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

Naoki Sakuyama[1,3], Motohiro Kojima[2], Shingo Kawano[1,3], Tetsuo Akimoto[3], Norio Saito[1], Masaaki Ito[1], Atsushi Ochiai[2]

Division of Colorectal Surgery[1] and Pathology[2], National Cancer Center Hospital East, Chiba, Japan, and Juntendo University Graduate School of Medicine[3], Advanced Clinical Research of Cancer, Tokyo, Japan;

Address correspondence and reprint requests to:

Atsushi Ochiai: MD

Division of Pathology, National Cancer Center Hospital East, Kashiwa, Japan;

Tel: +81-4-7133-1111

FAX: +81-4-7131-9960

E-mail: aochiai@east.ncc.go.jp

Division of Pathology, National Cancer Center Hospital East, 6-5-1 Kashiwanoha, Kashiwa City, 277-8577, Chiba, Japan.

Short title: **Histopathology in rectal cancer treatment**

Manuscript word count: **3381 words**

ABSTRACT

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 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.

Keywords: rectal cancer, preoperative chemoradiotherapy, neoadjuvant chemotherapy

INTRODUCTION

Standard treatment in rectal cancer is surgical resection concomitant with preoperative chemoradiation therapy (CRT). [1] [2] [3] 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] [5] [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 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-fluorauracil 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 6 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. All resected surgical specimens were fixed in 10% formalin. Tumor tissue was longitudinal sliced serially in 5mminterval and embedded in paraffin. . Four-μ m section from paraffin blocks were stained by HE, and were evaluated independently by 2 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. 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 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 firstly 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 5 cancer cells. After choosing one field where budding was the most intensive, a budding count was made in the field measuring 0.785 mm^2 using a ×20 objective lends. A field with 5 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 mm^2 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

138 Systems, Tucson, AZ, USA). Monoclonal anti-human α-SMA antibody (Dako, Glostrup,
139 Denmark) was used at a dilution of 1:100, and the conditions for antigen retrieval and
140 primary antibody incubation were set at 91°C for 8 minutes and 35°C for 60 minutes,
141 respectively. Anti-human CD31 antibody (Dako, Glostrup, Denmark) was used at a
142 dilution of 1:200. Antigen retrieval and primary antibody incubation were performed at
143 95°C for 8 minutes and 35°C for 60 minutes, respectively. Monoclonal anti-rabbit CD3,
144 anti-mouse CD20, and anti-mouse CD68 antibody (Dako, Glostrup, Denmark) were
145 used and the conditions for antigen retrieval and primary antibody incubation were set
146 at 95°C for 8 minutes and 35°C for 64 minutes, respectively. The slides were
147 photographed by using a NanoZoomer Digital Pathology Virtual Slide Viewer system
148 and were subjected to morphometric analysis.
149 We chose 3 hot spots from the WM-tumor-area and BM-tumor-area and 6 points in total
150 were used for the evaluation of the immunohistochemical slides. The azan-positive
151 areas and α-SMA-positive areas were calculated using the tracing algorithm of the
152 WinROOF version 6.5 software (Mitani Corporation, Tokyo, Japan). The azan-positive
153 areas and α-SMA-positive areas in ×40 pictures were taken and calculated, using each
154 color-detecting algorithm of the software. [16] The numbers of CD31-positive vessels in
155 ×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). [16]

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).

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 2 groups. All patients in the CRT

group underwent intersphinteric 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 6 cycles and the rate of downstaging was 59.1%. The ypT and ypN downstaging rates were 25% and 59.1%, respectively, and 4 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).

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.

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 (Figure 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 (Figure 4b).

Histochemical and immunohistochemical features

Immunochemical 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.. ~~On the other hand, the difference between NAC and control group was not statistically significant. ($P < 0.05$)~~ 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.

