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REVIEW ARTICLE

Micrometastasis in lymph nodes of colorectal cancer

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Abstract

Colorectal cancer (CRC) is one of the most common cancers worldwide. Postoperative adjuvant chemotherapy is recommended for node-positive stage III patients. A systematic meta-analysis reported that the presence of micrometastases in regional lymph nodes (LNs) was associated with poor survival in patients with node-negative CRC. Because most data employed in the meta-analysis were based on retrospective studies, we conducted a prospective clinical trial and concluded that stage II is a transitional zone between stage I and stage III, where CRC tumors continuously increase the micrometastasis volume in LNs and proportionally raise the risk for tumor recurrence. The one-step nucleic acid amplification (OSNA) assay is a simple and rapid technique to detect CK19 mRNA using the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method. Using the OSNA assay, we and colleagues reported that the upstaging rates of pStages I, IIA, IIB, and IIC were 2.0%, 17.7%, 12.5%, and 25%, respectively, in 124 node-negative patients. Survival analysis indicated that OSNA positive stage II CRC patients had a shorter 3-y disease-free survival rate than OSNA negative stage II CRC patients. In 2017, AJCC TNM staging (the 8th version) revised the definition of LN metastasis in colon cancer and it is stated that micrometastasis should be considered as a standard LN metastasis. To our surprise, this revision was based on a meta-analysis to which our previous study on micrometastasis largely contributed. The remaining questions to be addressed are how to find micrometastases efficiently and whether postadjuvant chemotherapy is effective to prevent disease recurrence and to contribute to longer survival.

KEYWORDS
CEA, colorectal cancer, micrometastasis, OSNA

1 | INTRODUCTION

Colorectal cancer (CRC) is one of the most prevalent cancers in the world. Prognosis depends on treatments, and tumor characteristics, and risk factors related to the backgrounds of patients.[1] Metastasis to the regional lymph nodes (LNs) is a crucial prognostic factor and it has been globally adopted in the tumor-node-metastasis (TNM) staging system of the American joint Committee on Cancer (AJCC)[2] and International Union Against Cancer (UICC),[3] which predicts outcomes of CRC patients. LN metastasis is also used in decision making of treatments.[4]

The MOSAIC (Multicenter International Study of Oxaliplatin / Fluorouracil/ Leucovorin in the Adjuvant Treatment of Colon Cancer) study demonstrated the benefit of oxaliplatin in addition to infusional 5-FU and LV in node-positive stage III colon cancer patients, but no survival benefit is proven in node-negative stage II colon

cancer patients.[5] Therefore, postoperative adjuvant chemotherapy is recommended for node-positive stage III patients. However, it has been suspected that a subset in the node-negative stage II patients may benefit from postoperative adjuvant treatment.[6,7]

Various risk factors have been raised thus far. The current American Society of Clinical Oncology (ASCO) Guidelines define T4 primary disease, inadequately sampled nodes, poorly differentiated histology, or perforation as high-risk factors for disease relapse in stage II.[8] According to the current European Society for Medical Oncology (ESMO) Guidelines, on the other hand, adjuvant chemotherapy is recommended in stage II patients who have the following tumor characteristics: pT4 tumor, LNs sampling <12, poorly differentiated tumor, lymphovascular invasion, perineural invasion, tumor obstruction, or perforation.[9] However, a retrospective study by O'Connor et al demonstrated that 75% of the 24 847 stage II cancers had one or more poor prognostic features of perforation, T4 stage, poor histology, or others, and that a survival benefit from postoperative adjuvant chemotherapy was not observed even with any poor prognostic features.[10] The findings suggest that such prognostic factors are not always able to predict the patients' survival benefit by adjuvant treatment. Therefore, it is important to find out certain risk factors that can classify a subgroup of patients at risk for disease relapse and benefit from adjuvant chemotherapy in stage II patients. As one of such factors, we focused on micrometastasis to LNs. As the object of this article, I introduce our research progress, including the detection methods, clinical relevance with regard to patients' prognosis, and provide a future perspective, especially with its relation to postadjuvant chemotherapy.

