-Original Clinical Research-

RT–PCR technique for the intra-operative assessment of breast sentinel lymph nodes – Is this the way forward?

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Abstract

Background

Sentinel lymph node biopsy (SLNB) is the current standard of care for lymphatic staging in early breast cancer patients. The combination of a SLNB with an intra-operative diagnosis of the SLNB enables a single staged procedure, avoiding a delayed axillary lymph node dissection (ALND) in node positive patients. The current conventional pathological techniques are resource intensive, require expert personnel and are not routinely used in many breast units.

Methods

GeneSearch™ BLN assay is a RT-PCR technique calibrated to detect SLN metastases of >0.2mm. It is designed to detect 2 clinically validated genes Mammaglobin (MG) and Cytokeratin – 19 to provide a rapid diagnosis.The SLN identification was by the combined technique using radiocolloid and patent blue dye. The excised SLNs were sliced in their short axis at intervals of 1.5 mm to 2 mm. The sections were numbered and alternate sections were processed for histopathology and RT-PCR assay.The study was conducted in 2 phases. The first phase was to evaluate the accuracy and feasibility of RT-PCR assay as an intra-operative diagnostic test. The second phase was the real time application of the results to inform decision regarding immediate ALND.

Results

This prospective study comprised of 166 patients with 164 females and 2 male patients. 86 patients had left breast cancer, 75 patients had right breast cancer and 5 patients had synchronous bilateral breast cancers. A total of 171 SLNB procedures were done and 271 SLNs were excised at an average of 1.58 SLNs per procedure. Only a total of 266 SLNs were included in the analysis as the RT-PCR assay results of 5 SLNs were invalid. All the SLNs underwent RT-PCR assay and the results were compared to the final histology. The results were recorded on per patient and per node basis. The initial validation phase of the study included 53 patients and the RT-PCR assay was performed on 92 SLNs from these patients. The sensitivity and specificity on a per patient basis was 100% and 87.1% respectively. On a per node basis, the sensitivity and specificity was 93.7% and 92.1% respectively. The average processing time for 1 SLN during this phase was 31.6 min and 47.4 min for 2 SLNs.

During the 2nd phase of the study, the actioning phase, 113 patients were recruited analysing 174 SLNs from these patients. The sensitivity and specificity of the RT-PCR assay on a per patient basis was 92.3% and 94.3% respectively. On a per node basis, the sensitivity and specificity was 94.1% and 96.4% respectively. The turn around time was significantly better at 21.5 min for 1 SLN and 32.6 min for 2 SLNs. Combining both phases, the sensitivity and specificity on a per patient basis was 95% and 92% respectively. The sensitivity and specificity on a per node basis was 94% and 94.9% respectively.

Conclusions
RT-PCR assay is a reliable intra-operative diagnostic technique for the detection of clinically relevant breast SLN metastases. It is a good alternative to conventional histopathological tests with better sensitivity but comparably less specificity.

Keywords
RT–PCR; Intra-operative assessment; Sentinel lymph nodes; Breast

INTRODUCTION
Axillary lymph node (LN) status is the single most important prognostic factor in breast cancer patients. Techniques to stage the axilla has significantly shifted in the last decade to minimally invasive procedures with sentinel lymph node biopsy (SLNB) being the current standard of care for lymphatic staging in early breast cancer patients. [1-5] It has established itself as an accurate and a minimally invasive modality for assessing axillary LN status in patients with clinical and radiological node negative axillae. As only 1/3rd of early breast cancers metastasise to the LNs, it avoids routine axillary lymph node dissection (ALND) with its associated morbidities in the remainder of the patients. [6]

The current American Society of Clinical Oncology (ASCO) guidelines recommend completion ALND for patients with SLN metastases of >0.2 mm. [7] If this can be detected intra-operatively, a second operation can be avoided in node positive patients. It avoids the technical difficulties of a repeat surgery in the axilla, a second anaesthetic and has economic benefits. Adjuvant treatment can be started early and facilitates immediate reconstruction in suitable patients offering psychological benefits.

