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The pH Levels of Sentinel Lymph Nodes and the Probability of Cancer Involvement in Breast Cancer Patients: a Cross-Sectional Study

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ABSTRACT

Background: Cancer cells in lymph nodes can undergo metabolic changes, producing acidic byproducts. Our previous experimental results demonstrated significant pH differences between involved and free lymph nodes (LNs), potentially attributed to the release of these acidic byproducts. However, the accuracy of frozen sections is influenced by pathologists' expertise and limited response time. To address these limitations, we developed a simple pH sensing method.

Methods: We conduct a cross-sectional study on non-neoadjuvant breast cancer patients (n= 34) from Shohada Tjarish Hospital and Azar Surgical Clinic to check the agreement between the pH value of sentinel lymph nodes (SLNs) and their pathologic score. To assess the pH assay's validity in detecting metastasized cancer cells in lymph nodes, the sensitivity, specificity, and accuracy of the sensor were calculated using Permanent pathology of LNs as the reference standard.

Results: The results showed that cancer SLNs had pH≤ 7, while free LNs had pH > 8 (P-value <0.001). The sensitivity and specificity of the data were 89% (95% CI (0.73-1.05)) and 82% (95% CI (0.64-1.00)), respectively.

Conclusion: Hence, in the absence of a frozen section, such a simple measurement may assist the surgeon in deciding whether to dissect more LNs or not.

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INTRODUCTION

It is well-known that in breast cancer, the lymph node is the earliest invasion site which may be affected by tumor cells due to the close contact between the tumor and SLN through lymphatic vessels.[1]

Many references assumed the involvement of SLN as the first step of disease systemic invasion and progression.[2-4] Hence, many attempts were dedicated to finding the involved SLNs.[5-11] Besides the conventional approved method, other interesting methods for the detection of metastatic cancer cells in lymph nodes have been reported like flow cytometry[12], RT-PCR[13], Raman spectroscopy[14], microwave spectroscopy[10], and Electrical Impedance Spectroscopy (EIS).[11,15,16] However, some of these methods are time-consuming and expensive, and most of them are not clinically calibrated.

Also, many methods were developed for pre-surgical scoring of LNs; still, the surgeons are in doubt about ruling out the involvement of reactive LNS during surgery, especially after touching them intra-operatively. On the other hand, many centers have not frozen section facilities to inform the surgeon about the state of SLNs.[17,18] Such information may be crucial in continuing the surgical plan.

Missing the involved Auxiliary LNs (ALNs) or over-dissection of normal ALNs are both destructive mistakes due to non-precise data and intervention procedures.[19,20]

Previously, we showed that a pH sensing procedure could intraoperatively check the pH of LN fluid. We presented an experimental pathological calibration between the pH values and cancer involvement in LNs.[18] In this paper, we designed a cross-sectional study to check the validation of the intra-operative LN pH sensing approach on 34 non-neoadjuvant breast cancer patients. The reference standard for checking the validity of the results was the permanent pathology of LNs. pH recording is a method for detecting the acidic content produced by cancer cells' metabolism in LNs. This simple measurement can assist the surgeon in making informed decisions about whether to dissect more LNs in the absence of a frozen section.

METHODS

Study design

This study is a cross-sectional design. To achieve a sensitivity of more than 67.9% and a specificity of 99.3% using the pH method, a sample size of 29 is needed with a statistical power of 80% and a margin of error of 0.05.[21] Considering 10% dropout, the total sample size required is 33 patients. The pH assay measurements were conducted on the lymph nodes of 34 female patients diagnosed with breast cancer who were recommended for lymph node dissection to establish diagnostic calibration based on lymph node pH changes. Patient recruitment was conducted at two medical centers, Shohada Tjarish Hospital and Azar Surgical Clinic in Iran, from February to June 2023. After being informed about the procedure and its implications, the patients provided consent. The project received approval from the Institutional Review Board (IRB) and the Research Ethics Committee (REC) under the ID IR.SBMU.CRC.REC.1401.022.