DISCCUSION

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.[17] 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. [18]

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. [19] 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. [20] [21] [22] [23] Reduction of immune cell infiltration in patients who received preoperative CRT was also reported. [24] 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. [24] 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 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.[7] 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-ART results may be associated with prognosis for preoperative CRT [10], 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

No author has a conflict of interest to disclose.

FIGURE LEGENDS:

Figure 1 (**a**) Low magnification view of hematoxylin and eosin (HE)-stained section. (**b**) Morphometric analysis used NanoZoomer Digital Pathology. (**a**) Area of residual tumor (ART) was measured by tracing the outline of the tumor nests (black line). When the size of the tumor was larger than 32 mm, we separated the slide and measured size. The border between the ART within the muscular layer (WM-ART) and the ART beyond the muscular layer (BM-ART) was measured by machine. The WM-ART was determined as the ART inside the inferior margin of the muscular layer, and BM-ART was measured as the ART outside the inferior margin of the muscular layer. If the muscular layer had not been identified or was replaced by inflammation, necrosis, and fibrosis, a connecting line between the muscular layers was drawn on the picture. In those cases, the area inside the line was measured as WM-ART and the area outside the line was measured as BM-ART. The total ART consisted of both areas.

Figure 2 Histological evaluation of the immunostaining of rectal tumors.

(a) Examples of immunostained CD3+ T cells (brown) are shown. (b) CD20+ B cell (brown) are shown. (c) Blood vessels are stained by CD31. The number of vessels was counted manually as CD31-immunopositive luminal structures detectable at a magnification of ×20. (d) CD68+ macrophages are shown. The number of various positive cells was counted manually as CD3 (T cell), CD20 (B cell), and CD68 (macrophage) detectable at a magnification of ×40 on the hot spot. (e, f) The azan positive area was shown with the visualized area stained aniline blue. (e) *Bright green* in this image was identified using the color-detecting algorithm of the Winroof Version 6.5 software (Mitani Corporation, Tokyo, Japan). (f) (g, h) The α-SMA positive area (g) was identified as *bright green* using the color-detecting algorithm of the software (h).

Figure 3 Pattern of downstaging differences between the NAC, preoperative CRT, and control groups. Ratio of Down T and Down N with each treatment. * $P < 0.05$

Figure 4 Patient ART and depth.

(a) T-ART, total area of residual tumor; WM-ART, within muscular layer area of residual tumor; BM-ART, beyond muscular layer area of residual tumor.

(b) Evaluation of area of residual tumor (ART) and depth compared to the ratio with

349 100% in control group.

350 * $P < 0.05$

351

352 **Table 1** Patient characteristics.

353 ISR, intersphinteric resection; cT, clinical T stage; cN, clinical lymph node metastasis;

354 ypT, pathological T stage; ypN, pathological lymph node metastasis.

355

356 **Table 2** Histological features.

357 Ly, lymphovascular invasion; V, vein invasion; PN, perineural invasion

358 *$P < 0.05$

359

360 **Table 3** Immunohistochemical features.

361 α-SMA, α-smooth muscle actin

362 *$P < 0.05$ **$P < 0.001$

363

364

365

366

REFERENCES

1.Bosset JF, Calais G, Daban A, et al. Preoperative chemoradiotherapy versus preoperative radiotherapy in rectal cancer patients: assessment of acute toxicity and treatment compliance. Report of the 22921 randomised trial conducted by the EORTC Radiotherapy Group. *Eur J Cancer*. 2004;40(2):219-24.

2.Sauer R, Becker H, Hohenberger W, et al. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med*. 2004;351(17):1731-40.

3.Sebag-Montefiore D, Stephens RJ, Steele R, et al. Preoperative radiotherapy versus selective postoperative chemoradiotherapy in patients with rectal cancer (MRC CR07 and NCIC-CTG C016): a multicentre, randomised trial. *Lancet*. 2009;373(9666):811-20.

4.Rullier E, Zerbib F, Laurent C, et al. Intersphincteric resection with excision of internal anal sphincter for conservative treatment of very low rectal cancer. *Diseases of the Colon & Rectum.* 1999;42(9):1168-75.

