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RESEARCH

# Prognostic value of whole lymph node molecular analysis in colorectal cancer: a prospective multicenter study

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## Abstract

Lymph node (LN)-negative colorectal cancer (CRC) patients may be understaged by conventional hematoxylin and eosin for its low sensitivity for detecting LN micrometastasis (LNM). The one-step nucleic acid amplification assay (OSNA) has shown superior performance in detecting LNM. To date, OSNA studies in CRC have analyzed part of the LN tissue with OSNA and part with H&E, introducing a tissue allocation bias for LNM detection. We aimed to evaluate the prognostic significance of the whole LN molecular analysis using OSNA in CRC patients. This prospective multicenter study analyzed LNs from stage I-III CRC by both cytology smears (CS) and OSNA, the latter analyzing the whole LN tissue and reported as the Total Tumor Load (TTL). X-tile software was used to determine the optimal TTL threshold. Cox proportional hazard and Kaplan–Meier estimation were used to assess the prognostic significance of TTL for both cancer-specific survival (CSS) and recurrence-free survival (RFS). 158 CRC patients were included, with 156 eligible for survival analysis. A TTL of 15,000 copies/μL was identified as the optimal cut-off, stratifying CRC patients into low and high risk for both CSS and RFS ($P < 0.05$). Conversely, CS pN-positive patients with TTL ≤ 15,000 copies/μL had comparable long-term outcomes to CS pN-negative patients ($P > 0.05$). A TTL threshold of > 15,000 copies/μL has prognostic value as it identifies CRC patients with significantly reduced CSS and RFS. The clinical implementation of the whole LN analysis by OSNA allows accurate patient risk stratification and optimizes postsurgical management.

**Keywords** Colorectal cancer · OSNA · Lymph node metastasis · Prognosis

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## Introduction

Colorectal cancer (CRC) represents the second leading cause of cancer-related mortality worldwide, with a steadily increasing incidence [1]. Despite therapeutic advances, the prognosis of CRC is largely dependent on early detection and accurate staging [1]. In this regard, CRC screening programs have substantially improved the identification of early stage tumors (stages I-II; pT1-4 N0), which account for > 70% of all screened CRC, thereby reducing its mortality rate [2, 3]. Nevertheless, the assessment of lymph node metastases (LNM) remains essential for determining prognosis and guiding therapeutic decisions, since postoperative adjuvant chemotherapy is routinely recommended for stage III CRC patients and for some high-risk stage II patients [4, 5]. Yet, the current gold standard for lymph node (LN) evaluation relies on histopathological examination with hematoxylin and eosin (H&E) staining, which may be limited due to its lack of sensitivity in detecting micrometastasis with minimal LN tumor burden, as it analyzes less than 1% of the LN tissue [6]. The presence of LN micrometastasis is related to poor survival in CRC patients [7]. To overcome this limitation, the one-step nucleic acid amplification (OSNA) assay has emerged as a promising approach for detecting LNM. The OSNA assay represents a rapid, objective, cost-effective, and standardized PCR–based technique [8, 9], which allows a quantitative detection of the amount of cytokeratin 19 (CK19) mRNA present in the LNs, by using the reverse transcription loop-mediated isothermal amplification (RT-LAMP). Originally used in breast cancer sentinel LNs, OSNA has demonstrated high sensitivity and specificity in detecting LNM, contributing to a more accurate evaluation of the LN status and improving patient's therapeutic management [10–15]. In gastric cancer and other solid tumors, OSNA has further proven its utility in detecting LN occult metastases with H&E [16–21], reinforcing its potential as a valuable prognostic tool.

To date, CRC studies involving OSNA were constrained by tissue allocation bias (TAB), wherein only part of the LN tissue was analyzed by OSNA, while the rest of the LN underwent standard H&E processing for conventional pN staging. This partial LN analysis with OSNA inevitably yielded false-negative results and underestimated the amount of LN metastatic tumor burden. However, this limitation might be solved by analyzing the entire LN tissue with the OSNA assay, as it is routinely performed in breast cancer [10, 11]. In a previous study, we demonstrated that cytology smears (CS) allowed for a pN stage on freshly dissected LNs. In addition, we observed that CS had similar performance to the OSNA assay in detecting LNM [22]. In this study, we aimed to use the whole LN for the OSNA analysis, and to establish a prognostic cut-off of the amount of tumor burden within the LNs, or Total Tumor Load (TTL), for an accurate CRC patient risk stratification.