Inclusion and Exclusion Criteria

All patients diagnosed with breast cancer, regardless of gender, age, and surgical history, were recommended for lymph node dissection based on the surgeons' opinions (Sentinel lymph node biopsy (SLNB) or Auxiliary lymph node dissection (ALND)) as registered in the study. The patients included those undergoing both breast-conserving surgery (BCS) and mastectomy. The study focused on non-neoadjuvant chemo/hormone therapy candidates (n=34), excluding neo-adjuvant cases.

Lymph node pH evaluation system

This system consists of a micro-syringe filled with sterile Deionized (DI) water and a syringe needle embedded with litmus paper. After injecting 50µl of DI water into the LNs, a syringe needle embedded with litmus paper was entered into the LNs. Then the color of litmus paper was checked to determine the acidity or basicity of the lymph node fluid based on FAO/hypoxia glycolysis metabolism of cancer cells in LNs. The test can be repeated with three needles, and if even one showed a pH lower than 7 (as experimentally calibrated), it was considered that the LN was involved.[18,22-24]

Measurement protocols

After opening the surgical field, litmus paper (ColorpHast® pH Test Strips, Merck, Germany) was inserted in the three different points of the individual LNs after dropping 50µl DI water to make hemogenic fluidic ambient in the LN.

After 10 seconds, the color of the paper was recorded, and due to the pH diagram, a score about the normal or cancer involvement state of the LN based on the pathology-calibrated classifications reported in a previous study[18] was assigned to the LNs (Figure 1).

All the histological and immunohistochemical staining assays on LNs were carried out blindly to check the pH-based scoring accuracy of LNs. All of the tests were performed under the license of the Ethics Committee of Shahid Beheshti University of Medical Science with the informed consent of the candidate patients.

Statistical analysis

The patients' characteristic data was presented as means ± standard deviation (Mean ± S.D.). Receiver operating characteristic curve (ROC) analysis was also used to calculate the area under the curve based on the pathology as a reference standard for checking the validity of the results using SPSS software (ver.26). Also, to assess the significance of the differences between the two experimental groups (free and involved lymph node), a statistical analysis was performed using Student's t-test using GraphPad Prism Software (ver.9). A P-value lower than 0.05 was considered statistically significant.

