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COMPARISON OF ONE-STEP NUCLEIC ACID AMPLIFICATION (OSNA) VS FROZEN SECTION BIOPSY (FSB) FOR ASSESSMENT OF SENTINEL NODES (SLN) IN EARLY BREAST CANCER: A PROSPECTIVE STUDY

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INTRODUCTION:

The treatment trend in breast cancer is shifting from maximum tolerable to minimum effective treatment[1]. Axillary lymph node dissection (ALND) causes significant morbidity in women including upper-extremity lymphedema, pain, shoulder range of motion impairment, and numbness or paraesthesia[2,3].

Sentinel lymph node (SLNB) biopsy is an alternate to ALND for negative axillary nodes. Intra-operative evaluation of sentinel nodes (SLN) allows complete ALND during the primary surgery itself, hence, obviating the need for a second surgery[3,4]

Frozen section biopsy of SLN has lower accuracy, is time consuming and requires additional technical and medical resources. The other major limitations of rapid frozen sections are two:

1) Sampling error: Because of the need for rapid diagnosis during surgery, a few sections may be examined[5].

2) Nuclear Artefacts: Slight nuclear aberrations may occur as a result of the freezing process that may interfere with diagnosis of non-infiltrating ductal and lobular malignancy[5].

Molecular approaches as real-time PCR have been shown to have high sensitivity and specificity compared to histopathological examination of SLNs .OSNA is an intra-operative molecular assay searching for CK19 mRNA as a surrogate of metastatic cells [6]. OSNA has been proven more accurate, valid, and reproducible for intra-operative evaluation of SLN. The advantages include examining the whole lymph node by a standardized procedure with lesser inter-observer variability. It has been validated, and standardized in SLN assessment in various studies[7-14]. In this study, we compared the results of OSNA and frozen section for intra-operative detection of lymph node detection in early breast cancer.

Material and methods

This experimental study was conducted in The Department of Surgery and Surgical Oncology in collaboration with Department of Pathology and Centre for Advanced Research at King George's Medical University, Lucknow, India. Ethical clearance was obtained vide letter no.: ECR/262/Inst/UP/2013/RR-16. 36 patients consented to participate in the study, node negative breast cancer patients were included in the study. SLNs were detected using 1% methylene blue, 5-15 ml of methylene blue was injected sub-areolar followed by thorough massage for 20 minutes. Nodes were bisected into two halves, each half was sent for FSB and OSNA. Any number of CK19 mRNA copies detected in SLNs was considered positive for OSNA. Results of both were compared with Histopathology (HPE).

ALND was performed if frozen was reported positive for metastasis, however, isolated OSNA positivity in the absence of frozen positivity, was followed by ALND after positive HPE reports.

Sampling Techniques:

SLNs were defatted after sentinel lymph node biopsy, and were split into 2 slices. One slice was sent for the FSB and further processed by formalin- for fixed

tissue histological examination. The other slice was used as the specimen for the OSNA assay.

FSB: After noting the Gross size of Lymph node and gross examination, the lymph node was bisected and cut at different levels. Tissue was fixed in Cryomatrix (Thermo^{TM}) on a cryocasette which was then placed in cryotome at adjusted chamber temperature of -30^{o} C and cryoblate temperature of -40^{o} C. When the block was ready, 10-15 serials (3-4 µm) were taken and stained with hematoxylin and eosin dye using progressive rapid protocol and examined.

ALND was performed when lymph node metastasis was reported on FSB.

OSNA: Expression analysis of CK-19 gene using quantitative real-time PCR (OSNA) in SLN, taking β-actin as a reference gene. RNA was extracted for each tissue using the Trizol method, and first strand cDNA was synthesized by annealing 2 µg of RNA with 0.5 µg of oligo d(T)18 primer in a total reaction volume of 20 µl using M-MLV reverse transcriptase and incubated at 37 °C for 1 h. The reaction was stopped by heating at 95 °C for 5 min. Validation of CK-19 gene primers was checked by gradient PCR and band intensities for different tissues were observed in agarose gel electrophoresis with β-actin as control.

Data was analyzed using IBM SPSS Statistics 20 software. Unpaired Student's t-test was used for quantitative variables when the data were normally distributed. For comparing categorical data, Chi-square test was performed. Continuous variables are presented as mean ± standard deviation, and categorical data are presented as numbers and frequencies.