## Materials and methods

### Study design and participants

This prospective multicenter study was conducted from 2019 to 2024 across six tertiary hospitals. The study was approved by the ethical committee of all centers (Registration number HCB/2012/7324 ER-03, on December 11, 2019) and carried out according to the Declaration of Helsinki, STARD and STROBE guidelines [23, 24]. The inclusion criteria were patients ≥ 18 years with histologically confirmed CRC who underwent curative-intended surgical treatment without neoadjuvant therapy. Preoperative MRI or CT imaging confirmed non-metastatic CRC (stages I-III, AJCC 8th edition) in all patients. Exclusion criteria included metastatic disease, synchronous tumors, familial adenomatous polyposis syndrome, intraluminal stents, inflammatory bowel disease-associated carcinoma, or the presence of other malignancies. Follow-up was performed according to the standard protocols for CRC at each institution. Informed consent was obtained from all patients, and all data were handled pseudo-anonymously to ensure confidentiality.

The primary endpoints of this study were cancer-specific survival (CSS, time from surgery to cancer-related death) and recurrence-free survival (RFS, time from surgery to tumor relapse). Secondary endpoints were the sensitivity, specificity, concordance rate, positive predictive value (PPV), and negative predictive value (NPV) of OSNA versus CS.

### Lymph node processing and analysis

The surgical specimen was immediately delivered to the pathology department after surgery, and LN dissection was performed within 45 min. Firstly, the mesocolon or mesorectal fat was separated from the colon or rectum wall. Then, LNs were harvested from the fat tissue with sterile instruments and the fat tissue around each LN was removed, bisecting each LN longitudinally to perform CS by making circular movements on pre-treated glass slides. Each slide was air-dried for CK19 immunocytochemistry (ICC) staining of epithelial tumor cells, which enabled us to establish the pN stage according to the number of positive LNs. Next, both LN halves were put into a microcentrifuge tube, thereby enabling the analysis of the whole LN by OSNA. The same process was performed with each harvested LN. Multiple LNs were pooled in a single tube, with a maximum weight of 600 mg, and stored at −80 °C for deferred OSNA analysis using the pooling method, as described by Rakislova et al. [25]. After LN harvesting, the surgical specimen was subjected to standardized gross description, preparation, and overnight formalin fixation, according to the standard protocols at each participant center. Occasionally, re-examination of post-fixation tissue resulted in the identification of extra LNs, which were exclusively assessed by standard H&E and contributed to the final pN staging.

### One-step nucleic acid amplification (OSNA) assay

The OSNA assay is a standardized, quantitative, and rapid molecular technique, which uses the RT-LAMP at 65 ℃ for CK19 amplification. Unlike conventional RT-PCR, it does not require mRNA extraction or purification. It involves three key steps, LN homogenization, centrifugation, and CK19 mRNA amplification [26]. Each tube contained various pooled LNs, which were homogenized using the RP-10 homogenizer (Sysmex, Kobe, Japan). Then CK19 mRNA amplification was conducted using the RD-210 analyzer (Sysmex, Kobe, Japan) following the manufacturer's manual. The OSNA results were obtained in 11 – 30 min and reported as the TTL, which is defined as the sum of all CK19 mRNA copies/μL present in all freshly dissected LNs of a surgical specimen. A TTL of $\geq 250$ copies/μL is the OSNA assay positive detection value [27].

### CK 19 immunocytochemistry

The slides containing CS were air-dried, fixed in 96° alcohol for 10 min. Then, CK19 ICC was performed without antigen retrieval, following a standard protocol using CK19 antibody (CK19 mouse monoclonal, clone RCK108; pre-diluted. Agilent, Santa Clara, California, USA). Positive immunostaining was defined as membranous staining, with or without cytoplasmic staining, in isolated or clustered epithelial cells. Additionally, immunohistochemistry was performed in all primary tumors to ensure its positivity [28].

### Estimation of sample size

PASS software (version 2021, NCSS LLC, USA) was employed. The parameters included a 90% sensitivity and a 96% specificity of OSNA, as reported in prior studies [29, 30], as well as a 37.0% prevalence of pN + derived from the SEER (Surveillance, Epidemiology, and End Results) registry, with $\alpha = 0.05$ (two-sided) and a statistical power of 90%. The results suggested that a minimum of 41 patients were needed for the diagnostic test, including 15 pN + cases.

### Statistical analysis

Continuous variables were reported as median and interquartile range (IQR). Group comparisons utilized the Mann–Whitney *U* test (two groups) or the Kruskal–Wallis *H* test (multiple groups). Categorical variables were described as absolute and relative frequencies, with statistical comparisons performed using the chi-square or Fisher's exact test. A Jonckheere trend test was performed to ascertain the distribution of TTL medians against ordered variables (tumor stages, pN stages, etc.). The agreement between OSNA and CS was analyzed by Cohen's kappa and McNemar's test. The optimal TTL cut-off was determined by X-tile software (version 3.6.1, Yale University, USA) according to the minimal *P* value, suggesting the greatest group disparity in survival probability [31]. Restricted cubic spline regression (RCS) analyses were used to examine potential nonlinear relationships between log-transformed TTL and unfavorable outcomes. Univariate and multivariate cox proportional hazards models were performed to identify independent predictors for CSS and RFS. Variables with $p < 0.1$ in univariate cox analysis entered multivariate analysis. Sex and age were included in all multivariate cox models, as they are fundamental and non-modifiable characteristics routinely controlled in epidemiological studies. A Firth penalized likelihood method was utilized in multivariate cox analysis to account for low event rate [32, 33]. Kaplan–Meier curves with log-rank tests were applied for survival comparisons. The Firth-corrected logistic regression analysis was conducted to identify independent predictors related to TTL. A $p$ of $< 0.05$ was considered statistically significant. All statistical analyses were performed using R (Version 4.2.2), unless otherwise indicated.