2 | MICROMETASTASIS IN CRC

CRC patients without LN metastasis are considered to be at low risk for recurrence. They should be cured by surgical resection alone. However, it is reported that ~30%–40% of node-negative stage II patients subsequently develop disease recurrence.[11] Tumor spread after curative resection of CRC may initially occur in LNs, blood, and bone marrow, each of which is undetected by standard clinical staging techniques but many studies have shown the presence of occult cancer metastasis in the LNs of CRC patients.[6,12]

It is often difficult to detect micrometastasis by the conventional examination of one slide of a hematoxylin and eosin (H&E)-stained section. It has been shown that the detection methods with immunohistochemistry (IHC) or reverse-transcription polymerase chain reaction (RT-PCR) provide evidence of the presence of micrometastasis in node-negative CRC. Cytokeratin (CK), the specific marker of epithelial cells, has been widely used for the IHC examination of micrometastasis in CRC.[13–17] IHC can identify the existence of micrometastatic cells at a frequency of 17%–39% of the cases with histologically negative LNs, but this method has a limitation because it takes time and effort to examine numbers of slides. Alternatively, tumor-specific genes, eg, CK19, CEA, and CK20 have been used for amplification by RT-PCR in patients with a variety of malignancies, including CRC.[18–24] This genetic detection method maintains high sensitivity, but in turn false positive is a problem.

In Table 1, the progress of our study on micrometastasis to LNs in CRC is summarized. I first introduce one of our studies by Miyake et al.[7] We examined 237 LNs from 11 CRC patients who underwent curative resection (stage I–III) by immunostaining of CK and RT-PCR for carcinoembryonic antigen (CEA) and CK20 mRNAs, and conventional histological examination (H&E). We then constructed an anatomical map of LNs to clarify the extent of micrometastasis in each CRC case. Histological examination detected that 20 of 237 LNs contained metastatic cells, and they were all positive by both IHC and RT-PCR. Of the 217 histologically negative LNs, 14 (6.5%) contained micrometastases by IHC with one slice examination, and 57 (26.2%) were positive for at least one of the two genetic markers CEA and CK20 by RT-PCR. Anatomical mapping of regional LNs of all patients indicated that micrometastases were dispersed not only at the pericolic LNs but often at distant LNs. A clinical follow-up study showed that two patients developed recurrence within 1 y after surgery and that both of them had RT-PCR-positive micrometastases in not less than 70% of LNs examined. In addition, these patients had frequent micrometastases at distant LNs, ie, those around the root or along the inferior mesenteric artery. Through this study, we are convinced that we cannot neglect micrometastasis in CRC patients.

Rahbari et al. reported a systematic meta-analysis on micrometastases in regional LNs of 4087 patients with node-negative CRC.[25] This analysis included 39 studies and showed that the presence of micrometastases in regional LNs was associated with poor overall survival (hazard ratio [HR], 2.20; 95% confidence interval [CI], 1.43–3.40), and disease-free survival (HR, 2.24; 95% CI, 1.57–3.20). The subgroup analyses also showed that molecular tumor-cell detection was an independent prognostic factor. However, because most studies employed in the meta-analysis were retrospectively performed, it was emphasized that prospective studies are required.

3 | THE ONE-STEP NUCLEIC ACID AMPLIFICATION-BASED NOVEL MOLECULAR TECHNIQUE

A histological diagnosis of postoperative LN metastasis is usually performed by microscopic examination of H&E-stained sections with the largest cross-section area of the LN. In this method, however, micrometastases are overlooked due to their minimal size. Although IHC and an RT-PCR detection technique are available in the laboratory, they have not yet been applied in clinical practice because of the time-consuming problem and their complexity.