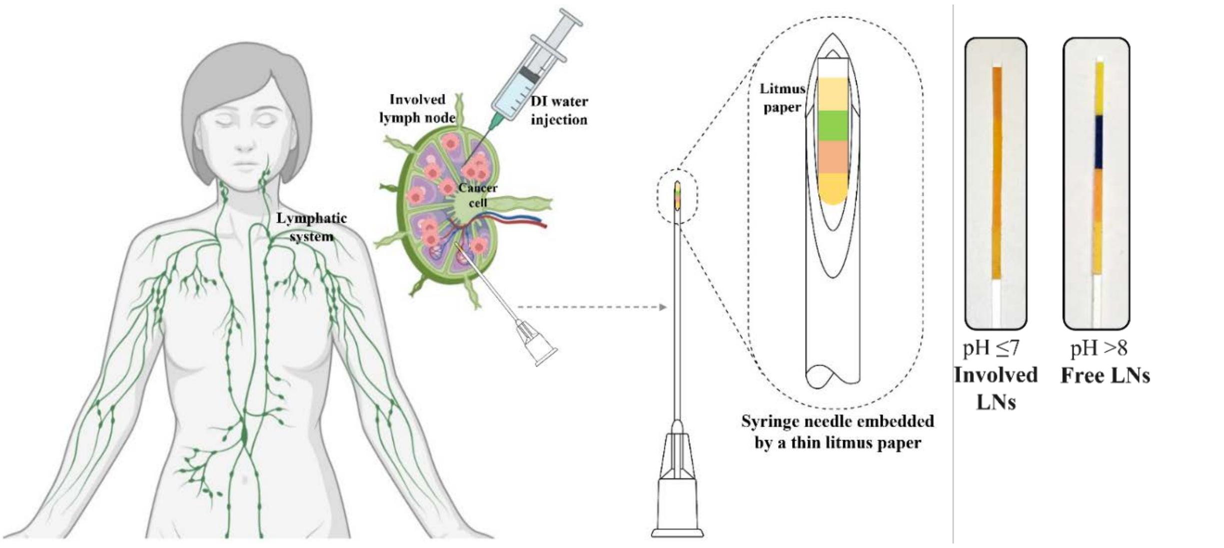

Figure 1. The schematic of the pH measurement procedure for detecting cancer cells in the suspicious lymph nodes during surgery

RESULTS

Study Participants' Characteristics

All female breast cancer patients who received recommendations for lymph node dissection were enrolled in the study. The recruited cases' mean age and tumor size were 45.85±10.23 years and 23.85±14.46 mm, respectively. Moreover, the grades of the tumor, nodal status, and hormonal parameters (evaluated by the pathologist) are presented in Table 1.

Permanent pathology of the tumors showed 31 (91%) invasive ductal carcinoma, 1 (3%) invasive ductal carcinoma with Mucinous, 1 (3%) Tubular carcinoma with in-situ carcinoma, and 1 (3%) Invasive micropapillary carcinoma (IMPC) with invasive ductal carcinoma. Moreover, all patients (n=34) had undergone surgery without pre-surgical therapy. SLNB was performed on 14 (41%) patients, ALND was carried out on 15 (44%) patients, and 5 (15%) patients underwent both SLNB and ALND.

pH values in free and involved LNs

Our previous study showed pH >8 in normal and ≤ 7 for cancer-involved LNs. It means that normal LNs have a buffer and non-acidic pH while cancer LNs have a stronger acidic pH. Figure 2a shows the comparative diagrams of the pH measurement and pathology responses of 63 healthy and cancerous lymph nodes (Supplementary Table 1).

Also, 38% (n=24/63) of LN samples had a pH equal to or less than 7, which is in the range of involved LN categorization. As for the pH scores of the involved LNs, 67% (n=16/24) of those cases were confirmed by histopathology (Figure 2a, Supplementary Table 3).

Table 1. Basic information of patients included in the study.

All patients (n=34)			
Age (years)	45.85±10.23	Tumor size (mm)	23.85±14.46
>50	8 (24%)	<10 mm	4 (12%)
35 to 50	22 (64%)	10 to 20 mm	11 (32%)
< 35	4 (12%)	>20 mm	19 (56%)
Cancer type		Nodal Status	
IDC	31 (91%)	N0	19 (56%)
IDC+ Mucinous	1 (3%)	N1(a)	12 (35%)
Tubular Carcinoma	1 (3%)	N2-3	3 (9%)
IMPC+IDC	1 (3%)		
Tumor grade		Hormone Receptors	
I	11 (32%)	ER$^+$/PR$^+$/HER2$^+$	6 (18%)
II-III	23 (68%)	ER$^+$/PR$^+$/HER2$^-$	28 (82%)
DIN category	0 (0%)		
Surgery		TNM	
SLNB	14 (41%)	T1miN0M0	1 (2.9%)
ALND	15 (44%)	T1aN0M0	2 (5.9%)
SLNB+ALND	5 (15%)	T1bN0M0	4 (11.8%)
		T1cN0M0	5 (14.7%)
		T1cN1M0	2 (5.9%)
		T1cN1aM0	1 (2.9%)
		T2N0M0	7 (20.6%)
		T2N1M0	5 (14.7%)
		T2N1aM0	3 (8.8%)
		T2N3aM0	1 (2.9%)
		T3N1aM0	1 (2.9%)
		T3N2aM0	2 (5.9%)

* Invasive micropapillary carcinoma (IMPC)

Moreover, 61% (n=39/63) of LN samples had a pH of more than 8, which is in the range of free LN categorization. As for the pH scores of the free LNs, 95% (n=37/39) of them were confirmed by histopathology (Figure 2a, Supplementary Table 3).