5.Kohler A, Athanasiadis S, Ommer A, et al. Long-term results of low anterior resection with intersphincteric anastomosis in carcinoma of the lower one-third of the rectum: analysis of 31 patients. *Dis Colon Rectum*. 2000;43(6):843-50.

6.Nishizawa Y, Saito N, Fujii S, et al. Association between anal function and therapeutic effect after preoperative chemoradiotherapy followed by intersphincteric resection.

386 *Digestive surgery*. 2012;29(5):439-45.

387 7.Nishizawa Y, Fujii S, Saito N, Ito M, et al. Differences in tissue degeneration between

388 preoperative chemotherapy and preoperative chemoradiotherapy for colorectal cancer.

389 *Int J Colorectal Dis*. 2012;27(8):1047-53.

390 8.Chau I, Brown G, Cunningham D, et al. Neoadjuvant capecitabine and oxaliplatin

391 followed by synchronous chemoradiation and total mesorectal excision in magnetic

392 resonance imaging-defined poor-risk rectal cancer. *J Clin Oncol.* 2006;24(4):668-74.

393 9.Trakarnsanga A, Gonen M, Shia J, et al. Comparison of tumor regression grade

394 systems for locally advanced rectal cancer after multimodality treatment. *J Natl Cancer*

395 *Inst.* 2014;106(10):1-6.

396 10.Kojima M, Ishii G, Yamane Y, et al. Area of residual tumor beyond the muscular

397 layer is a useful predictor of outcome in rectal cancer patients who receive preoperative

398 chemoradiotherapy. *Pathol Int*. 2009;59(12):857-62.

399 11.Dworak O, Keilholz L, Hoffmann A. Pathological features of rectal cancer after

400 preoperative radiochemotherapy. *Int J Colorectal Dis*. 1997;12(1):19-23.

401 12.Rullier A, Laurent C, Vendrely V, et al. Impact of colloid response on survival after

402 preoperative radiotherapy in locally advanced rectal carcinoma. *Am J Surg Pathol.*

403 2005;29(5):602-6.

13.Shia J, McManus M, Guillem JG, et al. Significance of acellular mucin pools in rectal carcinoma after neoadjuvant chemoradiotherapy. *Am J Surg Pathol.* 2011;35(1):127-34.

14.Ueno H, Murphy J, Jass JR, et al. Tumour 'budding' as an index to estimate the potential of aggressiveness in rectal cancer. *Histopathology*. 2002;40(2):127-32.

15.Shia J, Guillem JG, Moore HG, et al. Patterns of morphologic alteration in residual rectal carcinoma following preoperative chemoradiation and their association with long-term outcome. *American Journal of Surgical Pathology.* 2004;28(2):215-23.

16.Sugimoto M, Takahashi S, Kojima M, et al. What is the nature of pancreatic consistency? Assessment of the elastic modulus of the pancreas and comparison with tactile sensation, histology, and occurrence of postoperative pancreatic fistula after pancreaticoduodenectomy. *Surgery*. 2014;156(5):1204-11.

17.Hanahan D, Weinberg RA. Hallmarks of cancer: the next generation. *Cell*. 2011;144(5):646-74.

18.Alvarez R, Musteanu M, Garcia-Garcia E, et al. Stromal disrupting effects of nab-paclitaxel in pancreatic cancer. *British journal of cancer.* 2013;109(4):926-33.

19.Gao J, Knutsen A, Arbman G, et al. Clinical and biological significance of angiogenesis and lymphangiogenesis in colorectal cancer. *Dig Liver Dis*.

422 2009;41(2):116-22.

423 20.Galon J, Costes A, Sanchez-Cabo F, et al. Type, density, and location of immune

424 cells within human colorectal tumors predict clinical outcome. *Science*.

425 2006;313(5795):1960-4.

