**TITLE**

Evaluation of the Metasin assay for intraoperative assessment of sentinel lymph node metastases in breast cancer.

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**KEYWORDS**

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**ABSTRACT**

**Aims**: Sentinel lymph node (SLN) biopsy is the preferred surgical technique for staging the axilla in clinically node negative breast cancer. Accurate intraoperative staging allows for the immediate performance of an axillary clearance in node-positive patients. We assessed the Metasin assay for the intraoperative analysis of SLNs in a prospective evaluation of 250 consecutive patients undergoing intra-operative SLN analysis at the Breast Unit, University Hospital, Southampton, UK.

**Methods**: Metasin uses a real time quantitative polymerase chain reaction (RT-qPCR) to detect two markers of metastasis:-, cytokeratin 19 (CK19) an epithelial marker and mammaglobin (MGB) a breast specific marker. Metasin results were compared to the results from routine paraffin block Histopathology.

**Results**: Metasin was robust, with a failure rate of <1%, and demonstrated excellent accuracy and reproducibility. The average turnaround time for the Metasin assay was 42 minutes, the largest variable being the number of nodes assayed. A total of 533 SLNs were evaluated with 75 patients testing positive for MGB and/or CK19. Based on the analysis of individual SLNs the overall concordance between Metasin and Histology was 92.3% (sensitivity 88.7%, specificity 92.9%). When adjusted for tissue allocation bias the concordance was 93.8% (sensitivity 89.8%, specificity 94.6%). In this evaluation 57/250 patients (23%) proceeded to axillary clearance based on Metasin results and were considered spared a second operative procedure.

**Conclusions:** Metasin has proven to be an accurate, reproducible and reliable laboratory test. The analysis time is acceptable for intraoperative use, and in comparison to routine Histology demonstrates acceptable concordance, sensitivity and specificity.

**INTRODUCTION**

Sentinel lymph node biopsy (SLNB) is the preferred technique for staging the axilla during breast surgery.[1-3] It is associated with clinical outcomes equivalent to axillary dissection,[4] reduced morbidity and improved quality of life.[5]

The current published UK recommendation is that patients with a positive SLNB undergo further axillary treatment.[3,6] Intraoperative assessment of the sentinel lymph node (SLN) will stage the axilla, allowing an immediate axillary clearance to be performed in node positive patients. This avoids a second operation in these patients and averts possible delays to adjuvant chemotherapy or radiotherapy.[4] In addition this should be of cost benefit for the UK National Health Service (NHS).[7]

Traditional histological techniques for intraoperative analysis are frozen section analysis and touch imprint cytology. These are highly specific, approaching 100%, but the sensitivity is variable at around 60 – 70% and the reliable detection of micrometastases is a particular issue.[8-10]

Molecular tests have the potential to be even more sensitive than the “gold standard” paraffin blocked based Histology techniques, not least because they allow the sampling of the whole SLN biopsy.[10] Two molecular tests are available for analysing SLNs in breast cancer: Metasin [11] and the OSNA RD-100i system (Sysmex, Kobe, Japan).[12,13] Metasin is similar to the GeneSearch BLNA assay (Veridex LLC, Warren, NJ, USA), which is no longer commercially available.[14-17] It uses a real time quantitative polymerase chain reaction (RT-qPCR) to detect two markers of metastasis, cytokeratin 19 (CK19) an epithelial marker and mammaglobin (MGB) a breast specific marker. To ensure the validity of the RNA extraction porphobilinogen deaminase (PBGD) is used as a control. With publication of Metasin primer and probe sequences, laboratories are now able to use commercially available reagents and a choice of PCR platforms to validate the method for their own use.[11] Metasin has been evaluated against Genesearch and compared to routine histological techniques.[11]

The OSNA RD-100i system is a fully automated assay using reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) detecting CK19 only.

NICE diagnostic guidelines published in 2013 recommended whole lymph node analysis using the RD-100i OSNA system as an option for detecting SLN metastases during lymph node surgery.[18] The guidelines further recommended that robust evidence of the Metasin test should be demonstrated in clinical practice. This paper outlines our experience providing such evidence in clinical practice.

**METHODS**

**Study design and patient selection**

Between April 2013 and July 2014, 250 consecutive patients with breast cancer underwent axillary evaluation according to NICE guidance with axillary ultrasound and FNA as appropriate.[3] Those with clinically and radiologically negative axillae proceeded to sentinel node biopsy and intra-operative analysis with Metasin. Patients were counselled with regards to the implications of discordant results between Histology and the Metasin assay. All patients specifically consented to intra operative analysis and subsequent axillary clearance based on the results of Metasin.