Use of an accurate and reliable intra-operative detection method would translate SLNB from just being a staging procedure to a synchronous therapeutic procedure as ALND can be performed in node positive patients at the same time. Current available intra-operative diagnostic methods of touch imprint cytology (TIC) and frozen section (FS) histology are resource intensive, require highly specialised personnel and have imperfect sensitivities and specificities with inter-observer variations. [8-10] They are also time consuming and can potentially delay theatre lists. There is a need for a rapid, easy-to-use, intra-operative diagnostic method which is accurate and validated for clinical use. Research methods like Elastic scattering spectroscopy and Raman spectroscopy although very promising; are still in the preliminary stages to be safely employed in routine clinical practice. [11]

Real time polymerase chain reaction (PCR) techniques simultaneously amplify and quantify the targeted DNA. It is combined with reverse transcription (RT-PCR) for the detection and quantification of specific mRNAs in tissues, e.g. detection of tumor specific mRNAs in the SLN. The amplified product is measured after each PCR cycle and the data generated is then analysed by computer software providing relative gene expression or mRNA copy number.

GeneSearch™ (Veridex, LLC) Breast Lymph Node (BLN) assay is a RT – PCR, rapid molecular diagnostic technique designed to detect clinically relevant SLN metastases of >0.2 mm. It uses real time RT-PCR to detect 2 clinically validated genes Mammaglobin (MG) and Cytokeratin – 19 to provide a rapid diagnosis. It is also calibrated to detect clinically relevant metastases of >0.2 mm to avoid an unnecessary ALND. These markers were selected from 21,000 different markers with MG being a breast specific and CK-19 being an epithelial cell marker. The quantitative cut offs were established following a large United States study by Blumencraz, *et al.* and was approved for routine use by the United States Food & Drug Administration (US FDA) in conjunction with permanent section histology. [12,13] It has been validated by many other trials within the United Kingdom and other European centers. [14-17]

We present our prospective study looking into the accuracy and feasibility of using GeneSearch™ BLN assay as a routine intra-operative diagnostic technique to detect SLN metastases to facilitate 1 step ALND.

MATERIAL AND METHODS
Following approval by the research ethics committee, all patients scheduled to undergo SLNB after the multi-disciplinary team (MDT) discussions were included in this study. This included male and female patients with early

breast cancer (T1/T2) proven to be node negative both clinically and radiologically. Written informed consent was obtained from every participant adhering to the protocol approved by the local research ethics committee.

Study Design

The study was conducted in 2 phases. The initial phase was conducted to validate the GeneSearch™ BLN assay, to check the accuracy and assess turn around times. The feasibility of this assay as an intra-operative assessment tool was assessed in this validation phase. The results of the BLN assay were only recorded and not acted upon, as in a stand alone SLNB procedure. However, if clinically suspicious lymph nodes were detected in the axilla during the SLNB procedure, an immediate ALND was performed with prior informed consent.

Following the results of this phase and after MDT meetings, it was decided that the BLN assay was a reliable intra-operative diagnostic test as compared to the conventional histopathological techniques of TIC and FS histology. It was used real time to inform decision regarding immediate, one-step ALND in assay positive patients with prior patients' consent.

Sentinel Lymph Node Biopsy

The SLN identification was by the combined technique using radiocolloid technetium-99 and patent blue dye as recommended by the UK 'NEW START' training programme. [18,19] We always aimed to perform SLNB prior to the breast surgery to prevent cross contamination of tissue elements. Each excised SLN was subjected to RT-PCR assay and permanent section histopathological examination (HPE).

Node Preparation for BLN Assay and HPE

The biopsied SLNs were taken fresh to the histopathology lab where they were measured, stripped of attached tissues and sliced in their short axis, 'bread loaved' at intervals of 1.5 to 2 mm. The sections were numbered and alternate sections processed for histopathology and BLN assay. Histology specimens were formalin fixed and embedded.