Results:

Mean age of patients was 47.9±11.9 years. 53% patients were pre-menopausal group and 47% were post-menopausal **(Table 1).** 32 patients had stage I and 4 had stage II disease. Infiltrating ductal carcinoma was most common subtype in all except 2 who had DCIS. 23 patients were ER, PR+, 13 were ER,PR negative. 19 were Her2+. 13 patients underwent breast conservation surgery and 23 had MRM. 32 patients underwent ALND, in 4 patients a redo axillary surgery was performed. **(Table 2)**

OSNA had a Sensitivity of 88.2%; specificity of 100%; positive predictive value (PPV) of 100%; and negative predictive value (NPV) of 90.5% and accuracy- 94.4% **(Table 3)**. Frozen section biopsy was found to have sensitivity-76.5%; specificity-94.7%; positive predictive value (PPV)-92.9%; and negative predictive value (NPV)-81.8% and accuracy of 86.1%.**(Table 4)**

From 17 positive nodes, OSNA detects metastasis in 15 nodes and 2 nodes were false negative while FSB detects 13 positive and 4 false negative nodes.
All of the 19 negative nodes were reported negative on OSNA but 1 was reported false positive on FSB. **(Table 5)**

The results of OSNA were found more similar to histopathology in comparison to FSB. Kappa value of 1 implies perfect agreement of data values with the standards. K-value of OSNA was found to be 0.88 and that of FSB was 0.82. **(Table 6)**
The sensitivity of OSNA was 88.2%, significantly higher in comparison to FSB (76.5%) ($p<0.05$))

Cost analysis:

Since ours is a government run institute, the cost of treatment is much cheaper than any other private sector. Also the hospital rates are much cheaper number of days of hospital stay is not an additional burden to patient. We calculated the cost of using OSNA as a diagnostic modality and compared it with using FSB as an intraoperative technique. The cost of OSNA at our hospital amounts to INR 2500 (38 Dollars) per patient while that of frozen section was 100 INR (1.4 dollar). The cost of HPE and IHC is INR1000 (14 dollars) per patient. Compared to other studies, where the cost of OSNA is twice that of FSB[15], in our study it was much higher. **(Table 7)**
We calculated the mean stay of those patients who underwent ALND, both in the same surgical procedure and in a second procedure. In the first group, the mean stay was 4 days, 6.2 in the second group, which was calculated as the sum of the two admissions. According to data provided by the financial department of our hospital, the mean cost per day of a hospital stay is INR 250 (4 dollars) per patient.. The cost of the second surgical procedure, attributable to the False negativity of the FSB would be additional 250 INR (4 dollars)per patient.
Cost effectiveness of OSNA may be an important consideration, especially in less developed countries like India. Guillen et al[15]. performed a cost-benefit analysis and concluded that OSNA reduced the number of admission days, duration of surgery and achieved savings per patient.

Table 1: Menstrual status of cases

Menstrual Status	N	Percent
Pre-Menopausal	19	52.7%
Post-Menopausal	17	47.2%

Table 2: Clinico-pathological features of cases

Mean age, years (range)	47.9 (33-69)
Tumor stage	
Tis	0
T1	6
T2	26
T3	4
Histological Type	
Invasive ductal carcinoma	*34*
Invasive lobular carcinoma	0
Ductal carcinoma in-situ	2
Other cancer type	0
Histological Grade Grade I Grade II Grade III ER/PR status	 14 17 5
Positive	23
Negative	13
Her-2 neu status	
Positive	19
Negative	17
Breast surgery	
BCS/WLE	13
Simple mastectomy	23
Axillary Surgery SLNB ONLY RE-DO ALND	 32 4

Table 3: Results of OSNA vs HPE for sentinel nodes

	HPE	
OSNA	**Positive**	**Negative**
Positive	15 (88.2%)	0 (0%)
Negative	2 (14%)	19 (100%)
TOTAL	100%	100%

Table 4: Results of FSB vs HPE for sentinel nodes

	HPE	
FSB	**Positive**	**Negative**
Positive	13 (57%)	1 (0%)
Negative	4 (43%)	18 (100%)
TOTAL	100%	100%

Table 5: Comparison of Histopathology Results with Intraoperative OSNA and FSB for Sentinel nodes