426 21.Lim SH, Chua W, Cheng C, et al. Effect of neoadjuvant chemoradiation on

427 tumor-infiltrating/associated lymphocytes in locally advanced rectal cancers. *Anticancer*

428 *Res.* 2014;34(11):6505-13.

429 22.Kasajima A, Sers C, Sasano H, et al. Down-regulation of the antigen processing

430 machinery is linked to a loss of inflammatory response in colorectal cancer. *Hum Pathol*.

431 2010;41(12):1758-69.

432 23.Forssell J, Oberg A, Henriksson ML, et al. High macrophage infiltration along the

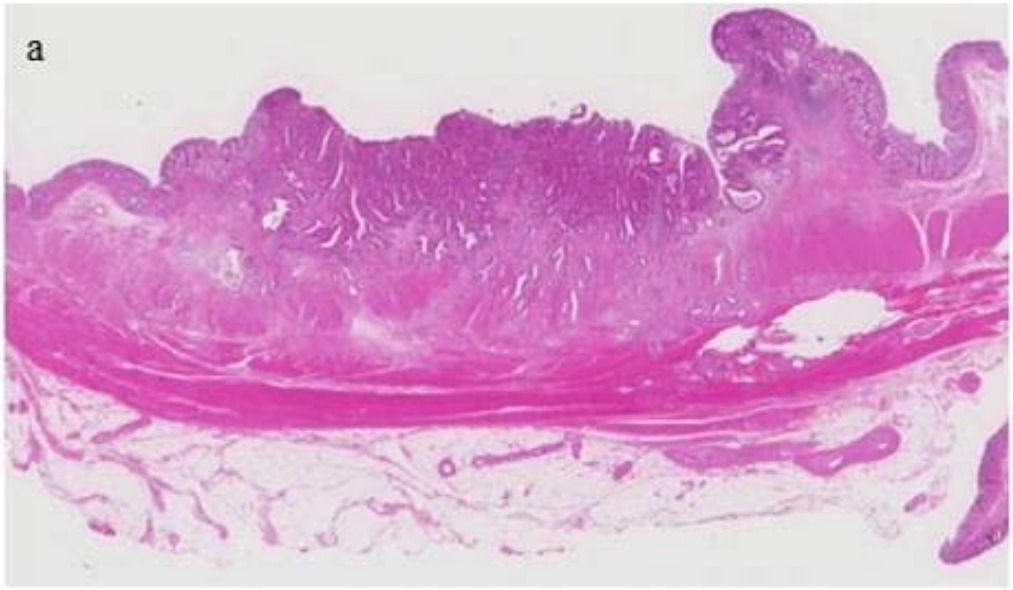
433 tumor front correlates with improved survival in colon cancer. *Clin Cancer Res*.

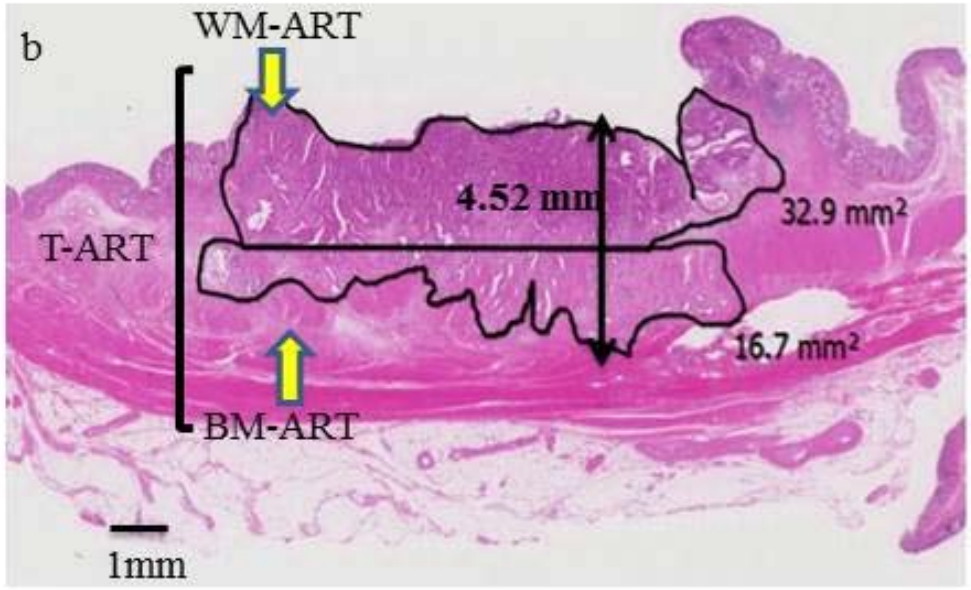
434 2007;13(5):1472-9.

