

Second evaluation of the mesenteric tissue after ethanol fixation improved the total and metastatic number of lymph nodes in colorectal resections

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ABSTRACT


Context: There is a correlation between prognosis of the colorectal carcinomas and the number of retrieved and metastatic lymph nodes (LNs) from mesentery/mesorectal region. At least 12 LNs must be sampled for accurate evaluation of patients. A number of factors related to surgeon, pathologist, patient and disease could affect the total LN number. For maximizing LN yield, pathologist can use ancillary methods, as fat clearance and special solutions. **Aims:** This study investigates the effect of second evaluation after ethanol fixation on total and metastatic LN number and assesses factors that influence the dissected LN number. **Materials and Methods:** 177 colorectal resections were refixed with ethanol for a night, after standard LN sampling. Mesentery/mesorectal tissue was reevaluated for missed LNs. Results were statistically analyzed, *P* values <0.05 were considered significant. **Results:** Mean LN number increased from 26 to 30 (median: 20 to 25, *P* < 0.001) after ethanol fixation. Fourteen cases had additional metastatic LNs after reevaluation of the fat tissue and 5 of them upstaged. 22.5% (44/177) of the patients had <12 LNs before ethanol fixation and this decreased to 14.3% (26/177) after ethanol fixation. Resection type and length, tumor localization, size and histologic degree, pT and neoadjuvant therapy (*P* < 0.001) had an impact on the LN number (*P* = 0.034 for histologic degree, *P* = 0.02 for pT, *P* < 0.001 for others). **Conclusions:** Carrying out a second evaluation with ethanol fixation increased total and metastatic LN number and could lead upstage of pN. Ethanol fixation is cost-effective, easy accessible and applicable method; it may improve accuracy of LN assessment and staging, which are important for patients' outcome.

KEY WORDS: Colorectal carcinoma, ethanol, fat clearance, lymph node, lymph node sampling

INTRODUCTION

Colorectal carcinoma (CRC) is the most common tumor of the gastrointestinal tract. Survival and prognosis of CRC is improved when the number of harvested lymph nodes (LNs) identified in resection specimen increase. According to the current classifications; staging and therapy options depend on the involvement of LNs; and LN metastasis is one of the major prognostic factors.^[1] Many associations recommend 12 LNs as a minimum number for accurate and reliable staging.^[1-3]

A number of clinicopathological factors have influence on nodal count. These are patient age, gender, obesity, surgeons', pathologists' and pathology assistants' experience and skill, surgical technique, time spent for and ancillary dissection techniques used for

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retrieving LNs, tumor site, differentiation, stage and neoadjuvant therapy.^[4]

Regarding pathology related factors, sampling the LNs in the grossing room is critical. Pathologists', residents' or assistants' experience and time are important factors. The standard LN sampling method is serial sectioning of the mesenteric tissue, visual inspection and palpation. With this method, especially small LNs can be overlooked. For achieving not only sufficient but also maximum LN

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number in gross room, various ancillary techniques have been reported. These target to clear mesenteric fat for a straightforward sighting of LNs. Many different methods such as fat clearance, Schwartz solution, GEWF (glacial acetic acid, ethanol, water and formalin) solution, fat dissociation method, are defined in the literature. Although all methods have some disadvantages such as time consumption, toxicity, specialized preparation and cost, eventually they achieved to increase total and metastatic LN number.^[5-12]

The purpose of this study was to evaluate ethanol fixation effect on the number of harvested and metastatic LNs in colorectal resections and to identify factors associated with the total LN number.