**Surgical management**

SLNB was performed according to New Start guidelines.[2] Prior to surgery 6-40MBq 99mTc-labelled nanocolloid (Nanocoll; GE Healthcare, Slough, UK) was injected intradermally into the peri-areolar tissue and lymphoscintigraphy performed. The timing of the injection and that of the surgery determined the dose of 99mTc. Following anaesthesia, if used Patent V Blue dye (Laboratoire Guerbert, Paris, France) was injected into the sub-areolar tissue. SLNs were trimmed to remove excess fat. During the Metasin assay the surgeon performed the breast tumour resection.

**Metasin assay**

The Metasin assay was performed as described previously.[11] In brief, SLNs were sliced at 2mm intervals in accordance to minimum dataset guidelines.[19, 20] Alternate slices were tested in the Metasin assay and the remainder of the tissue underwent routine Histology examination. For the Metasin assay the slices were weighed and homogenised into RLT buffer (Qiagen®) containing 1% 2-mercaptoethanol (Sigma-Aldrich). The buffer volume was adjusted to standardise the amount of SLN per extraction. RNA was extracted using the RNeasy Mini Kit (Qiagen®) and the products run in the Metasin assay on the Cepheid SmartCycler (Cepheid. Sunnyvale, California, USA).[11] The SmartCycler performs 38 PCR cycles in 27 minutes. The Metasin assay analyses three marker genes, CK19, MGB and PBGD in a multiplex reaction. Known patient positive and negative controls and a non-target control are run with each assay to ensure the validity of the results. All test samples, to give a valid result must have a Cq <36 for PBGD. The assay is called invalid if the controls fail.

Patient results were classified as positive – macrometastasis, positive – micrometastasis, and negative, based on the Cq values for MGB and CK19 given in

Table 1.

**Table 1.** Interpretation of Metasin results

|  |  |  |
| --- | --- | --- |
|  | **Cq Value** | **Interpretation** |
| **MGB** | >32.7 | Negative |
| ≥ 27.9 and ≤ 32.7 | Micrometastasis |
| <27.9 | Macrometastasis |
| **CK19** | > 31.7 | Negative |
| ≥ 25.1 and ≤ 31.7 | Micrometastasis |
| < 25.1 | Macrometastasis |

Reference ranges supplied by the Cellular Pathology Department,

Queen Alexandra Hospital, Portsmouth.

To enable a turnaround time audit, we recorded the time of sample receipt and the time the result was telephoned to the surgeon (N=250).

**Histology**

Formalin fixed paraffin section histopathology was the “gold standard” against which the Metasin assay was assessed. SLN slices were fixed and embedded in accordance with standard laboratory procedures. After initial trimming, 2μm sections were cut at 6 levels, with 100μm between each level. Sections were stained with H&E and examined by a consultant breast pathologist. Where there was discordance between the Histology and Metasin (Histology -ve, Metasin +ve) further histological examination of the SLNs was performed to minimise tissue allocation bias (TAB).[18] A further 6 sections at 100μm intervals were examined. This process was repeated until a metastasis was identified and size classified or until exhaustion of the tissue block. The histological criteria for macrometastasis are tumour deposits of greater than 2mm and for micrometastasis between 0.2 and 2mm.[19, 20]

**RESULTS**

**Patient demographics**

250 consecutive SLNB operations are detailed. 162 patients had a wide local excision, 83 a mastectomy of which 7 where bilateral and 2 patients had re-excisions. The median age was 60 years (range 33 - 86 years). The patients’ tumour characteristics are illustrated in table 2.

**Table 2**. Patient demographics and histology tumour characteristics for 250 patients undergoing Metasin intra-operative analysis of sentinel lymph nodes.

Patient characteristic N % Total

Histopathological DCIS 15 6.0 250

tumour type IDC 174 69.6

 Invasive lobular 16 6.4

 Invasive mucinous 6 2.4

 Invasive papillary 1 0.4

 Invasive tubular 4 1.6

 Mixed 34 13.6

Histopathological Positive - macrometastasis 39 15.6 250

lymph node status Positive – micrometastasis 19 7.6

 Negative 192 76.8

Tumour TNM stage Tis (DCIS) 15 6.0 250

 T1 154 61.6

 T2 73 29.2

 T3 8 3.2

Tumour grade NA (DCIS) 15 6.0 250

 G1 49 19.6

 G2 121 48.4

 G3 65 26.0

Oestrogen receptor status Positive 209 83.6 250 Negative 41 16.4

Progesterone receptor status Positive 67 26.8 250

 Negative 41 16.4

 NA/NT 142 56.8

HER-2 status\* Positive 33 13.2 250 Negative 194 77.6

 NA/NT 23 9.2

DCIS, ductal carcinoma in situ; IDC, invasive ductal carcinoma (no specific type); NA/NT, Not applicable/Not ascertained; HER-2 status assessed by immunohistochemistry and where appropriate silver in-situ hybridisation (SISH); TNM, classification of malignant tumours, UICC, 7th Edition, 2009. \* Not routinely performed in patients > 80 years old.