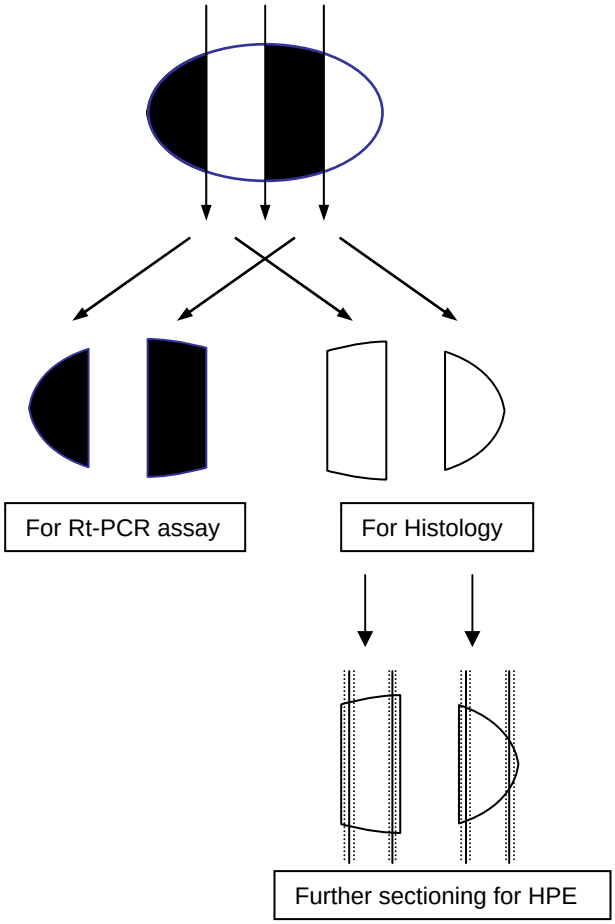

Figure 1 Node preparation for RT-PCR assay & histopathological examination.

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RT-PCR Assay

The assay was conducted by trained personnel as per the GeneSearch™ BLN assay instructions. The fresh, alternate node slices from the 'bread loaving' were homogenised and the RNA purified by processing each node separately. The purified mRNA was amplified by the reverse transcription PCR technique and simultaneously quantified in real time. The number of amplification cycles and cycle time were captured by the software as a qualitative result (positive or negative) for each node based on cut off values for >0.2 mm metastasis for the 2 markers MG (cycle threshold value Ct $\leqslant$31) and CK-19 (Ct$\leqslant$30). The node was considered positive if either or both MG and CK-19 were positive.

For quality control, internal and external controls are employed to avoid spurious results. Internal negative control is by Porphobilinogen deaminase, which detects mRNA from a constitutively expressed gene. External controls are avoiding general contamination of the tissues, monitor reagent quality, PCR set up and instrument performance. Assay cut offs are chosen to call <0.2 mm metastases as negative based on the Ct values of MG and CK-19.

Histopathological Examination

As per our institution protocol, each section for HPE was further sliced at intervals of 150 – 200 μm (as in Figure 1) and underwent standard Haematoxylin & Eosin (H&E) staining. The results were reported as per the TNM classification by an experienced histopathologist blinded to the BLN assay results.

Comparison of Results

The results of the assay were then compared to the permanent section histology on 'per node' and 'per patient' basis. The SLNs with discordant results underwent further sectioning and immunohistochemistry (IHC) for comprehensive assessment during the feasibility phase of the trial. Although, different tissue samples were used for the RT-PCR assay and permanent section histology assessment, the results of this study are expressed as 'sensitivity' and 'specificity' for ease of understanding.

The time taken for the assay was noted to determine the feasibility of BLN assay as an intra-operative diagnostic test to avoid delays and wastage of precious theatre time.

RESULTS

Patient Demographics and Tumor Characteristics

During the period from November 2007 to July 2010, 166 patients were recruited in this prospective study comprising of 164 female and 2 male. In this study, 86 patients had left breast cancer, 75 patients had right breast cancer and 5 patients had synchronous bilateral breast cancers. In effect, 171 SLNB procedures were done in 166 patients. 271 SLNs were analyzed using the RT-PCR assay at an average of 1.58 SLNs per procedure. But 5 SLNs were excluded from the study as the results were invalid due to improper stripping of the perinodal fat. The patient demographics and tumor characteristics are as in the table below.

Results of the Feasibility Phase

The initial validation phase consisted of 53 patients. 92 SLNs from these patients underwent RT-PCR assay. The results were as follows.

The concordance rates on per patient and per node basis were 92.5% and 92.4% respectively. On a per patient basis, the positive predictive value (PPV) was 73.7% and the negative predictive value (NPV) was 100%. On a per node basis, the PPV was 71.4% and NPV was 98.6%.

The average processing time for 1 SLN was 31.6 min and for 2 SLNs, it was 47.4 min. The assay time was constant at 20 min.

In this validation phase, although 14 patients had involved SLNs on final histology, 19 patients underwent ALND of which 9 were immediate and 10 were delayed ALNDs. In the 9 patients who had immediate ALND performed on clinical suspicion, 3 patients did not have any metastases in the axillary nodes by either RT-PCR assay or permanent section histology.