One-step nucleic acid amplification (OSNA) assay is a technique using the reverse-transcription loop-mediated isothermal amplification (RT-LAMP) method for gene amplification. Since this method can directly analyze the supernatant of a homogenized LN solution without the mRNA purification process, it offers rapidity and simplicity of detection of LN micrometastases. OSNA has been in clinical practice for the diagnosis of axillary LN metastases in breast

TABLE 1 Summary of research progress

2002	Retrospective investigation on occult metastasis to LNs of CRC[7,23,29]
	Occult metastasis to LNs were detected at 54.7% by IHC and at 29.6% by CEA-based RT-PCR. The RT-PCR showed a prognostic value.
2004	Translational research: Establishment of LN sampling[35]
	RNA was stable in lymph nodes for up to 3 h after surgical resection. RNA was well preserved in RNA later at −20 °C for 3 wk
2013	OSNA feasibility test.[28]
	The OSNA test was approved by the Japanese Ministry of Health, Labour and Welfare in 2013. The OSNA assay did not produce false-positive results and a judgment performance of the OSNA assay was comparable to a 2-mm interval histological examination
2016	I. Clinical relevance of micrometastasis volume (MMV) in stage II CRC.[36]
	The prospective clinical trial demonstrated that MMV determined by qRT-PCR of CEA mRNA was a useful marker in identifying patients who are at high risk for recurrence of stage II CRC.
	II. OSNA-assisted molecular staging.[31,42]
	The upstaging rates of pStages I, IIA, IIB, and IIC were 2.0%, 17.7%, 12.5%, and 25%, respectively. Later it was revealed that the CRC patients upstaged from stage II to stage III had a significantly shorter 3-y disease free survival rate
2017	Revision of AJCC TNM staging.[2]
	The 8th edition of the AJCC TNM Cancer Staging Manual announced that micrometastases have been defined as clusters of 10 to 20 tumor cells of clumps of tumor ≥0.2 mm in diameter. Our early work contributed this revision[29]
2020~	Observational clinical trial started to assess the relevance of chemotherapy (UMIN000037532)
	Adjuvant chemotherapy vs no treatment in OSNA-positive stage II CRC

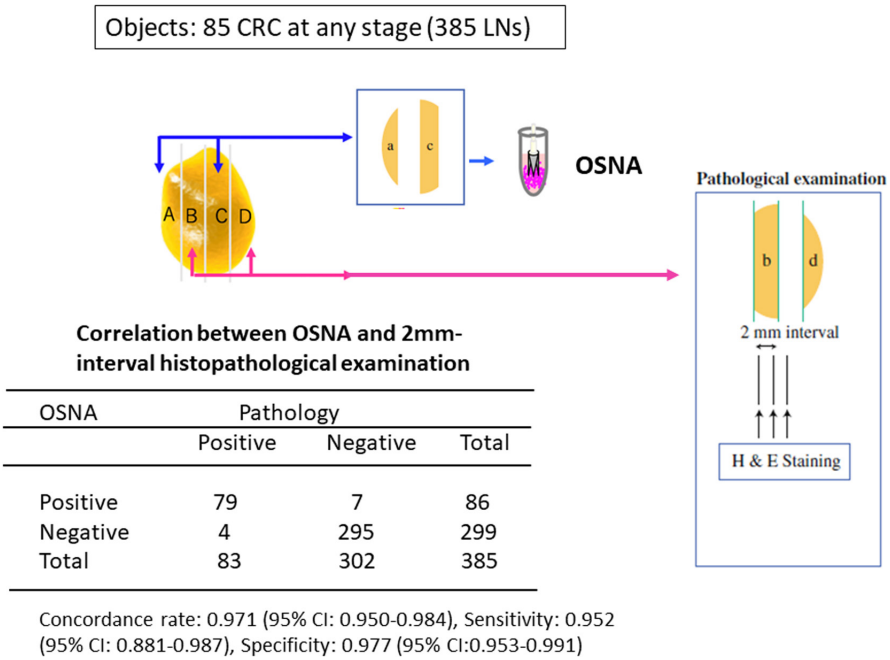
cancer, saving a second surgery for patients requiring an axillary clearance.[26,27]