MATERIALS AND METHODS

Patients with colorectal carcinoma, underwent any type of colorectal resection over a 3-years period were routinely handled. Each specimen was left to formalin fixation, then, after sampling tumor, surrounding tissue and surgical margins, dissecting of the LNs was performed according to this procedure: First, parallel sections, as thin as possible, were performed to mesentery/mesorectal tissue. Lymph nodes that could be seen by naked eye were sampled entirely. For smaller ones, fatty tissue was palpated and small ones were sampled entirely, also. After the manual dissection, the mesentery fat tissue was put in a 96% ethylene alcohol (Vinprom Peshtera, Bulgaria) fixation until the next day. Then all the fatty tissue was reevaluated for missed LNs by the staff that made the first dissection [Figure 1]. All picked up LNs were evaluated on H and E stained slides; total and metastatic number of LNs harvested before (standart technique) and after ethanol fixation, the diameters of the LNs found after ethanol fixation were noted.

Patients' demographic data, type of surgery, surgeon, and neoadjuvant therapy were collected from the hospital system. Tumor site and size, resection length, histologic grade, pT, pN, therapy effect (according to AJCC 7th edition) were recorded from pathology reports, retrospectively. Correlation between total LN number and patients age, gender, material length, tumor size, site and grade, neoadjuvant therapy was also investigated.



Figure 1: Residual small lymph nodes can be seen easily after ethanol fixation. Inset: Closer view of the lymph node

Statistical analysis

Statistical analyses were done with Wizard for mac (Version 1.8.16 (182)). One-way ANOVA and the Student's *t*-test, Mann-Whitney U test and the Kruskal-Wallis tests were used for statistical analysis. Differences with *P*value <0.05 were considered statistically significant.

RESULTS

Total 177 colorectal specimens resected for adenocarcinoma were evaluated. All patients' demographic and pathologic data are summarized in Table 1.

There was a relationship between dissected LN number and resection type, length, tumor site and size, pT and histologic degree. In anterior resections, less number of LNs was detected (*P* < 0.001), while right hemicolectomies and subtotal colectomies had more LNs (*P* < 0.001). Tumors located in ascending colon and caecum had more LN (*P* = 0.006 and

Table 1: Demographic distribution of all cases (n=177)

Parameters	Cases
Age	60 years old (minimum: 26, maximum: 87, median: 61)
Gender, <i>n</i> (%)	Male: 113 (62.1); female: 69 (37.9)
Material type, <i>n</i> (%)	Total colectomy: 6 (3.3); subtotal colectomy: 8 (4.4); right hemicolectomy: 46 (25.3); left hemicolectomy: 3 (1.7); anterior resection: 114 (62.6); abdominopelvic resection: 5 (2.7)
Surgeon specialty, <i>n</i> (%)	Colorectal surgeon: 166 (91.2); noncolorectal surgeon: 16 (8.8)
Tumor type, <i>n</i> (%)	Adenocarcinoma: 149 (81.9); mucinous carcinoma: 14 (7.7); no viable tumor: 7 (3.8); other: 12 (6.5)
Tumor site, <i>n</i> (%)	Caecum: 24 (13.2); ascending colon: 13 (7.1); hepatic flexura: 6 (3.3); transvers colon: 3 (1.6); splenic flexura: 7 (3.8); descending colon: 2 (1.1); sigmoid colon: 37 (20.3); rectum: 81 (44.5); anus: 2 (1.1); no viable tumor: 7 (3.8)
Length of the specimen	Mean: 38 cm (minimum: 9 cm, maximum: 124 cm, median: 30 cm)
Tumor size	Mean: 4.9 cm (minimum: 0 cm, maximum: 17 cm, median: 4.3 cm)
Histologic grade, <i>n</i> (%)	Low: 152 (88.9); high: 19 (11.1)
Neoadjuvant therapy, <i>n</i> (%)	No: 117 (64.3); yes: 65 (35.7)
Neoadjuvant therapy response (according to AJCC), <i>n</i> (%)	Grade 0: 7 (10.8); grade 1: 12 (18.4); grade 2: 23 (35.4); grade 3: 23 (35.4)
pT, <i>n</i> (%)	pT0 (no viable tumor and high grade adenoma): 9 (4.9); pT1s: 2 (2.2); pT1: 2 (2.2); pT2: 22 (12.1); pT3: 98 (53.9); pT4a: 36 (19.8); pT4b: 9 (4.9)
pN, <i>n</i> (%)	pN0: 104 (57.1); pN1a: 24 (13.2); pN1b: 22 (12.1); pN1c: 3 (1.7); pN2a: 13 (7.1); pN2b: 16 (8.8)
Total LN number	Mean: 30 (minimum: 4, maximum: 102, median: 25)
Total metastatic LN number	Mean: 2 (minimum: 0, maximum: 17, median: 0)