## Results

### Patient characteristics

One hundred and sixty-six CRC patients from six tertiary hospitals were prospectively included between 2019 and 2024. All freshly dissected LNs were analyzed by two methods, CS and OSNA. We excluded three patients with in situ carcinoma, and five patients who had only positive LNs dissected after formalin fixation that could not be evaluated by OSNA, resulting in 158 patients with pathologically confirmed stages I-III CRC (AJCC 8th edition) (Supplemental Fig. 1). Thirty-three patients received adjuvant chemotherapy, of those, 17 showed OSNA positivity, and 16 were OSNA negative. Patient's average follow-up was 31.0 months (SD ± 17.6; range 0.5 – 63.2 months). Demographic and clinicopathological characteristics are summarized in Table 1.

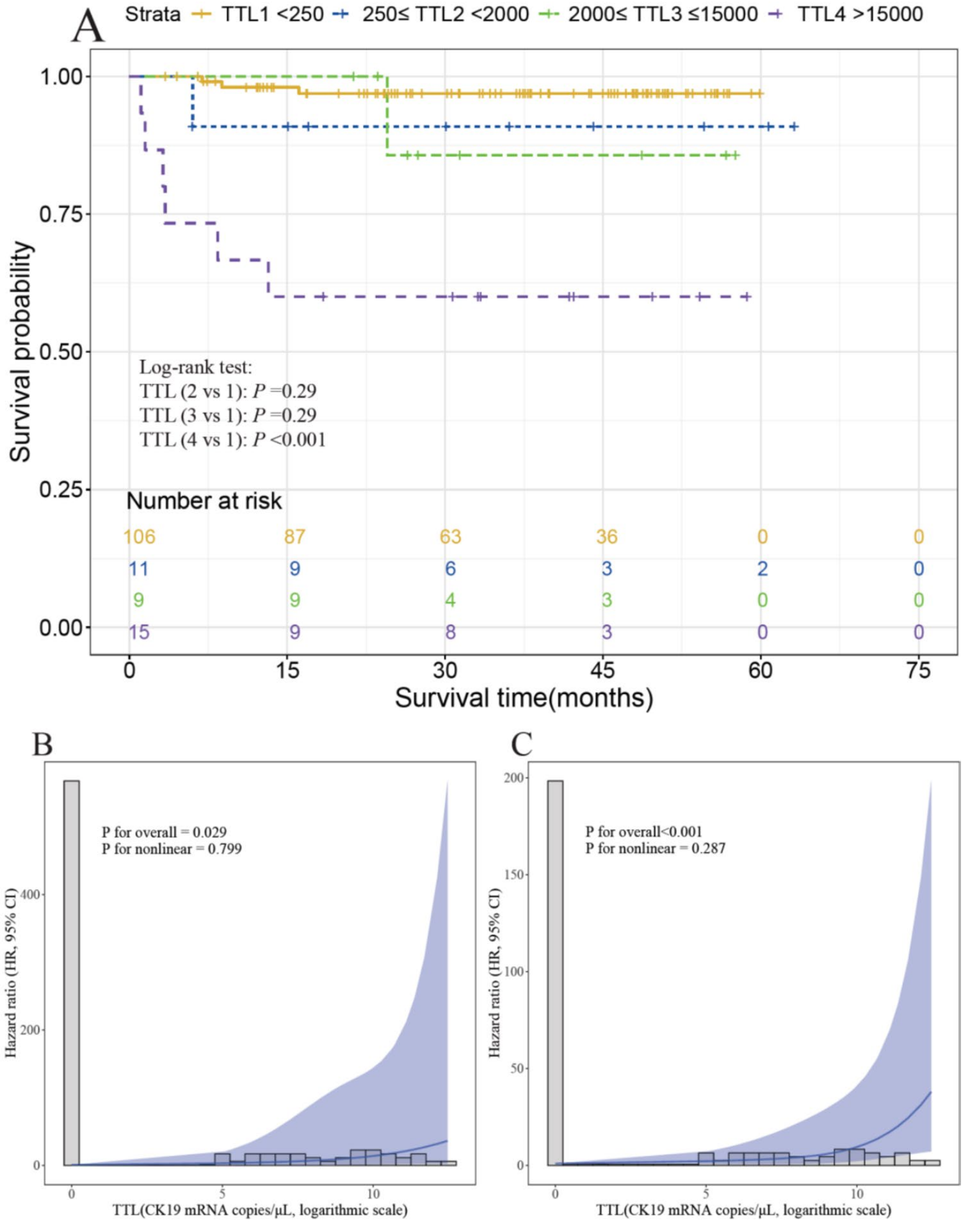

**Fig. 1** Prognostic relevance of TTL in colorectal cancer patients. **A** Kaplan–Meier estimates of RFS according to several TTL subgroups. **B** Unadjusted restricted cubic spline regression analyses of relationships between log-transformed TTL and cancer specific death. **C** Unadjusted restricted cubic spline regression analyses of relationships between log-transformed TTL and tumor recurrence. The shaded areas denote the 95% CIs. RFS indicates recurrence-free survival; TTL, total tumor load (cytokeratin 19 mRNA copies/μL)