Final HPE	**OSNA**		**FSB**	
Number of sentinel nodes	**Positive**	**Negative**	**Positive**	**Negative**
Positive (17)	15	2	13	4
Negative (19)	0	19	1	18
Total (36)	**15**	**21**	**14**	**22**

Table 6: OSNA vs FSB: Sensitivity and Specificity in comparison with histopathology (HPE)

	OSNA	**FSB**
Sensitivity (%)	88%	76%
Specificity (%)	100%	95%
K-Value	0.88	0.82
P-Value	0.001	0.013
Level of Agreement	Very Good	Good

Table 7: Cost analysis per patient

Number of patients	Cost (INR)(per patient)			
	OSNA	FSB	IHC	HPE
36	2500	100	900	100

Discussion

Node-negative breast cancers can be effectively managed by SLNB, hence decreasing the morbidity of ALND[16]. Markers as CK19, mammaglobin, carcinoembryonic acid, MUC1, prolactin-induced protein have been examined for detection of lymph node metastasis in many studies[17]. Based on the result of these studies, CK19 has been shown to have highest sensitivity and specificity[17]. OSNA is a reverse-transcriptase amplification-based assay that detects the presence of metastases in lymph nodes by measuring the expression of CK-19.

Various studies have documented the superiority of OSNA over FSB in detecting SLN metastasis with sensitivity of OSNA varying from 80% to 95% and of

FS from 60% to 92%[18-23]. The sensitivity of FSB in our study was comparable to other studies which have documented the sensitivity of FSB in the range of 60%- 92%[4-5]. This wide variation in sensitivity may be explained by many reasons[5]. Due to uneven distribution of tumor foci within a LN, partial node evaluation may not give positive results. Node section taken from superficial layers may miss metastatic cells present in deeper layers. FSB results may also be affected by variable expertise and experience in tissue sampling and processing by examining pathologists and technicians.

OSNA has the advantage of proper calibration and pre-analytic preparation, which is not possible with FSB. Godey et al.[11] in their study, found no significant difference between OSNA and HPE. In their study, OSNA was positive in 24.4% of patients, compared to HPE which was positive in 24.8%. However, these results were applied for large tumors (T2-T3). In case of smaller tumors (T1), same study concluded that OSNA detected more positive SLNs than conventional histopathology (15.8% vs. 0%) [11]. Our results collaborate with the results of other studies where we found the sensitivity of OSNA to be 88.2. Results of OSNA in our study also collaborate better with HPE results (kappa value=0.88).

Several studies have shown that smaller primary tumors cause nodal micro-metastases than macro-metastases[7,] Lymph node micro metastases can be reported false-negative on FSB (Vrande et al)[4]. In our study, we considered positive nodes as only those which showed increased expression of CK-19 expression assuming that normal cells do not express CK-19. <2% lymph node metastatic cells do not express CK-19[26]. We didn't include expression of CK-19 in terms of copies/µL. Therefore, this study is unable to evaluate OSNA in terms of detecting micro-metastasis. In most of the studies [24-25] . OSNA was found to be superior to FSB for detecting micro-metastasis.

The clinical importance of detecting sentinel lymph node micro-metastasis is a controversy till date[24-25] .Many studies reported the risk of non-sentinel lymph node involvement to be 15% to 20% in patients with sentinel lymph node micro-metastasis detected by histological examination [27, 31]

Since all cases included in the present study have HPE of invasive Ductal carcinoma, it does not compare the accuracy and utility of OSNA and FSB for detecting SLN metastasis in case of lobular carcinomas. However, many studies showed superiority of OSNA over FSB in both ductal and lobular carcinomas [9, 22,23,25,32].

In the present study, two patients were reported as false negative on OSNA assay, they also stained negative for CK-19 on IHC. The reason may be the low or no expression of CK-19 in those metastatic cancer cells. Bartek et al.[29]reported a 0% incidence for breast cancer, and Parikh et al[30] reported 20.5% incidence of breast cancer for young premenopausal women, in patients with low or no CK 19 expression.

Conclusion:

The molecular evaluation of SLN can be very useful since standardisation and reproducibility can be easily obtained. OSNA assay provides satisfactory results with the advantage of standardisation and reproducibility. The OSNA assay can thus be used as an alternative tool for examining metastasis in SLNs.

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