AJCC: American Joint Committee on Cancer; LN: Lymph node

$P < 0.001$, respectively) but rectum and sigmoid colon located tumors had less LN ($P < 0.001$ and $P = 0.006$, respectively). Lymph node number was correlated with pT; pT2 tumors had less LN than pT3 and pT4 tumors ($P = 0.02$). Low-grade tumors had less LN ($P = 0.034$). Neoadjuvant therapy had also effect on the LN number ($P < 0.001$). Besides, when there was any degree of response to the neoadjuvant therapy, LN number decreased, compared with the no therapy response ($P = 0.03$). There was not any correlation between total LN number and surgeons' subspecialty, patients' gender and age, and tumor type.

When LNs were sampled by traditional palpation method, mean number of dissected LN was 26 (range: 2-99, median: 20). Seventy (39.5%) of the cases had metastatic LN with a mean number of 2 (min-max: 1-17). After second evaluation with ethanol fixation, the mean LN number increased to 30 (range: 4-102, median: 25); and 74 (41.8%) of the cases had newly found metastatic LNs [Table 2]. The second evaluation with the use of ethanol fixation resulted in increase in LN number ($P < 0.001$). The diameter of the missed LNs was ranged between 0.1 cm to 1.6 cm with mean and median value of 0.3 cm. After second evaluation with ethanol fixation, a total of 698 missed LNs were found in 156 cases. In 14 cases, 15 newly found LNs had metastasis. The probability of finding missed and metastatic LN in each specimen by second evaluation using ethanol fixation was 88% and 7.9%, respectively [Table 2].

Of these 14 cases with new metastatic LNs, 4 had neoadjuvant therapy. Also, in 3 of these 14 cases, pN was upstaged from pN0

Table 2: Second evaluation with ethanol effect on lymph node numbers and pN

	With traditional sampling	Found after second evaluation with ethanol fixation	Total
Total LN		<i>n</i> =177	
<i>n</i>	4534	698	5232
Mean	26	4	30
Median	20	3	25
Range	2-99	0-15	4-102
Metastatic LN		<i>n</i> =14	
<i>n</i>	291	25	316
Mean	4	2	4
Median	3	1	3
Range	1-17	1-4	1-17
pN			
N0	103		99
N1a	24		24
N1b	21		22
N1c	1		3
N2a	12		13
N2b	16		16
pN upstage			
N0-N1a			1
N0-N1b			1
N0-N1c			2
N1b-N2a			1

LN: Lymph node

to pN1a, pN0 to pN1b and pN1b to pN2a. Additionally, in 2 cases, tumor nodules were found and these patients upstaged from pN0 to pN1c [Table 2].

Sixty five (36.7%) patients had neoadjuvant therapy, in our study group. With traditional LN sampling method, mean total LN number was 15 (median: 12, ranging 2-66), after second evaluation with ethanol fixation; mean total LN number was 17 (median: 14, ranging 4-68). This increase was statistically significant ($P < 0.001$). Missed metastatic LNs were found in 4 cases; 1 LN in 2 cases, 2 LNs in 1 case and 3 LNs in 1 case.

With traditional sampling method, 41 (22.5%) of the cases had total number of LN less than 12, but after second evaluation with ethanol fixation, it decreased to 26 (14.3%) cases. Ten of these 26 cases had no additional LNs despite second evaluation and ethanol fixation.