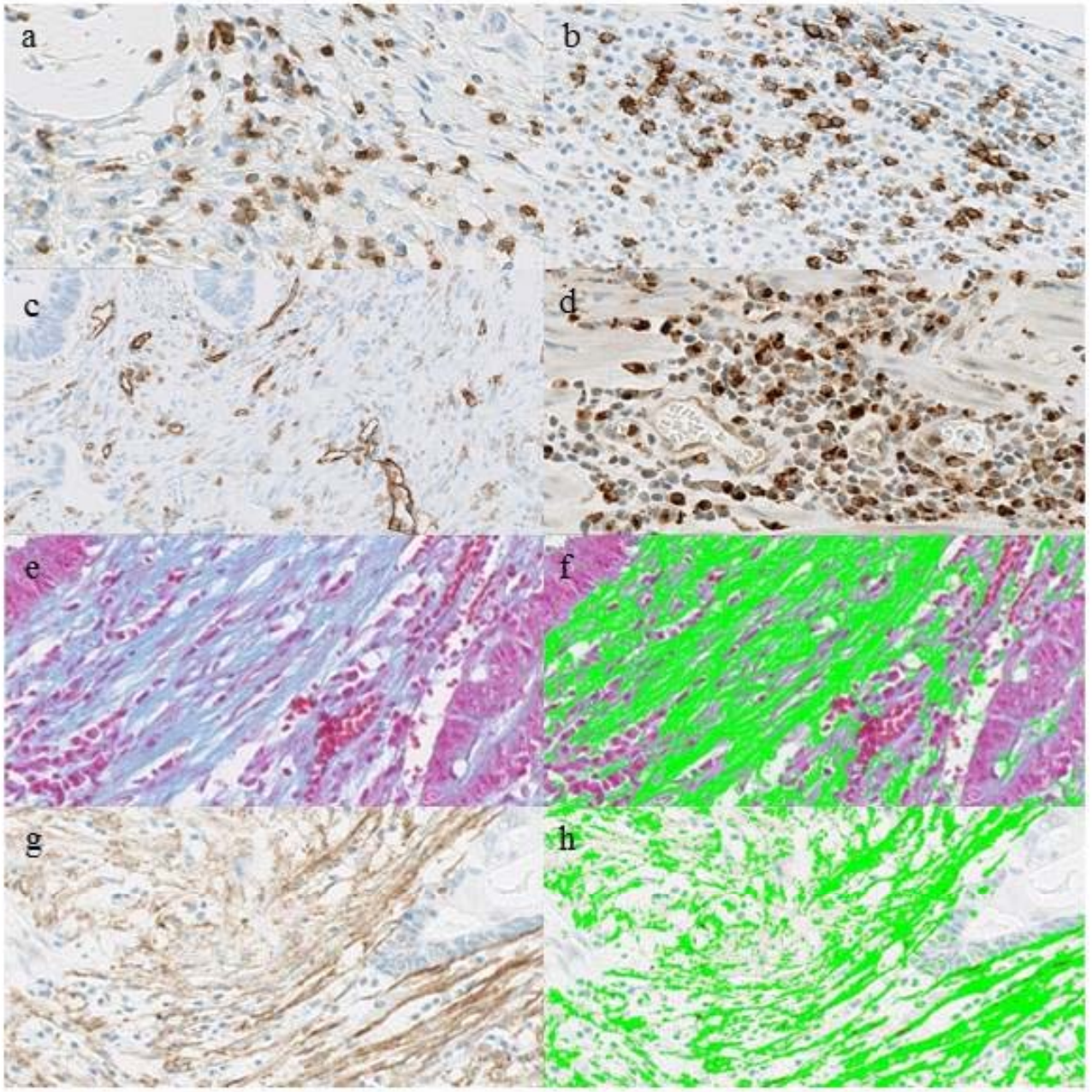
435 24.Mantovani A. B cells and macrophages in cancer: yin and yang. *Nat Med*.