In a multicenter clinical study in Japan, Yamamoto et al clarified the diagnostic power of OSNA for LN micrometastases in CRC using cytokeratin 19 (CK19) mRNA as a molecular marker.[28] The OSNA assay was performed on 121 LNs dissected from 14 early-stage CRC patients (pStage 0 or I) or from patients with four benign colorectal disease and it was confirmed that the OSNA assay did not produce false-positive results for histologically negative LNs (OSNA-positive: 0/121). Moreover, 385 LNs were collected from 85 CRC patients at any stage to examine whether the OSNA assay would represent equivalent performance to the histopathological examination (Figure 1). Although the false-negative reaction (ie, pathology positive/OSNA negative) were noted in 4 of 385 LNs (1.04%), possibly derived from allocation bias (uneven location of metastatic tumor cells), the OSNA assay exhibited a concordance rate of 0.971 (95% CI: 0.950–0.984) against the 2-mm interval histopathological examination, with a κ-value of 0.916 (95% CI: 0.868–0.965), confirming the high equivalence between the two methods. Thus, this study demonstrated that a judgment performance of the OSNA assay was comparable to a 2-mm interval histological examination of three slices of H&E sections. Based on these findings, the OSNA-based molecular detection technique was approved as a novel diagnostic kit for LN micrometastases of CRC by the Japanese Ministry of Health, Labour and Welfare in 2013.

4 | RETROSPECTIVE STUDIES ON MICROMETASTASES IN CRC

It is important to recognize the distribution of micrometastasis in the regional LNs of CRC. Although several studies showed the existence of micrometastasis in LNs of CRC, little is known about accurate localization and frequency. Our colleague, Noura S et al, immunohistochemically assessed localization and frequency of micrometastasis in CRC using the pancytokeratin monoclonal antibody AE1/AE3.[29] We found that the frequency of micrometastasis in node-negative CRC patients increased as the slices increase from one to two to five, through observation of the 4-μm-thick LNs sections. With five slices, micrometastases were present in 49.1% of histologically LN-negative patients. As a pattern of micrometastasis, isolated tumor cells (ITC: single or multiple) and cluster of tumor cells were noted (Figure 2). When we assessed the disease recurrence stratified by the pattern, ie, None, ITC, or Cluster, the patient with Cluster rather than ITC tended to experience cancer relapse in LN-negative patients ($P = .09$, Figure 2), although the number of CRC patients was insufficient to make a conclusion at that time.

As molecular tests become popular, RT-PCR is often utilized to detect micrometastasis using cancer-specific genetic markers such as CEA, CK19, and so on. A retrospective study for the presence of micrometastases was performed using both IHC and CEA-specific RT-PCR in 64 N0 CRC patients by Noura S et al.[23] Micrometastases were detected in 19 (29.6%) of 64 patients by RT-PCR and in 35 (54.7%) of 64 patients by IHC. Survival analysis indicated that patients exhibiting a positive band for CEA mRNA had a significantly worse prognosis than those who were RT-PCR-negative, with respect to both disease-free and overall survival ($P = .027$ and .015, respectively). On the other hand, by IHC analysis the presence of tumor cells including ITC did not affect patient outcome. By multivariate Cox regression analysis, micrometastasis detected by RT-PCR was retained as an independent prognostic factor. These data