**Technical performance**

In performing 250 Metasin assays (539 nodes) we had two technical failures, a rate of 0.8%. One was a failure in RNA extraction (PBGD Cq >36) and the second was a fault on the Cepheid SmartCycler. In these cases the patient’s treatment was based on the Histology results. On repetition Metasin gave results concordant with Histology. Across a broad range of tests performed in the Molecular Pathology Laboratory our in-house failure rates for PCR, RT-qPCR and RNA extraction are 2.7%, 4.9% and 0.65% respectively (unpublished data).

**RNA extraction and cDNA estimation**

The 539 lymph nodes assayed by Metasin weighed on average 632mg (range 10mg – 9.4g). The average yield of RNA was 7.5ug ± 6.8 (1SD) (range 0.75 – 32.2ug) as measured @ 260nm (NanoDrop®). This broad range of RNA concentrations gave an average PBGD Cq of 25.1 ± 1.6 (1SD) (range 20 – 32.4) in the Metasin assay. These figures suggest that the PBGB element of the Metasin reaction is limited by the PCR conditions and/or the reagents available in the multiplex reaction.

**Metasin assay - Intra/Inter-batch variation**

To assess Intra-batch variation a single positive patient sample was analysed 32 times using a single batch of Metasin master mix. The results for the PBGD, MGB and CK19 markers are shown in Table 3.

**Table 3.** Metasin assay Intra-batch variation (N=32)

|  |  |  |  |
| --- | --- | --- | --- |
| **Marker** | **Average Cq** | **±1 SD** | **Range** |
| **PBGD** | 26.34 | 1.02 | 24.4 – 29.6 |
| **MGB** | 19.90 | 0.48 | 19.2 – 21.2 |
| **CK19** | 19.36 | 0.27 | 18.8 – 19.9 |

To assess Inter-batch variation a single positive patient sample, aliquoted and stored at -80oC, was analysed in 73 consecutive Metasin assays using different batches of Metasin master mix. The results for the PBGD, MGB and CK19 markers are shown in Table 4.

**Table 4.** Metasin assay Inter-batch variation (N=73)

|  |  |  |  |
| --- | --- | --- | --- |
| **Marker** | **Average Cq** | **±1 SD** | **Range** |
| **PBGD** | 23.76 | 0.68 | 22.7 – 25.7 |
| **MGB** | 23.85 | 0.45 | 23.0 -24.9 |
| **CK19** | 18.87 | 0.31 | 18.3 – 19.8 |

**Turnaround time audit**

We received 1 – 6 nodes per patient with a mean value of 2.17 nodes per patient. The average turnaround time (TAT) was 41.93 minutes ± 6.91 (1SD) (range 27 – 72 minutes). Each additional node tested added an average 5 minutes to the TAT and Metasin negative patients took an average 2 minutes longer to process. Results are detailed in Table 5.

**Table 5.** Metasin assay turnaround times (N=250). Error bars are used to illustrate the range of turnaround times.

 **Comparison of Histology and Metasin**

1 node 2 nodes 3 nodes 4 nodes 5 nodes 6 nodes All nodes

(N=76) (N=96) (N=51) (N=22) (N=3) (N=2) (N=250)

Metasin -ve

Metasin +ve

For this analysis patients were defined as Metasin positive (macrometastasis or micrometastasis) if they are MGB and/or CK19 positive according to the values in Table 1. 75 patients were Metasin positive (30%). Of these 6 were positive for MGB (2.4%), 26 were positive for CK19 (10.4%) and 43 were positive for both markers (17.2%).

The results comparing the patients Metasin assay results to routine Histology are shown in Table 6.

**Table 6:** Comparison of Histology and Metasin results. Positive v Negative.

 (N = 250)

|  |  |  |
| --- | --- | --- |
|  |  | **Histology** |
|  |  | Positive | Negative |
| **Metasin** | Positive | 53 | 22 |
| Negative | 5 | 170 |

Comparing the Histology results to the Metasin assay (N=223), 89.2% of patients gave concordant results, 53 patients (21.2%) were Histology positive and Metasin positive (Hist +ve/ Met +ve) and 170 (68 %) were Histology negative and Metasin negative (Hist –ve/ Met –ve). 27 results were discordant. 22 (8.8%) were Histology negative but Metasin positive (false positive) and 5 results (2%) were Histology positive but Metasin negative (false negative).