The rate of pH scoring confirmation (either free or involved) by histopathology in cases with the age of ≥50 years old was 56%, while in <50 years old cases, the rate was 84%.

This pH measurement method showed 89% sensitivity (95% CI (0.73-1.05) and 82% specificity 82% (95% CI (0.64-1.00) in detecting cancer cells metastasized to lymph nodes.

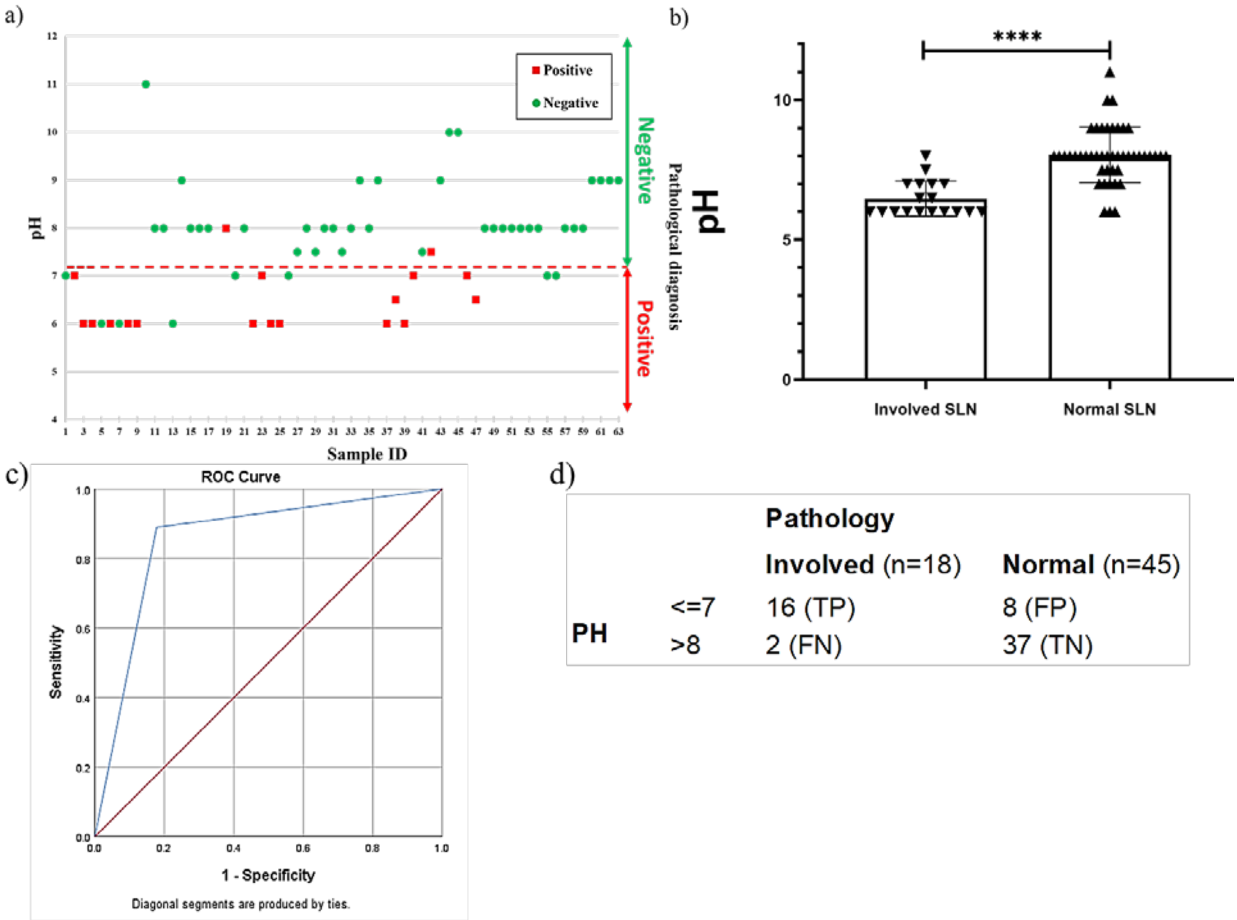

Figure 2. a) Diagram of lymph node pH for 63 LN samples of 34 patients was evaluated by pathology as a reference standard, b) Differences in the mean pH values between involved and free LNs groups were highly significant (P-value < 0.001), c) ROC curve, d) a 2x2 table of TP, TN, FP, and FN counts

As a result, to evaluate the accuracy of the diagnostic test study, ROC was used to compare each of them with the reference standard test (Pathology). As shown in the ROC curve, the area under the curve is 0.86 (CI 95% 0.75-0.96), which shows that this diagnostic test has a proper discrimination or predictive ability (Figure 3). It also has an acceptable balance of sensitivity and specificity.

DISCUSSION

A crucial step in designing a new assay for real-time evaluation of lymph nodes (LNs) with pathological calibration is to understand the metabolic and proliferation pathways of cancer cells in the LN environment. Several reports have indicated that cancer cells that migrate to or invade LNs undergo a shift in their metabolism towards fatty acid oxidation (FAO).[25,26] However, other studies have suggested that cancer cells in the LN environment exhibit both hypoxia glycolysis and FAO metabolism(Figure 3).[22-24]

According to some references, cancer cells within involved LNs are surrounded by an abundance of lipids, which create a lipid-rich LN environment. The saturation of bile acids and their activation through vitamin D receptors (VDR) can trigger the activation of the yes-associated protein (YAP), the main effector of the Hippo signaling pathway involved in cell proliferation and apoptosis regulation. This activation leads to a shift from hypoxia glycolysis to FAO for adenosine triphosphate (ATP) synthesis.[6,7] The absence of a lipidic hilum is considered one of the radiological hallmarks of a suspicious LN.[28] Conversely, other reports have shown the presence of glucose transporter 1 (GLUT1) and Hypoxia-inducible factor (HIF)-1&2 alpha transcriptomes and proteins in cancer cells within lymph nodes, confirming that glutamine consumption is the main process for ATP production.[26] Regardless of which metabolism is dominant, both pathways result in the acidification of the LN environment (Figure 3).

