoperative CRT and NAC. There was a more particular effect on ypT from preoperative CRT and on ypN from NAC. This result was reflected by different systemic effects between preoperative CRT and NAC. It might be thought that the influence of CRT is limited only to local tissue, that is, tumor tissue and the lymph nodes around the tumor, while NAC might be effective both for tumor tissue and distant lymph node metastasis.

Next, our result revealed that different therapies give a histologically different effect on the primary tumor. In addition to more a prominent effect on ART, preoperative CRT more preferably affected BM-ART. These results suggested that not only the amount, but also the distribution of the residual tumor is affected by the type of the therapy. Furthermore, the amount and the distribution of fibrosis, and the vascular and immune cell population density of tissues, are different between preoperative CRT and NAC. Therefore, different therapies give a different effect on the cancer microenvironment. The cancer microenvironment consists of fibroblasts, vascular and immune cells, and constitutive cells. Our results suggest the effect on the cancer microenvironment is dependent on the variety of therapy.

Fibrosis has been reported as a basic histological feature after preoperative CRT; we also found marked fibrosis in patients who received preoperative CRT. In addition, we found fibrosis is also influenced by the type of therapy. Recently, some drugs have been reported to disrupt cancer stroma, and fibrosis may not be a common feature to all preoperative therapy.

As for vasculature, preoperative CRT showed fewer CD31 positive vessels, which may suggest powerful suppression of angiogenesis. Gao et al. reported that there are many vessels in the surface area of colorectal tumors. In our study, the preoperative CRT may have inhibited angiogenesis predominantly in the surface area of the tumor. As for immune cells, patients who received preoperative CRT showed significantly fewer T and B lymphocytes than those in any of the other groups and the reduction rate of ART was larger than that seen in any other group. Immune cells have been reported to be associated with postoperative convalescence and clinical outcome. Reduction of immune cell infiltration in patients who received preoperative CRT was also reported. In addition, our results revealed that the degree of immune cell suppression and distribution in the tumor was dependent on the therapeutic protocol. Immune cells are an important element of the tumor microenvironment. A recent study revealed that immune cells in the tumor microenvironment orchestrate with other stromal components, including fibroblast and vascular component cells, to accelerate tumor progression. We found that preoperative CRT and NAC reduce ART. However, the effect for the tumor in NAC may be different from that seen after preoperative CRT, which can be

|                  | NAC       | CRT       | Control  | P value (NAC vs CRT) | P value (CRT vs Control) | P value (NAC vs Control) |
|------------------|-----------|-----------|----------|----------------------|--------------------------|--------------------------|
| Azan-positive WM ratio % | 41.50 ± 13.95 | 50.60 ± 14.76 | 33.24 ± 9.45 | <0.001** | <0.001** | <0.001** |
| Azan-positive BM ratio % | 50.30 ± 6.87 | 55.41 ± 11.68 | 36.86 ± 9.53 | <0.001** | <0.001** | <0.001** |
| α-SMA-positive WM ratio % | 14.90 ± 8.17 | 11.56 ± 7.45 | 20.03 ± 6.79 | <0.001** | =0.010* | <0.001** |
| α-SMA-positive BM ratio % | 13.00 ± 7.25 | 15.29 ± 6.65 | 14.75 ± 5.98 | =0.024* | 0.05 | 0.526 |
| Vessel (CD31) WM density/×20 | 42.41 ± 18.93 | 29.39 ± 13.13 | 38.03 ± 18.06 | <0.001** | <0.001** | =0.048** |
| Vessel (CD31) BM density/×20 | 33.11 ± 13.81 | 30.81 ± 16.65 | 33.28 ± 13.79 | <0.027* | 0.015* | 0.916 |
| Macrophage (CD68) WM density/×40 | 40.29 ± 14.73 | 50.51 ± 22.02 | 46.39 ± 21.37 | <0.001** | 0.109 | =0.006* |
| Macrophage (CD68) BM density/×40 | 49.04 ± 16.80 | 36.17 ± 17.59 | 76.07 ± 25.30 | <0.001** | <0.001** | <0.001** |
| T cell (CD3) WM density/×40 | 89.81 ± 50.32 | 73.41 ± 36.78 | 101.01 ± 51.60 | =0.002* | <0.001** | 0.071 |
| T cell (CD3) BM density/×40 | 90.97 ± 45.28 | 59.75 ± 34.80 | 113.95 ± 55.69 | <0.001** | <0.001** | <0.001** |
| B cell (CD20) WM density/×40 | 54.33 ± 71.49 | 12.98 ± 22.12 | 71.79 ± 99.48 | <0.001** | <0.001** | 0.09 |
| B cell (CD20) BM density/×40 | 45.17 ± 52.57 | 24.02 ± 43.32 | 87.06 ± 100.78 | <0.001** | <0.001** | <0.001** |

*P < 0.05  
**P < 0.001; α-SMA, α-smooth muscle actin.
dependent on the different biological mechanism induced by each different therapeutic protocol.

Finally, histological tumor regression grade after preoperative CRT is represented by fibrosis and residual tumor, which contribute to the patient’s prognosis. Our results of therapeutic-protocol-dependent tumor histology seemed to suggest a question of whether regression grade after preoperative CRT can be applied for other therapeutic protocols. Preoperative CRT has been reported to induce severe anal dysfunction, and NAC can be an alternative strategy that preserves better postoperative anal function. However, fibrosis, a histological feature effect on regression grade for CRT, is dependent on the therapeutic protocol. Therefore, the histological assessment method used for preoperative CRT may not be acceptable when applied for another therapeutic protocols, and its utility should be confirmed in detail.

As for limitations in this study, the number of cases is small. Because this study did not follow up the patients for many years, a comparison of the correlation between convalescence and the preoperative treatment method was impossible. ART in BM-AKT results may be associated with prognosis for preoperative CRT, but the cases used in this study do not have a long enough follow up time to search for a prognostic marker; it will be necessary to investigate any possible correlation with convalescence in the future.

In conclusion, the systemic effects of preoperative CRT and NAC are different. Moreover, the histological features of the tumor after preoperative CRT and NAC are much different. ART and fibrosis are affected by the different preoperative therapies, and the utility of application of the assessment method for CRT for other treatments should be carefully investigated.

DISCLOSURE STATEMENT

None declared.

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