## LN analysis with OSNA *versus.* CS

A total of 3751 LNs were dissected from 158 surgical specimens, with a mean of 23.7 LNs per case. Of those, 2779 LNs (74.1%) were freshly dissected (17.6 LNs per case), whereas 972 (25.9%) LNs were dissected after formalin fixation and analyzed only by H&E (Table 1). Totally, 24.7% (39/158) patients had a TTL of ≥ 250 copies/μL, yielding a slightly higher positive detection rate (PDR) compared to CS (21.5%; 34/158). The TTL values among these patients ranged from 320 to 258,000 copies/μL, with an average of 30,056.9 copies/μL.

A high concordance rate (93.0%) between OSNA and CS was observed, as evidenced by the Cohen's Kappa (kappa = 0.804) and McNemar ($P = 0.132$) tests. Of the 34 CS + patients, 31 were OSNA positive. OSNA resulted positive in 6.5% (8/124) of CS − patients, with TTL values ranging from 320 to 7000 copies/μL. Overall, the sensitivity and specificity of OSNA compared to CS were 91.2% and 93.5%, respectively, with a PPV of 79.5% and a NPV of 97.5% (Table 2).

## An optimal TTL cut-off

Seventeen patients were excluded due to lost follow-up or death unrelated to cancer, resulting in a total of 141 patients included for the cut-off exploration (Supplemental Fig. 1).

**Table 1** Demographic and clinicopathologic characteristics

| Characteristics | No. (%) |
|---|---|
| Sex (male) | 90 (57.0) |
| Age (years), median (IQR) | 75 (64, 81) |
| Range (years) | 24–94 |
| Tumor size (cm), median (IQR) | 4.0 (3.0, 5.2) |
| Range (cm) | 0.3–14.0 |
| *Tumor grade* | |
| Low (G1-G2) | 135 (85.4) |
| High (G3-G4) | 23 (14.6) |
| *Tumor budding* | |
| Bd1 | 109 (69.0) |
| Bd2 | 35 (22.1) |
| Bd3 | 14 (8.9) |
| *Perineural invasion* | |
| Absent | 145 (91.8) |
| Present | 13 (8.2) |
| *Lymphovascular invasion* | |
| Absent | 126 (79.7) |
| Present | 32 (20.3) |
| *Tumor deposits* | |
| Absent | 154 (97.5) |
| Present | 4 (2.5) |
| Total LNs, median (IQR) | 23.0 (18.0, 29.0) |
| Range | 7–71 |
| Freshly obtained LNs, median (IQR) | 17.0 (13.0, 21.8) |
| Range | 4–59 |
| Post-fixation LNs, median (IQR) | 5.0 (2.0, 9.0) |
| Range | 0–30 |
| *pT stage* | |
| T1 | 15 (9.5) |
| T2 | 27 (17.1) |
| T3 | 84 (53.2) |
| T4 | 32 (20.2) |
| *pN stage* | |
| N0 | 123 (77.8) |
| N1 | 20 (12.7) |
| N2 | 15 (9.5) |
| *pStage* | |
| I | 37 (23.4) |
| II | 86 (54.4) |
| III | 35 (22.2) |
| TTL, median (IQR) | 0 (0, 192.5) |
| Range | 0–258000 |
| Mean | 7423.5 |
| OSNA positive | 39 (24.7) |
| *Adjuvant chemotherapy* | |
| No | 125 (79.1) |
| Yes | 33 (20.9) |

IQR, interquartile range; TTL, total tumor load (cytokeratin 19 mRNA copies/μL); OSNA, one-step nucleic acid amplification assay; LNs, lymph nodes

a Analysis based on a subset of patients due to missing data

b A TTL of not less than 250 copies/μL was considered positive for nodal metastases

**Table 2** Diagnostic performance of OSNA versus CS

| Assay evaluated | | CS | No | Concordance (95% CI) | Sensitivity (95% CI) | Specificity (95% CI) | PPV (95% CI) | NPV (95% CI) | Kappa index (95% CI) | McNemar's *p* value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CS- | CS+ | | | | | | | |
| OSNA | – | 116 | 3 | 158 | 0.930 (0.879, 0.965) | 0.912 (0.763, 0.981) | 0.935 (0.877, 0.972) | 0.795 (0.655, 0.952) | 0.975 (0.923, 0.989) | 0.804 (0.693, 0.916) 0.132 |
| | + | 8 | 31 | | | | | | | |