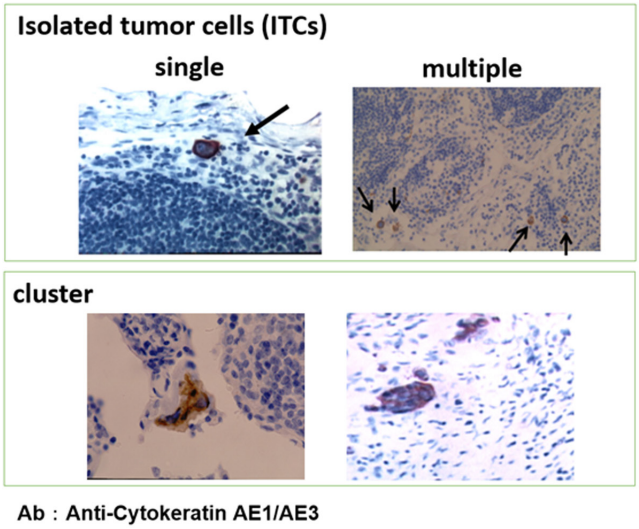


Correlation between OSNA and 2mm-interval histopathological examination

OSNA	Pathology		
	Positive	Negative	Total
Positive	79	7	86
Negative	4	295	299
Total	83	302	385

Concordance rate: 0.971 (95% CI: 0.950-0.984), Sensitivity: 0.952 (95% CI: 0.881-0.987), Specificity: 0.977 (95% CI:0.953-0.991)

FIGURE 1 Concordance rate between OSNA and histopathological examination lymph node processing and the relationship between OSNA and pathology are shown. Lymph nodes were divided at 2-mm intervals, and nonadjacent blocks were alternatively subjected to histopathological examination or the OSNA assay. Three sections prepared from the cut surfaces were stained with H&E. It was confirmed that the OSNA assay provided a judgment performance equivalent to that of 2-mm interval histopathological examination. Concordance rate: 0.971 (95% CI: 0.950–0.984), sensitivity: 0.952 (95% CI: 0.881–0.987), specificity: 0.977 (95% CI:0.953–0.991)



P=0.09

	None	ITC	Cluster
No. Node-negative CRC Case(n=55)	28	22	5
No. Recurrence	7	5	3

FIGURE 2 A pattern of occult metastasis in LNs of CRC. Isolated tumor cells (single: ×200, multiple: ×100) and clusters (×200) were detected by immunostaining with anti-cytokeratin AE1/AE3 antibody. Of 55 node-negative CRCs (stage I: 9, stage II: 46), recurrence tended to occur in CRC cases with clusters (3/5) compared with those with ITCs (5/22) (*P* = .09)

suggest that micrometastases in LNs detected by CEA-specific RT-PCR may be useful for predicting a high risk for relapse in stage II CRC patients.