Table 1 Patient demographics and tumor characteristics

Characteristic	n	%	Total
Sex			166
Female	164	99	
Male	02	01	
Age			
Range – 30-91 yrs			
Mean – 61.9 yrs			
Median – 61 yrs			
Laterality of the tumor			166
Left	86	52	
Right	75	45	
Bilateral	05	03	
Type of breast surgery			171
Wide local excision	109	64	
Mastectomy	62	36	
Tumor histology			171
IDC	123	72	
- Grade 1	14	11	
- Grade 2	34	28	
- Grade 3	37	30	
- Mixed type	38	31	
Lobular	19	11	
Mucinous	07	04	
Tubular	03	02	
Cribriform	07	04	
DCIS	03	02	
Micro invasive	03	02	
Mixed histology	06	03	
Tumor histology with positive nodes			40
IDC	29	73	
Lobular	05	12	
Mucinous	02	05	
Cribriform	01	03	
Mixed histology	03	07	
Presence of lymphovascular invasion			171
Yes	46	27	
No	114	67	
Not mentioned in the report	11	06	
LV invasion in positive nodes			40
Yes	21	52.5	
No	17	42.5	
Not mentioned	02	05	
Oestrogen receptor status			171
Positive	154	90	
Negative	17	10	
Progesterone receptor status			171
Positive	144	84	
Negative	27	16	
HER-2 receptor status			171
Positive	12	07	
Negative	159	93	

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Table 2 Results of the 1st phase (feasibility phase)

	Final histology (H&E)						
	Per patient basis				Per node basis		
		+	-			+	-
GeneSearch™ BLN assay	+	14	5		+	15	6
	-	0	34		-	1	70
	Total	53			Total	92	
Sensitivity	100%				93.7%		
Specificity	87.1%				92.1%		

Table 3 Results of the 2nd phase (actioning phase)

	Final histology (H&E)						
	Per patient basis				Per node basis		
		+	-			+	-
GeneSearch™ BLN assay	+	24	5		+	32	5
	-	2	82		-	2	135
	Total	113			Total	174	
Sensitivity	92.3%				94.1%		
Specificity	94.3%				96.4%		

Table 4 Results combining both phases

	Final histology (H&E)						
	Per patient basis				Per node basis		
		+	-			+	-
GeneSearch BLN assay	+	38	10		+	47	11
	-	2	116		-	3	205
	Total	166			Total	266	
Sensitivity	95%				94%		
Specificity	92%				94.9%		

Of the 5 patients reported to be positive on RT-PCR, but negative on final histology of the SLN, 2 patients underwent immediate ALND on clinical suspicion and there was one further axillary node that was involved in these 2 patients.

In this study phase, all SLNs reported as positive on RT-PCR assay but negative on the final histology underwent further sectioning and only 1 SLN was noted to contain a micrometastasis which has been included as a 'true positive' in the above table.

Results of the 2nd Phase (Actioning Phase)

Following the validation phase after progressing through the learning curves, a further 113 patients were recruited and the results are as follows. The assay results were acted upon after informed consent from the patients.

In this phase, the concordance rates on per patient and per node basis were 93.8% and 96% respectively. The PPV and NPV on per patient basis were 82.8% and 97.6%. On per node basis, the PPV and NPV were 86.5% and 98.5% respectively.

In a further 5 patients, the SLNs were reported to contain metastases by the RT-PCR assay, but were negative on the final histology. These patients underwent immediate ALND based on the RT-PCR assay and 2 patients had further involved axillary nodes.

The turn around times improved significantly in this phase of the trial to 21.5 min for 1 SLN and 32.6 min for 2 SLNs.

Combining both the phases of the trial, the results were as follows.

On permanent section histology, 40 (24%) patients had positive SLNs which were detected correctly by the RT-PCR assay in 38 patients with sensitivity ("positive concordance rate") of 95%. On a per node basis, the sensitivity ("positive

concordance rate") was 94%. 126 patients had node negative disease with 116 patients labelled correctly by the RT-PCR assay. The specificity (node negative concordance rate) on a per patient basis was 92% and on a per node basis was 95.3%.

41 SLNs were positive for macrometastases and 9 SLNs for micrometastases. The sensitivity of the RT-PCR assay in detecting macrometastases was (40/41) 97.6% and for micrometastases (7/9) was 77.7 %.

In the entire series, 38 patients underwent immediate ALND and 12 had delayed ALND. In 20 patients, the SLN was the only LN involved. In the remainder of the patients, the average number of LNs involved was 4.4 with a median of 3.

In 2 patients who had negative SLNs on RT-PCR assay, final histology showed the SLNs to contain micrometastasis.

DISCUSSION

Intra-operative detection of SLN metastases facilitating a one-step ALND in node positive patients has definite advantages to the patient, the surgeon and the institution that is offering this service. The current guidelines recommend completion ALND for SLN metastases >0.2mm. The intra-operative identification of this subset of patients using the conventional intra-operative diagnostic techniques of TIC and FS histology has always been a challenge due to logistic and resource issues. This, compounded by low sensitivities and inter-observer variability of these techniques has not found favour in many breast units across the country. The introduction of rapid molecular assay techniques for intra operative identification of SLN metastases has made the concept of one-step ALND possible without the need for expert histo/cytopathologists.