OSNA, one-step nucleic amplification assay; CS, cytology smears; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval

According to the X-tile software, a TTL of 15,000 copies/μL was identified as the optimal cut-off for predicting RFS, thereby dividing patients into low-risk (TTL ≤ 15,000 copies/μL) and high-risk (TTL > 15,000 copies/μL) subgroups (Supplemental Fig. 2). To further validate this cut-off, patients were manually classified into 4 subgroups based on three thresholds of 250, 2000, and 15,000 copies/μL. As shown in Supplemental Table 1, a TTL of 15,000 copies/μL remained the most appropriate cut-off, with the greatest $\chi^2$ value of 2.333. Kaplan–Meier curves indicated that a TTL of > 15,000 copies/μL was related to significantly reduced 3-year RFS (Fig. 1A).

## Correlation of TTL with histological factors

The TTL values exhibited a significant increase across tumor stages (Jonckheere-test $P < 0.01$) and pN stages (Jonckheere-test $P < 0.01$), with a mean of 93.9 copies/μL for pN0, 29,470.0 copies/μL for pN1, and 38,130.7 copies/μL for pN2. Moreover, the presence of lymphovascular invasion (LVI), perineural invasion (PNI), and tumor deposits (TD) were significantly associated with higher TTL values (Supplemental Fig. 3B, E, H). By univariate or multivariate analyses using Firth's method, we found that only LVI + was an independent predictor for