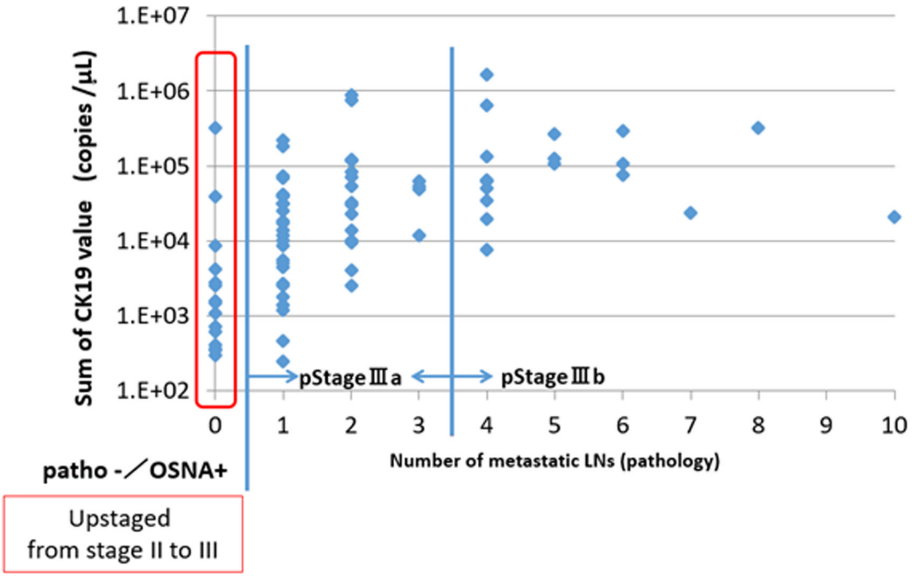
5 | PROSPECTIVE MULTICENTER STUDIES

Several studies reported the clinical benefit of OSNA in CRC.[28,30] Based on those findings, we prospectively performed an extended study to examine LN metastases of patients with CRC (n = 204 cases) and investigated the migration of clinical stage when OSNA was added to the standard pathological examination.[31] In this study, it was shown that the concordance rate between a one-slice H&E examination and OSNA assay was 95.7% (1842/1925 LNs), and the sensitivity and specificity of the OSNA assay were 86.2% (125/145) and 96.5% (1717/1780), respectively. Among 124 LN-negative patients, the respective upstaging rates of pStages I, IIA, IIB, and IIC were 2.0%, 17.7%, 12.5%, and 25%, respectively. Moreover, OSNA-positive patients had deeper invasion to the colonic wall and severe lymphatic invasion (*P* = .048 and *P* = .004, respectively). The sum of CK19 mRNA assessed by OSNA (TTL: total tumor load) values increased significantly as the node status increased when the Jonckheere trend test was used to compare TTL from each nodal stage (*P* = 1.3445E-07). TTL values of the cases upstaged from stage II to stage III by the OSNA method were largely overlapped on the range of those in stage III CRC, and these upstaged patients are likely to be at high risk for disease recurrence (Figure 3).

The prognostic value of molecular tumor cell detection in patients with LN-negative CRC was uncertain because of a lack of evidence from prospective studies.[12,32–34] In this context, Yamamoto et al conducted a prospective multicenter clinical trial after a translational study for a definite prognostic marker in patients with LN-negative stage II CRC.[35,36] In that study, molecular detection of CEA mRNA by qRT-PCR was employed to determine the optimal threshold of high risk for disease recurrence. A total of 296 patients with pathologic stage II CRC were analyzed. Multivariate Cox regression analyses revealed that a high micrometastasis volume (high MMV, n = 95) was an independent poor prognostic factor for 5-y disease-free survival (DFS; $P = .001$) and 5-y overall survival (OS; $P = .016$) (Figure 4). This prospective clinical trial clearly demonstrates that MMV determined by qRT-PCR of CEA mRNA is a useful marker in identifying patients who are at high risk for recurrence of stage II CRC. Furthermore, these data provide a concept that stage II is a transitional place between localized stage I and expanding stage III, where CRC tumors continuously increase MMV in LNs and the risk for tumor recurrence (Figure 5). LN metastasis in stage III CRC is a well-established predictive marker to ensure survival benefit by chemotherapy.[5,37] Considering the recurrence ratio of the high MMV group in stage II CRC patients was compatible to that in stage III, such groups may be an appropriate target for a postoperative adjuvant chemotherapy. We also found that MMV determined by CK19 mRNA level was also useful to discern high-risk stage II CRC patients in the same clinical settings. This suggested that OSNA targeting CK19 mRNA could be a simple and stable detection system for micrometastases.

6 | REVISION OF TNM CANCER STAGING

The 8th edition of the AJCC (American Joint Committee on Cancer) TNM Cancer Staging Manual announced that micrometastases have been defined as clusters of 10 to 20 tumor cells of clumps of tumor ≥0.2 mm in diameter and recommends that these micrometastases be considered as standard positive nodes.[38] This is particularly innovative because colon cancer patients with micrometastasis should receive adjuvant chemotherapy according to this revision. In the Cancer Staging Manual, it is stated that micrometastases rather than isolated tumor cells provide the prognostic value according to a meta-analysis by Sloothaak et al.[39] This was



Median value of sum of CK19 in each node stage patients

Pathological status	N(OSNA+)	Median (copies/μL)	Range (copies/ μL)	*P* value
pN0	14	1,550	300-320,000	
pN1	44	24,050	250-890,000	1.3445E-07
pN2	21	90,600	2,500-1,635,100	