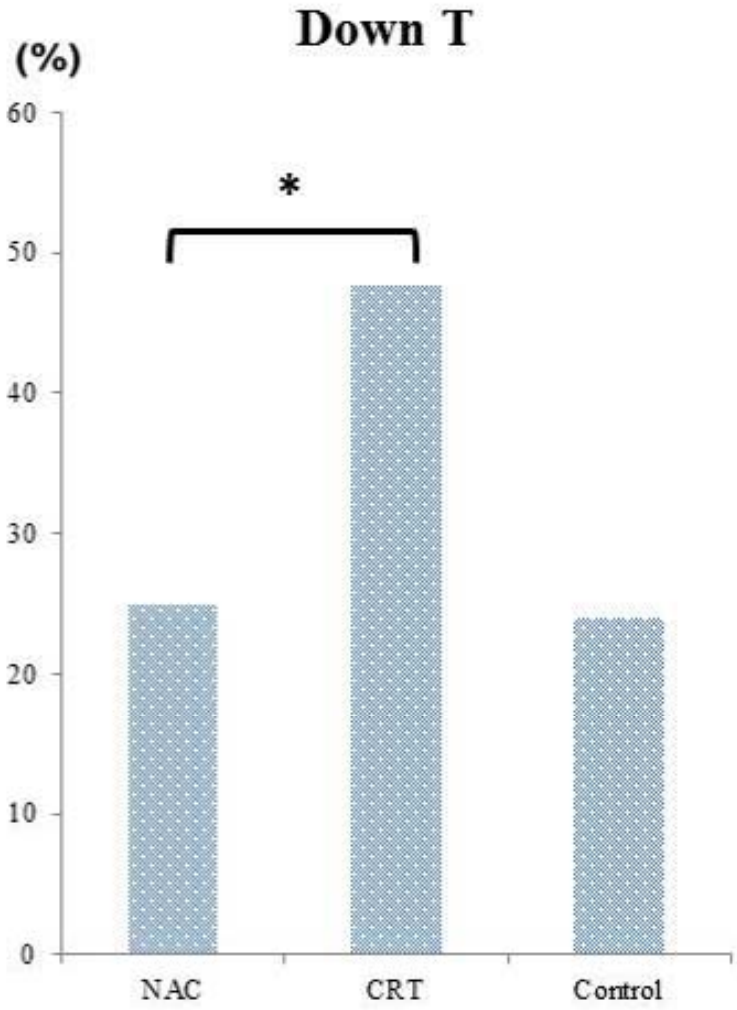
436 2011;17(3):285-6.