DISCUSSION

Correct staging of CRC is crucial for planning therapy and determining the prognosis. pT and pN, depending on the depth of tumor invasion and metastatic LN numbers, are critical for staging.^[1,3,4] Not only the metastatic LNs but also the total number of dissected LNs is important. Studies showed that approximately 20-25% of the node negative patients relapse and especially stage II patients with more number of LNs have better prognosis.^[13-15] The possible explanation is when more LNs are evaluated, the chance of finding positive node increases, and by lymphadenectomy, metastatic spread and tumor clearance could be achieved more successfully.^[6,16] Also, entity of lymphadenectomy can be used as a quality parameter for pathology laboratories.^[2,17]

Some authors believe that there should be no limit for the number of evaluated LNs.^[14,18] But for correct staging, the minimum/accurate LN number '12' was first established at the World Congress of Gastroenterology in 1990 and after that many guidelines, such as Collage of American Pathologists, referred this number.^[2,3]

In this study, retrieved LN number ranged between 4 and 102 with a mean of 30. We achieved to dissect 12 and more LNs in 85.7% (156/177) of the cases. Before second evaluation with ethanol fixation, the ratio was 77.5% (141/177). Compared with the reported series that ranged from 26.5% to 93.6%, our finding is one of the highest values.^[6,10,19] We can explain this by our dissection method. Because, in our routine practice, we aim to find as much as LNs we can, so that we perform second evaluation to mesenteric/mesorectal fat tissue after 1 night ethanol fixation. Ethanol fixation makes LNs easily visible in second evaluation.

Different variables can affect the harvested LN number. These can be divided into two categories as modifiable and nonmodifiable factors. Modifiable factors are surgeon's and pathologist's experience, surgical technique, ancillary dissection techniques and time spend for the retrieval by pathologists; and

nonmodifiabiles are patients age, gender, obesity and tumor site, stage and neoadjuvant therapy.^[4,20]

Most of the studies indicated that advanced age and rectum located tumor can affect LN number negatively while right colon location of tumor, grade and pathologic T stage can effect positively.^[15,16,21-25] There are conflict data on the effect of gender and obesity.^[2,13,16,25] In our study, age and gender of the patients, tumor type and pT did not correlate with the LN number. Similarly with the publications, there were less LN in anterior resection specimens and the tumors located at rectum and sigmoid colon ($P < 0.001$). There was positive correlation between length of the resection and tumor size with total LN number ($P < 0.001$, for both). Since rectum and sigmoid colon location and neoadjuvant therapy had negative effect on total LN number, we advise to seek LNs with additional techniques such as second evaluation, ethanol fixation for these patients.

Although surgeons' experience reported as an independent factor for number of removed LNs by some publications, in 3 studies comparing surgeons, there was no exact difference between dissected LN numbers and the experienced/inexperienced surgeon (eg. 13 vs 11 LN, 11 vs 9 LN and achieving >12 LN in the 86% and 83% of the patients).^[15,22,26-28] In our study, there were 2 surgeons performing the operations; one of them was a colorectal surgeon. Although case number of the noncolorectal surgeon was less ($n = 16$), there was no statistically significant difference between two of them ($P = 0.9$). Also, we couldn't find any difference between two surgeons when LN cutoff value was taken as 12 ($P = 0.2$).