FIGURE 3 Total CK values and number of metastatic LNs. One diamond dot represents the sum of CK19 mRNA (TTL: total tumor load) value in each case. TTL values gradually increased as the number of pathologically node-positive LNs increased. The median TTL values of pN0, pN1 (1-3 positive LNs), and pN2 (4 or more positive LNs) were 1550 copies/IL (300–320000 copies/IL), 24050 copies/IL (250–890000 copies/IL), and 90600 copies/IL (2500–1635100 copies/IL), respectively. OSNA-negative cases had a TTL fewer than 250 copies/IL. The TTL increased significantly ($P = 1.3445E-07$) as the node status increased. The Jonckheere trend test was used to compare TTL from each nodal stage. Note that TTL values of the cases upstaged from stage II to stage III by OSNA were largely overlapped in the range of those in stage III CRC

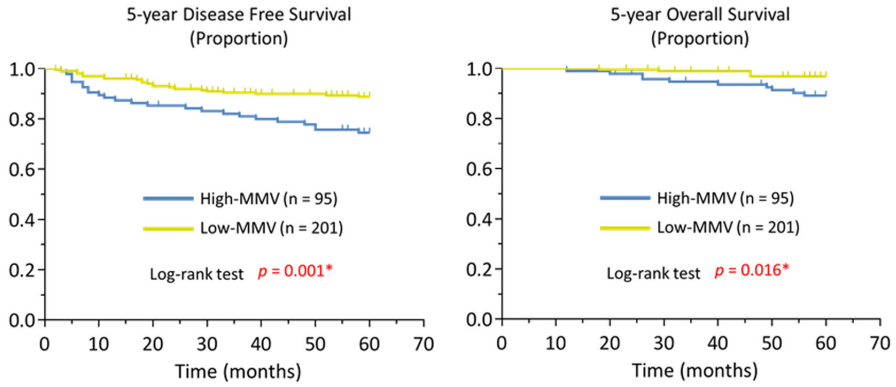


FIGURE 4 Survival curves stratified by high MMV and low MMV. Survival analyses indicated that the high MMV group had significantly worse 5-y DFS (74.7% vs 88.6%) and 5-y OS (89.5% vs 95.5%) as compared with the low MMV group ($P = .001, .016$). There was no difference in the mean follow-up period in high MMV and low MMV groups ($P = .8471$; mean ± SD, 86.0 ± 25.8 vs 79.9 ± 22.1 mo). The median follow-up period was 82.9 (12.6–133.2) mo and 81.4 (5.3–124.9) mo, respectively. Abbreviations: DFS, disease-free survival; OS, overall survival

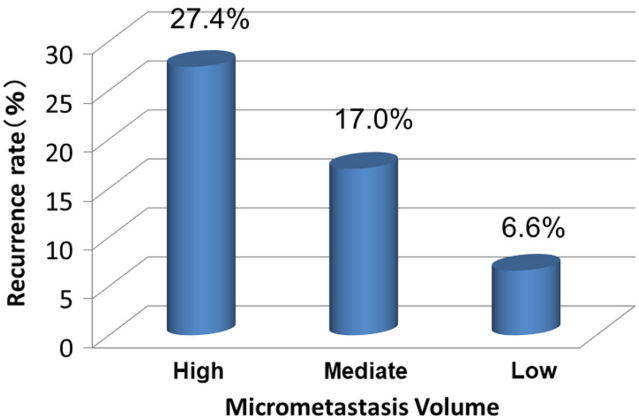


FIGURE 5 Micrometastasis volume and 5-y recurrence rate. A positive correlation was noted between micrometastasis volume and 5-y recurrence rate in CRC. The 5-y recurrence rate of high micrometastasis volume patients is almost compatible with that in stage III CRC patients