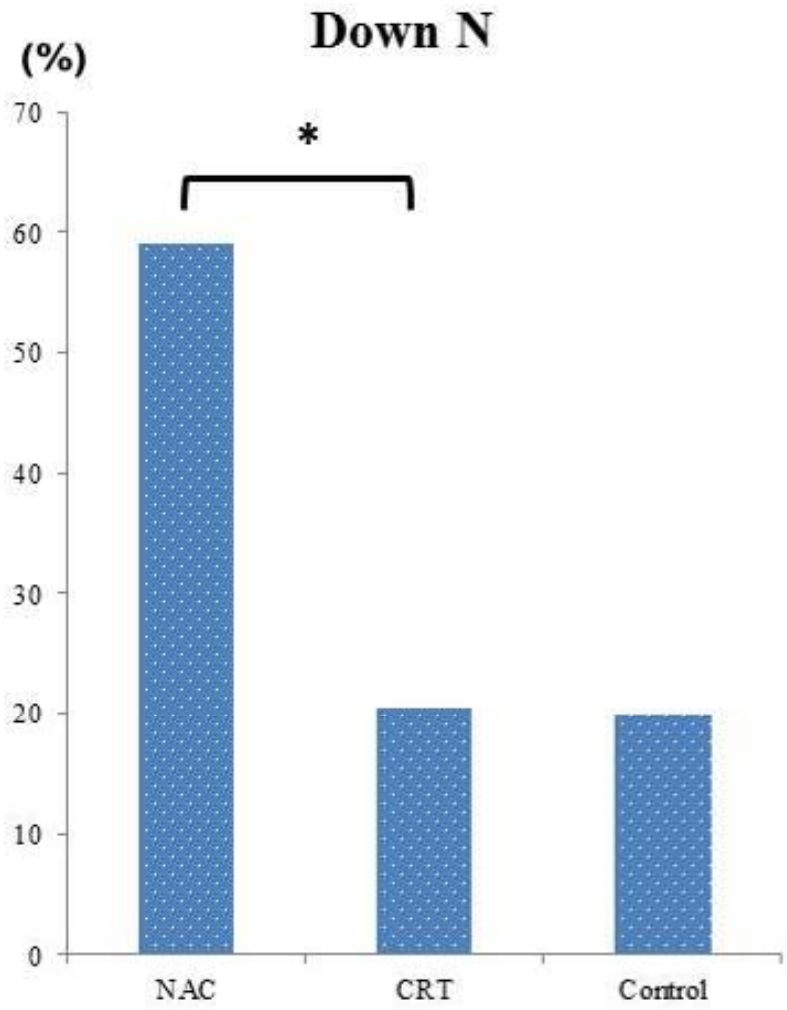
437


a


b
WM-ART
4.52 mm
32.9 mm²
T-ART
16.7 mm²
BM-ART
1mm


a
b
c
d
e
f
g
h

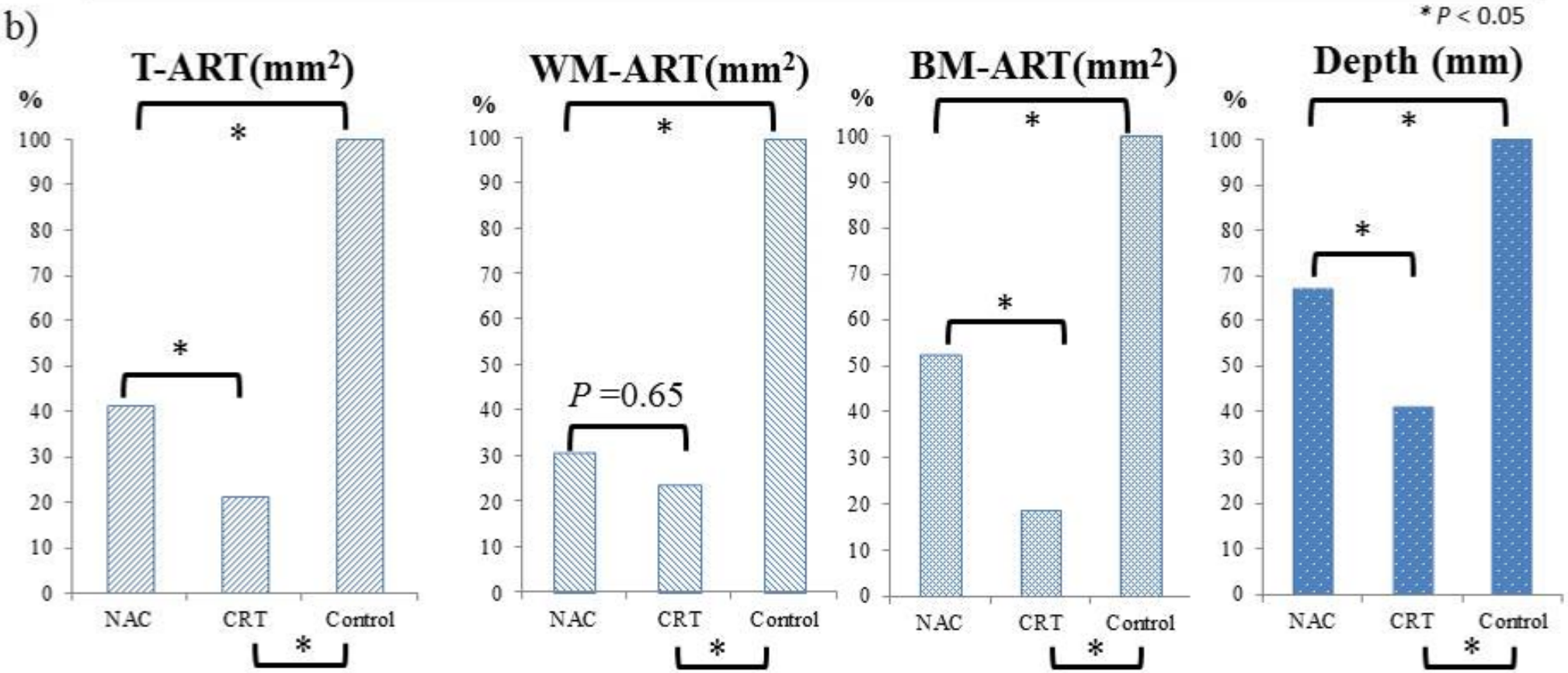

Down T
(%)
60
50
40
30
20
10
0
*
NAC
CRT
Control


Down N
(%)
70
60
50
40
30
20
10
0
*
NAC
CRT
Control

a)

ART	NAC 44	CRT 44	Control 50	*P* NAC vs CRT	*P* NAC vs Control	*P* CRT vs Control
T-ART (mm^2)	81.4 ± 100.0	42.1 ± 44.0	199.26 ± 125.07	*	*	*
WM-ART (mm^2)	31.5 ± 43.6	24.3 ± 31.0	103.63 ± 78.31	*P*=0.65	*	*
BM-ART (mm^2)	50.0 ± 78.2	17.8 ± 20.5	95.63 ± 93.68	*	*	*
Depth (mm)	6.3 ± 4.3	3.7 ± 3.1	9.04 ± 3.99	*	*	*

b)



	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 (%)			
ISR	34（77.2）	44(100)	37(74)
Other	10(22.8)	0(0)	13(26)
cT (0/1/2/3/4)	0/0/0/38/6	0/0/9/35/0	0/0/0/39/11
cN (0/1/2/3/4)	5/16/7/16/0	27/10/6/1/0	26/20/2/2/0
pT (0/1/2/3/4)	4/2/10/23/5	9/1/12/22/0	0/0/4/41/5
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/Ⅱ/ⅢA/ⅢB/Ⅳ)	0/0/5/14/21/4	0/6/19/9/10/0	0/0/21/21/8/0
pathology Stage (0/Ⅰ/Ⅱ/ⅢA/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)	0/11/25/3/5	0/3/17/15/9	
3/4(%)	8(18.2)	24(54.5)	-

	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%)	=0.40	<0.05*	<0.05*
PN	15(34.1%)	13(29.5%)	20(40%)	=0.65	=0.56	=0.29
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%) B: 1(2.3%) C: 2(4.5%)	A: 5(11.4%) B: 3(6.8%)	A: 0(0%) B: 3(6%)			
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	<0.05*
3 (>50%)	3	26	3			

	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*	=0.050	=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**	=0.090
B cell (CD20) BM density,/×40	45.17 ± 52.57	24.02 ± 43.32	87.06 ± 100.78	<0.001**	<0.001**	<0.001**