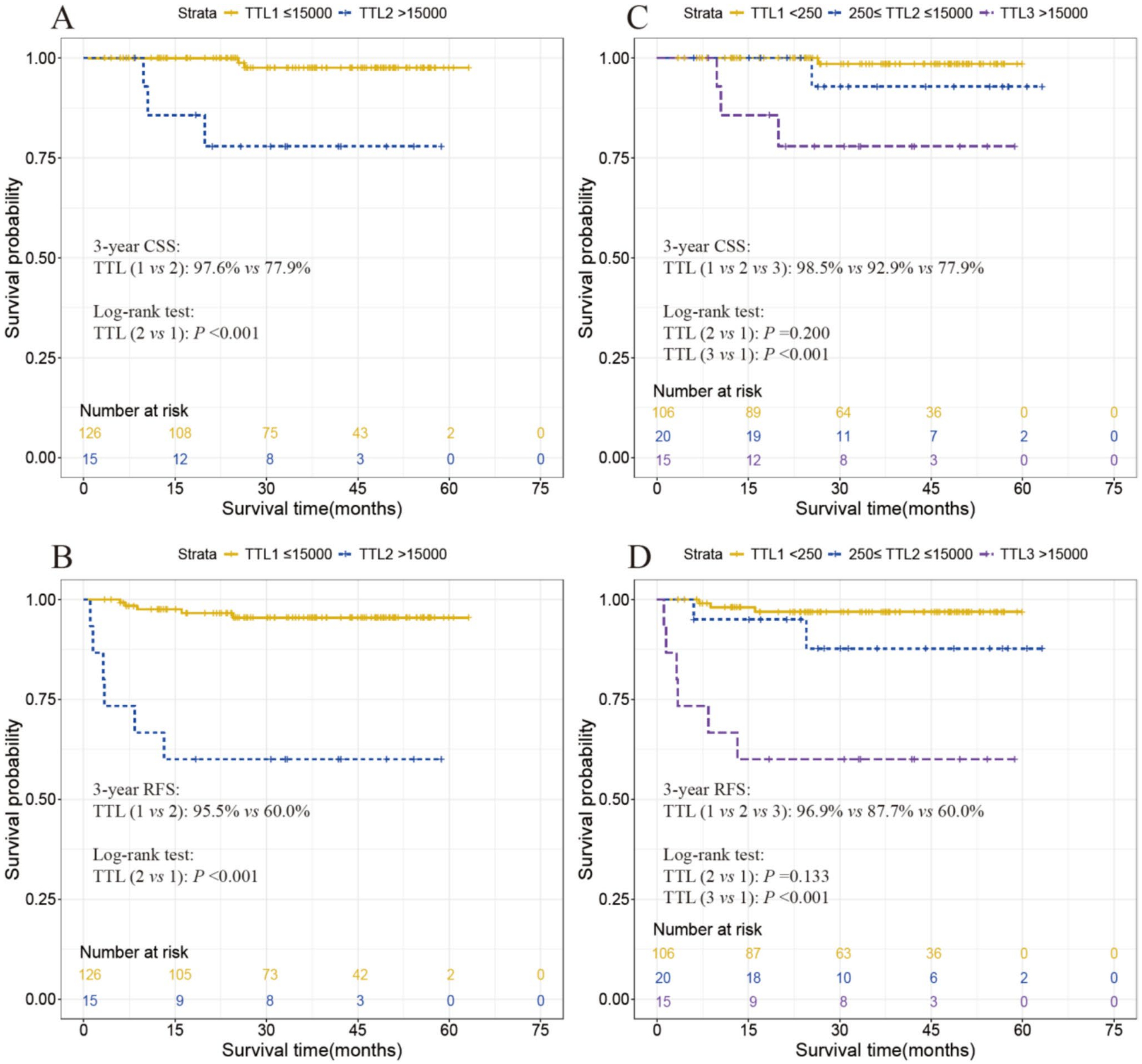

**Fig. 2** Kaplan–Meier estimates of CSS (**A**, **C**) and RFS (**B**, **D**) according to TTL subgroups; TTL, total tumor load (cytokeratin 19 mRNA copies/μL); CSS, cancer-specific survival; RFS, recurrence-free survival

LN involvement in patients with TTL > 15,000 copies/μL (Supplemental Table 2).

## Prognostic relevance of TTL

Of the 158 patients included, two patients were excluded for survival analysis due to a follow-up of less than one month. The average follow-up was 32.4 months (SD ± 16.6; range 1.0 to 63.2 months). Five patients died from cancer between 9.8 and 26.4 months after surgery. Four of them (80%) had TTL values > 15,000 copies/μL. The recurrence rates were 2.5% (3/118), 8.7% (2/23), and 40.0% (6/15) for patients with a TTL of < 250, 250–15,000, and > 15,000 copies/μL, respectively. On average, patients with recurrence showed a TTL 10.2 times higher than that of non-recurrent patients (46,503.6 versus. 4558.3 copies/μL; $P < 0.001$, data not shown).

RCS analyses demonstrated a linear dose–response relationship of log-transformed TTL with cancer-specific death ($P$ for nonlinear = 0.768) or tumor recurrence ($P$ for nonlinear = 0.245) in CRC patients (Fig. 1B-C). The results of Firth-corrected univariate and multivariate Cox regression analyses are shown in Supplemental Table 3 and Table 3, respectively. When analyzed as a continuous variable, elevated TTL levels were implicated in worse CSS and RFS ($P < 0.05$). When dichotomized at 15,000 copies/μL,

**Table 3** Multivariate Cox regression analyses according to OSNA and CS Results

| OSNA and CS results | No. (%) | Cox regression analysis for CSS | | | | Cox regression analysis for RFS | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | Model 1 | | Model 2 | | Model 3 | | Model 4 | |
| | | HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value | HR (95% CI) | P value |
| TTL (logarithmic scale) | 156 (100) | 1.27 (1.00, 1.61) | 0.015 | 1.27 (1.00, 1.62) | 0.019 | 1.30 (1.12, 1.52) | < 0.001 | 1.29 (1.11, 1.50) | < 0.001 |
| OSNA positive[a] | | | | | | | | | |
| No | 118 (75.6) | ref | | ref | | ref | | ref | |
| Yes | 38 (24.4) | 7.94 (0.92, 68.78) | 0.055 | 7.44 (0.87, 63.96) | 0.051 | 10.37 (2.34, 45.95) | 0.001 | 9.92 (2.23, 44.21) | 0.001 |
| TTL ≥ 6000 copies/μL | | | | | | | | | |
| No | 135 (86.5) | ref | | ref | | ref | | ref | |
| Yes | 21 (13.5) | 6.47 (0.71, 59.21) | 0.068 | 6.34 (0.72, 55.65) | 0.066 | 14.63 (3.37, 63.45) | < 0.001 | 12.90 (3.17, 52.59) | < 0.001 |
| TTL > 15,000 copies/μL | | | | | | | | | |
| No | 141 (90.4) | ref | | ref | | ref | | ref | |
| Yes | 15 (9.6) | 12.44 (1.33, 116.02) | 0.014 | 17.98 (1.80, 179.44) | 0.007 | 11.47 (2.84, 46.26) | < 0.001 | 10.59 (2.75, 40.81) | < 0.001 |
| TTL subgroups, copies/μL | | | | | | | | | |
| (~, 250) | 118 (75.6) | ref | | ref | | ref | | ref | |
| [250, 15000] | 23 (14.7) | 5.23 (0.38, 71.99) | 0.182 | 3.97 (0.28, 57.21) | 0.265 | 5.10 (0.78, 33.21) | 0.089 | 4.83 (0.73, 31.94) | 0.101 |
| (15,000, ~) | 15 (9.6) | 20.10 (1.44, 280.64) | 0.008 | 25.25 (1.77, 359.55) | 0.005 | 18.00 (3.63, 89.22) | < 0.001 | 16.53 (3.44, 79.44) | < 0.001 |
| TTL subgroups, copies/μL | | | | | | | | | |
| [0, 250) | 118 (75.6) | ref | | ref | | ref | | ref | |
| [250, 6000) | 17 (10.9) | 7.65 (0.57, 102.10) | 0.100 | 7.10 (0.52, 97.79) | 0.114 | 3.78 (0.44, 32.40) | 0.241 | 3.50 (0.39, 31.16) | 0.274 |
| [6000, ~) | 21 (13.5) | 9.66 (0.77, 121.95) | 0.038 | 9.06 (0.75, 109.16) | 0.060 | 18.29 (3.65, 91.55) | < 0.001 | 15.96 (3.34, 76.21) | < 0.001 |
| CS results | | | | | | | | | |
| CS- | 122 (78.2) | ref | | ref | | ref | | ref | |
| CS + | 34 (21.8) | 13.00 (1.18, 143.55) | 0.018 | 11.77 (1.07, 129.27) | 0.021 | 15.43 (2.85, 83.67) | < 0.001 | 13.90 (2.64, 73.06) | < 0.001 |
| Combine (CS results and the optimal TTL cut-off) | | | | | | | | | |
| CS- | 122 (78.2) | ref | | ref | | ref | | ref | |
| CS + (TTL ≤ 15,000) | 19 (12.2) | 8.33 (0.48, 144.19) | 0.122 | 5.47 (0.30, 98.98) | 0.216 | 7.35 (0.91, 59.44) | 0.055 | 6.64 (0.85, 51.67) | 0.065 |
| CS + (TTL > 15,000) | 15 (9.6) | 26.82 (1.82, 396.02) | 0.006 | 30.01 (2.03, 442.65) | 0.004 | 21.00 (3.93, 112.31) | < 0.001 | 19.27 (3.71, 100.12) | < 0.001 |