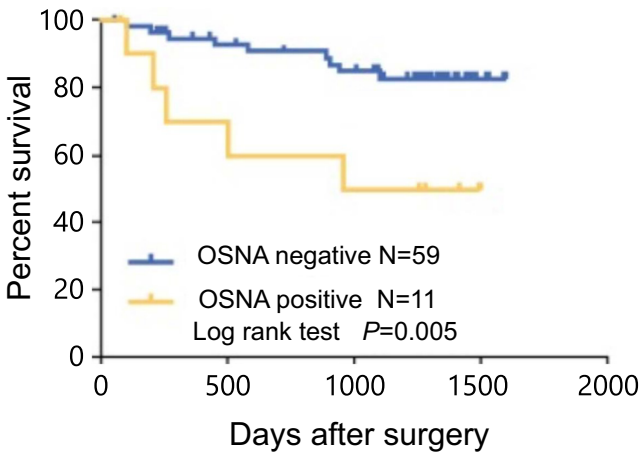


FIGURE 6 Survival rates of OSNA-positive and OSNA-negative patients in pStage II CRC patients. 3-y disease-free survival rates are shown

subsequently further confirmed by the same group.[40] To our surprise, our early research on immunohistochemical analysis of micrometastases and isolated tumor cells[29] remained in the last 8 out of 590 reports and played an important role in the meta-analysis. Therefore, it is a great honor for us to make prominent contribution to the TNM revision in AJCC.

7 | CAN MICROMETASTASES BE A TARGET FOR ADJUVANT CHEMOTHERAPY?

As explained above, the AJCC approved that micrometastasis should be considered a standard metastasis, but UICC has not adopted micrometastases as a standard metastasis in TNM staging. It is, I suppose, because some problems might remain to be addressed. First, a predictive factor for high-risk stage II CRC, eg, micrometastases, may not always be a true target of chemotherapy. It would be better to clarify this point before application of chemotherapy. Second micrometastases are so minute that pathologists rarely come across them in H&E-stained LN sections. In other words, application to chemotherapy depends on an incidental encounter of micrometastases. In this regard, OSNA takes advantage because it checks up a piece of LN as a bulk sample; thus, a stable detection ability will be warranted.[41] It should be emphasized that the OSNA assay provides a judgment performance equivalent to histopathological examination of three slices at a 2-mm-interval (Figure 1, Study 2). Thus, it is likely that OSNA test would contribute efficient detection of micrometastasis compared with the pathological routine survey with one slice.

Moreover, a recent multicenter study including our institute showed that OSNA-positive stage II CRC patients had a shorter 3-y DFS rate than OSNA-negative stage II CRC patients (Figure 6).[42] Taken together, it is considered that micrometastases in stage II CRC patients could be a sensitive marker for disease recurrence or

shorter OS. The last question remains as to whether such high-risk patients in stage II CRC would benefit from adjuvant chemotherapy or not. Nevertheless, we are rather confident that micrometastasis is a good target of chemotherapy because LN metastasis is the best proven target, ie, a gold standard for application of chemotherapy, and we previously found that ~15%–30% stage II CRC patients harbored a high micrometastasis volume that is compatible to the level at stage III. To conclude this issue, a large-scale prospective clinical trial is currently in progress in OSNA-positive stage II CRC patients, with and without chemotherapy (UMIN000037532: our observational research). Hopefully, this result will indicate obviously better prognosis in 3-y recurrence-free survival in the chemotherapy-applied group.

8 | CONCLUSION

In conclusion, it is almost clear at present that even micrometastases have an impact on stage II CRC patients' outcome. However, it is still underway to clarify whether a standard postadjuvant chemotherapy would prolong their survival or not. In addition, it is reported that a significantly diverse set of cancer cells and intratumor heterogeneity exist within a micrometastasis.[43] Therefore, it is expected that recent innovative techniques, including the single-cell RNA sequence, would unveil the characterization of tumor cells forming micrometastases in LNs.

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DISCLOSURE

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