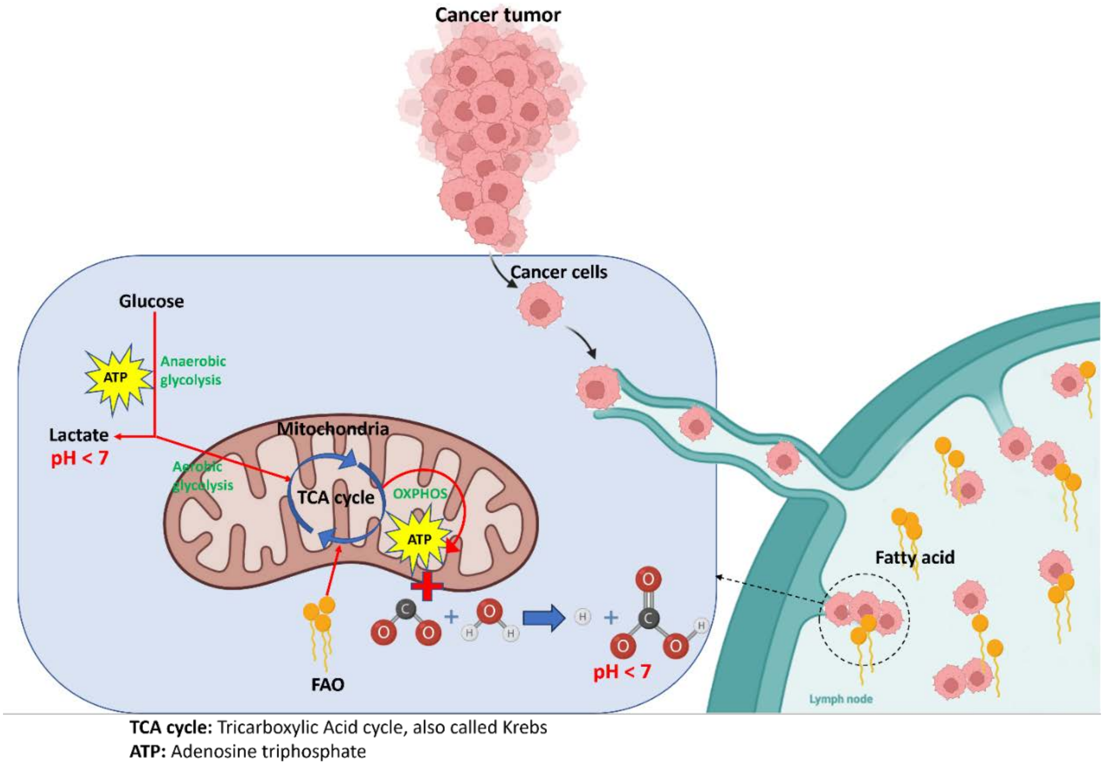

Figure 3. The schematic of the metastatic cancer cells' metabolism to the lymph node [25], [26], [22].

The value of pH sensing-based LN diagnosis lies in the fact that regardless of whether cancer cells in LNs undergo FAO or glycolysis, both pathways lead to the production of low pH agents such as hydrogen ions or pyruvic acid, which lower the pH of the LN to acidic values. Therefore, regardless of the dominant metabolism, pH-based diagnostic evaluation of LNs can be beneficial.

Our previous studies have shown significant differences in pH ranges between normal and involved LNs.[18] Involved LNs exhibited lower pH values, while normal LNs acted as buffers. Therefore, pH recording can be a helpful method for detecting the acidic content produced by cancer cells' metabolism in LNs.

In this study, a syringe needle embedded with litmus paper was inserted into LNs. Before inserting the paper, 50µl of DI water was injected into the LN. The pH measurement was performed on both the cortex and inner parts of the LNs. After a few seconds, the pH indicator was read and recorded using the color table of the litmus paper (ColorpHast® pH Test Strips, Merck, Germany). The pH scores of the LNs were then compared with the pathology results.

We observed a diagnostic sensitivity of 89% when comparing the pathology scoring of LNs with their pH scores. No significant agreement was found between the age of the patients and the matching rate of pH versus pathology of LNs. Similarly, there was no agreement between the immunohistochemical state of the tumors and the pH scores.

Based on the reported statistical data, consistent results are expected even with a larger sample size. However, further testing on more samples is necessary to confirm the device's performance as a complementary tool beside pathology.

CONCLUSION

In this paper, the new assay for real-time evaluation of LNs with pathological value was designed to detect metastatic cancer cells in the lymph node. Using this tool during surgery can greatly reduce the risk of removing healthy lymph nodes and improve the overall effectiveness of the procedure. Furthermore, it has the potential to provide a precise and immediate diagnosis, serving as an effective technique for assessing pH levels of LNs during surgery and eliminating the need for additional procedures.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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ETHICAL CONSIDERATIONS

The patients were enrolled and provided written informed consent according to an ethically approved protocol by the institutional review board of Shahid Beheshti University of Medical Sciences under Ethics Committee ID: IR.SBMU.CRC.REC.1401.022 in Breast Shohada Tjarish Hospital and Azar Surgical Clinic in Iran (Februray-June 2023).

DATA AVAILABILITY

The authors declare that all the other data supporting this study's findings are available within the article and from the corresponding author upon request. The authors also declare that all software codes supporting this study's results are available from the corresponding author upon request.

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