CSS, cancer-specific survival; RFS, recurrence-free survival; HR, hazards ratio; CI, confidence interval; OSNA, one-step nucleic acid amplification; TTL, total tumor load (cytokeratin 19 mRNA copies/μL); CS, cytology smears; ref, reference

[a] A TTL of not less than 250 copies/μL was deemed as positive for nodal metastases

Model 1 was adjusted for age (continuous), sex, lymphovascular invasion, and adjuvant chemotherapy

Model 2 was adjusted for age (categorical), sex, lymphovascular invasion, and adjuvant chemotherapy

Model 3 was adjusted for age (continuous), sex, tumor size (continuous), tumor grade, perineural invasion, lymphovascular invasion, and adjuvant chemotherapy

Model 4 was adjusted for age (categorical), sex, tumor size (continuous), tumor grade, perineural invasion, lymphovascular invasion, and adjuvant chemotherapy a TTL > 15,000 copies/µL showed significant associations with worse CSS and RFS in all models ($P < 0.05$). When trichotomized at both 250 and 15,000 copies/µL, multivariable-adjusted analyses revealed that a TTL of > 15,000 copies/µL remained an independent predictor for reduced CSS and RFS ($P < 0.05$), whereas TTL levels between 250 and 15,000 copies/µL were not significantly associated with poorer prognosis in any model ($P > 0.05$). Additionally, our results indicated that CS + patients with TTL > 15,000 copies/µL had worse CSS and RFS in all models ($P < 0.05$), while TTL ≤ 15,000 copies/µL failed to confer poorer prognosis to CS + patients ($P > 0.05$) (Table 3). Kaplan–Meier curves demonstrated significantly reduced 3-year CSS and RFS associated with a TTL of > 15,000 copies/µL in CRC patients (Fig. 2A–D, Supplemental Fig. 4A,B).

## Discussion

In this study, we aimed to investigate the prognostic significance of the analysis of the whole LN tissue with the OSNA assay in CRC patients. Our findings demonstrate that OSNA exhibits a high sensitivity for LNM detection, establishing an optimal TTL threshold of 15,000 copies/µL that stratifies CRC patients into low and high risk for both CSS and RFS with significant prognostic implications. In addition, CS pN-positive patients with TTL ≤ 15,000 copies/µL had comparable long-term outcomes to CS pN-negative patients, not related to worse CSS or RFS, therefore, not clinically relevant. We conclude that clinical implementation of whole LN analysis by OSNA improves CRC patient management.

The main advantages of the OSNA method include being highly sensitive, fast, quantitative, and automated [34]. Nearly two decades have passed since Tsujimoto et al. pioneered the use of the OSNA assay for the analysis of sentinel LN (SLN) in breast cancer patients [26], where it has demonstrated both predictive and prognostic value [10–15]. It was later extended to CRC and other solid neoplasms [16–22, 35–38]. Notably, it has shown superior performance to conventional H&E analysis [29, 30, 39, 40], equivalent to that of an intensive histology examination at 2 mm intervals [27]. Additionally, it has demonstrated its prognostic value in CRC [35, 36, 41]. Likewise, it may provide a novel paradigm for molecular node staging, as it reflects the amount of the metastatic tumor volume or tumor burden within the LN compartment, rather than the number of metastatic LNs.