The GeneSearch™ BLN assay was approved by the FDA to be used in conjunction with permanent section histology for intra-operative diagnosis of breast SLNs in the United States of America. Multi-centric trials using this commercially available assay were being conducted through out Europe. As this assay was well validated and standard protocols were used, we conducted the validation phase to resolve local and logistic issues within our breast unit with a view to act on the results after progressing through the learning curve.

Permanent section histology is considered as 'Gold Standard' and all other intra operative diagnostic techniques are compared against this standard. But many authors have disputed this as it has inherent sampling flaws. [13,14]Only 5% of the SLN is examined during routine histopathological examination of the SLN. However, the molecular assays examine 100% of the available tissue. For e.g. in this study half of one SLN in its entirety undergoes Rt-PCR assay and the rest of the SLN slices undergo thin sectioning of either sides at 150-200microns and examined comprising <5% of the SLN. Comprehensive HPE of the SLN can only be achieved by serial sectioning and IHC.

The study design of this trial does not allow the comparison of like to like tissues as different tissue samples undergo RT-PCR assay and HPE. Hence the use of 'sensitivity' and 'specificity' as statistical measures of the performance of the RT-PCR assay against HPE may not be reliable due to the reasons elicited above. To minimise this discrepancy, alternate SLN slices are used.

In our study of 166 patients, there was concurrence in the RT-PCR assay and HPE results in 38 (23%) and 116 (70%) patients with positive and negative SLNs, respectively with a total concordance rate of 93%. In 10 patients SLNs were positive on RT-PCR assay but negative on final histology. In 7 of these patients, further ALND showed 4 patients to have involved axillary nodes.

The reason for this discordance could be due to the fact that different tissue samples are assessed by the RT-PCR assay and the final histology and the fact that the SLN is the only involved node in 47 % of patients. Another possibility is the contamination of epithelial tissue in the LN sample resulting in false positive results. This can be avoided by performing the SLN biopsy prior to the breast surgery, formal training of the personnel performing the assay and adhering to strict quality control measures in the lab.

In 2 patients the RT-PCR assay was negative, but the final histology showed micrometastasis (<0.2mm). This small sized metastasis may not have been included in the SLN sample subjected for RT-PCR assay resulting in the discrepancy.

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During the feasibility phase of our study, 3 patients underwent immediate ALND based on clinical suspicion. The adoption of an accurate intra-operative diagnostic technique would have avoided this unnecessary ALND as the specificity of a surgeon's hands in detection of SLN metastases is low. [20]

During the validation phase of the trial, the turn around time for reporting the assay results were slow. This was because the lab personnel were going through the learning curve and had no time pressure, as the results were not acted upon. The delay was mainly in stripping the perinodal fat to avoid an invalid result. The turn around times improved significantly with the number of assays performed by the trained lab personnel and with the introduction of the 'real time' phase of the assay.

The SLN was always done prior to the main breast operation allowing time for the RT-PCR assay results to be back to inform decision regarding immediate ALND. The processing time significantly improved as the learning phase was passed. The uncertainty about the nodal status can affect operating theatre scheduling. Some flexibility and sensible listing of patients is required to avoid over or under running. Another potential drawback of intra-operative diagnosis of SLN is the psychological impact on the patient as they are unaware of the final outcome of the axillary surgery which can cause anxiety and apprehension.

The GeneSearch™ BLN assay has been trialled in other centres across UK and the results have been similar. [14,21] Although, GeneSearch™ assay has significantly better sensitivity compared to other conventional intra-operative diagnostic techniques for detection of SLN metastases in breast cancer, it has a comparatively lower specificity which is the greatest drawback. Its routine adoption is also limited due to the high start up costs, the need for reagents and health economic reforms in this tough economic climate. [21]

In our series, the RT-PCR assay avoided a second surgery of a completion ALND in 24 (14.5%) patients in the second phase of our trial resulting in significant overall benefits.

CONCLUSION

SLNB, being the current standard of lymphatic staging with ALND being the current recommended treatment for node positive patients, reliable intra-operative diagnosis of the SLN is the next logical step in managing early breast cancer patients. RT-PCR assay is a reliable technique which does not require expert histopathologist for interpretation and is a good alternative to conventional histopathological tests with better sensitivity but comparably less specificity.

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