Lymph node dissecting pathology staff, such as pathology assistants, residents and pathologists, could affect the number of sampled LN and some studies indicate that it is an independent factor as surgeons' experience.^[22,23,27,28] Time, educational training and skills are important factors intervening with this finding. Bomboat *et al.* and Kuijpers *et al.* showed that pathology assistants and first year pathology residents can achieve dissecting more LNs, especially in rectal resections, than experienced pathologists because they have more time, less distractions and may use ancillary techniques such as fat clearing solutions and intra-arterial methylene blue injection.^[14,25,26]

American Collage of Pathologists recommend to use ancillary techniques while or after manual sampling of the LNs especially if the LN number is less than 12, because the remaining mesentery tissue could contain additional LNs.^[3,8] There are several methods for this purpose, such as fat clearance, Schwartz solution, GEWF solution, fat dissociation method, transluminating the mesentery, entirely sampling the mesenteric tissue, extending the fixation time and dye injection. Solutions contain various proportions and combinations of some chemical agents, such as acetone, acetic acid, diethyl ether, ethanol, formalin, hydrochloric acid and xylene.^[5-7,10,11,19,29,30] With these methods, additional mean LN numbers varied from 1 to 48.^[11,19,29-32] But in some studies no statistically significant improvement was found when

compared to manual dissection.^[7,30] Increase in metastatic LN number and even upstaging of pN, ranging 1% to 33% of the cases, was also reported by authors.^[11,30-32] Size of the missed LNs ranged from 1 to 15 mm, usually 1-5 mm.^[7-9,11,12,19,29,31] However, there are some limitations for each procedure. These are time consumption (1-9 days), special preparation need, toxicity and additional cost.^[5-10,31] There is no consensus on which method is more efficient but all these methods aim to clear fat and visualize the LNs distinctly and easily without damaging the tissue. Among all the solutions and methods, GEWF is one of the most studied solutions in the literature. It is reported as a safe and efficient lymph node revealing solution, and also preparing is quick, cheap and easy.^[7,12,19]

Not only for achieving the minimum required number, but also finding the missing LNs and reaching the maximum LN number, we performed additional second evaluation with ethanol fixation to our colorectal resection specimens after standard LN sampling. Ethanol was easily accessible, cost-effective for laboratory, and was no need to prepare new solutions. In our study, mean and median number of LNs with traditional sampling was 26 and 20 (ranging 2-99), while after second evaluation with ethanol, mean and median number were 30 and 25 (ranging 4-102). This increase in LN number was statistically significant ($P < 0.001$). Usually, small LNs were found after second evaluation, with a mean size of 0.3 cm. Ethanol fixation made these remaining small LNs readily visible. Metastatic LN number also increased in 14 cases, in our study. Moreover, new tumor nodules were found in 2 cases. Eventually 3 cases (1.7% of all cases) upstaged by additional metastatic LNs and 2 cases (1.1% of all cases) upstaged pN0 to pN1c.

Neoadjuvant therapy also affects the LN sampling by posttherapy inflammation and fibrosis.^[17,20,33] Reported mean LN numbers ranged from 7 to 9.8 for patients treated with neoadjuvant therapy, and Marks *et al.* concluded that limit of 12 LN cannot be used for these patients.^[18,21,23,33] This decrease is statistically significant and more distinct when radiotherapy and chemotherapy are used in combination.^[12,13] This finding can also be interpreted as the positive response to the therapy. In our study, 35.7% (65/177) of the patients received neoadjuvant therapy. It was not surprising for us that 84.6% of the cases, which had less than 12 LNs, received neoadjuvant therapy. Mean LN numbers were less for patients who had neoadjuvant therapy than the patients that did not have neoadjuvant therapy but after second evaluation with ethanol fixation mean LN number increased from 15 to 17. This increase was statistically significant. In 4 cases having neoadjuvant therapy, we found additional metastatic LNs after second evaluation with ethanol fixation.

CONCLUSION

In conclusion, we highly recommend second evaluation of LNs with using additional visual enhancement techniques, such as ethanol fixation in routine practice for all case but especially in these conditions: i. anterior resection specimens, ii. patients taking neoadjuvant therapy, iii. when total LN number

is <12. With ethanol fixation, LNs, even the small ones, can be visualized easily. Second evaluation and ethanol fixation are effective, simple, cheap and accessible methods for all pathology laboratories.

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Conflicts of interest

There are no conflicts of interest.

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