The gold standard of nodal staging is based on the number of positive LNs identified with H&E, which has been included in most guidelines, such as the AJCC and UICC, as benchmarks for survival stratification and indication of postsurgical therapy. Nevertheless, the histologic evaluation of LNs is not exempt of false-negative cases, since < 1% of the total LN tissue is analyzed with H&E [6]. This is evidenced by the fact that approximately 20–25% of pN0 CRC patients may develop disease recurrence following curative-intended surgical resection [42, 43]. Previous studies involving OSNA in CRC analyzed only part of the LN tissue, as the whole LN analysis would preclude the standard H&E evaluation necessary for pN staging. Thus, TAB constituted a fundamental barrier to the implementation of OSNA as a molecular LN staging technique in CRC routine practice. In an attempt to improve this scenario, we have recently proposed the performance of CS on fresh LNs to obtain the number of positive LNs, therefore, the pN stage of CRC patients. This has paved the way toward the use of the whole LN tissue for OSNA analysis [22]. To the best of our knowledge, our study is the first one that uses the whole LN for OSNA analysis in CRC patients.

This study demonstrates a comparable diagnostic performance between OSNA and CS for detecting LNM in CRC patients. These results are in line with our previous study in an independent cohort of patients with comparable OSNA sensitivity and specificity [22]. Thus, although the sensitivity of CS and OSNA are similar, the TTL is related to CSS and RFS, conferring a reliable objective prognostic value, which enables the selection of high-risk patients who might benefit from postsurgical adjuvant therapy, and sparing low-risk patients from overtreatment.

Regarding breast cancer, where the OSNA analyzes the whole SLN, an established TTL threshold of 25,000 copies/µL allows to classify patients into low and high-risk for tumor recurrence, irrespective of the use of neoadjuvant treatment [10, 11]. Yet, such a widely accepted TTL threshold has not been determined for CRC patients. So far, several studies have indicated that TTL levels of ≥ 250 or ≥ 6,000 copies/µL could significantly confer worse prognosis to CRC patients [35, 36]. Nevertheless, in all these studies, only a part of the LN tissue was used for molecular analysis by OSNA, resulting in an underestimation of the real LN metastatic tumor burden, or TTL. Therefore, the optimal cut-off which has clinical prognostic value was unable to be determined.

To the best of our knowledge, this study establishes for the first time an optimal TTL threshold of 15,000 copies/µL, providing a robust approach for identifying those CRC patients at high risk of tumor relapse. Our results indicate that TTL levels of > 15,000 copies/µL are significantly associated with reduced CSS and RFS across all cox models. In addition, although CS has a high sensitivity for LN tumor detection, a CS pN-positive result does not state the amount of LN tumor burden and adds little information about its clinical significance. Notably, we observed that CS + patients with TTL ≤ 15,000 copies/µL showed comparable long-term outcomes to CS- patients, whereas CS + patients with a TTL of > 15,000 copies/µL had significantly reduced CSS and

RFS. This phenomenon is likely attributable to the detection of ITCs, corresponding to low TTL values.

There are several limitations in this study. Firstly, the OSNA analysis can only be performed on freshly harvested LNs. LNs identified following formalin fixation can only be assessed by H&E, contributing to the conventional pN stage. In future, an improved LN analysis technique should be developed to allow quantification of the amount of the LN tumor burden in formalin-fixed tissue. Secondly, the small sample size of our study prevented to separate a training set and a validation set, which may potentially compromise its reproducibility and generalizability. Thirdly, this study had a mean follow-up of 32.4 months, during which some patients might not have experienced endpoint events as in 5-year follow-up. However, we observed CRC-related deaths occurring at a mean of 18.4 months and tumor recurrences at a mean of 8.5 months, suggesting that the follow-up period in this study was adequate for assessing CSS and RFS.

## Conclusion

This is the first multicenter prospective study to evaluate the prognostic relevance of molecular analysis with the OSNA assay using the whole LNs tissue from CRC specimens. In addition to its high sensitivity for detecting LNM, we have also established an optimal TTL cut-off value of 15,000 copies/μL, which stratifies CRC patients into low and high risk for both CSS and RFS. We conclude that the clinical implementation of whole LN molecular assessment not only is of prognostic value, but it enables a fast turnaround time for patient risk stratification and personalized therapy.

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**Author contributions** MC and IA contributed to the conceptualization and study design. LQ, SL-P and IA contributed to acquisition, analysis, and interpretation of the data. LQ, SL-P, IA and MC contributed to the draft of the manuscript. IA, MC, KSG, MR-G, JRM, AR, IP, JT, IM, MMB, JM, RB and JC contributed to the manuscript critical review. LQ contributed to the statistical analysis. All authors reviewed the manuscript.

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**Data availability** No datasets were generated or analyzed during the current study.

## Declarations

**Competing interests** The authors declare no competing interests.

**Consent to participate** Informed consent was obtained from all individual participants included in the study.

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