**Node by node analysis**

A total of 539 nodes were analysed by Metasin. On subsequent Histology examination 6 of these contained no nodal tissue, leaving 533 nodes with both Histology and Metasin data. Concordant results were produced in 92.3% of the nodes (N=492) (71 Hist +ve/Met +ve and 421 Hist –ve/Met –ve). Of the 41 discordant nodes, 32 (6%) were false positive (Hist –ve/Met +ve) and 9 (1.7%) were false negatives (Hist +ve/Met –ve).

In the false positive cases (N=32) further deeper sections were examined histologically to minimise TAB. 8 lymph nodes were reclassified as positive. Two were reclassified as macrometastases, four as micrometastases and two as positive containing isolated tumour cells (ITC). The false negative cases (N=9) were retested in the Metasin assay and gave repeatable results.

Based on patient results, with adjustment for TAB, the concordance of Histology with Metasin is 92.4%. Based on analysing individual nodes, with tissue bias adjustment, the concordance is 93.8%.

Based on patient results, the sensitivity of the Metasin assay compared to Histology is 91.4% (95% confidence intervals (CI) 80.3 – 96.8%) and the specificity is 88.5%. (95% CI 83 – 92.5%). Metasin has a Positive Predictive Value (PPV) of 70.7% and a Negative Predictive Value (NPV) of 97.1%. When the patient results are adjusted for TAB, the sensitivity is 92.2% (95% CI 82 – 97.1%) and the specificity is 91.4% (95% CI 86.2 – 94.8%). The PPV is 78.7% and the NPV is 97.1%.

Based on the results for individual nodes, the sensitivity of the Metasin assay compared to Histology is 88.7% (95% CI 79.2 – 94.1%) and the specificity is 92.9% (95% CI 90.1 – 95%). Metasin has a PPV of 68.9% and a NPV of 97.9%. When the node results are adjusted for TAB, the sensitivity is 89.8% (95% CI 81 – 94.9%) and the specificity is 94.6% (95% CI 91.9 – 96.4%). The PPV is 76.7% and the NPV is 97.9%.

The Metasin assay can be used to classify results into three groups, macrometastasis, micrometastasis and negative based on the Cq values for the MGB and CK19 markers. The Metasin classifications for all 533 nodes were compared to Histology and the results shown in Table 7.

**Table 7:** Comparison of Histology and Metasin results. Macrometastasis v micrometastasis v negative. (N = 533)

|  |  |  |
| --- | --- | --- |
|  |  | **Histology** |
|  |  | Macro | Micro | Negative |
| **Metasin** | Macro | 48 | 6 | 6 |
| Micro | 6 | 11 | 26 |
| Negative | 2 | 7 | 421 |

The Metasin assay was 90% concordant when compared to the Histology (N=480). A significant set of 32 nodes, negative by Histology, were classified as positive by Metasin (6%). 6 nodes were macrometastases (1.1%) and 26 micrometastases (4.9%) by Metasin. This set of 32 nodes comprised of 22 patients samples.

A smaller, but equally significant, set of 9 nodes were positive by Histology and negative by Metasin (1.7 %). 2 nodes where macrometastases (0.4%) and 7 micrometastases (1.3%) by Histology. This set of 9 nodes comprised of 5 patients samples.

**Axillary clearances**

In this evaluation 57/250 patients (23%) proceeded to axillary clearance and were spared a second operative procedure. 40 patients (70%) had been classified as a macrometastasis by Metasin and 17 (30%) as a micrometastasis.

Histological examination of the axillary clearance lymph nodes (N=57) showed 29 patients (50.9%) positive by Metasin (24 macro and 5 micro) had positive clearance lymph nodes. 28 patients (49.1%) positive by Metasin (16 macro and 12 micro) had negative clearance lymph nodes. A further 18 patients were classified as having a micrometastasis by Metasin but did not proceed to axillary clearance.

Patients having a Metasin defined macrometastasis in their SLNB had a 60% incidence of metastasis in their axillary clearance nodes compared to those with micrometastasis, who had a 29.4% incidence.

**DISCUSSION**

Accurate intraoperative staging of the axilla during breast surgery allows for the immediate performance of an axillary clearance in node-positive patients and avoids a second operation in these patients. Node-negative patients avoid the morbidity associated with axillary clearance. In the routine clinical environment of a large UK breast unit, we describe the intraoperative analysis of SLNB using Metasin and compare the results to routine Histology.

Metasin is reliable with a failure rate of just 0.8%, comparing favourably with our routine RT-qPCR techniques (4.9%), a further study of Metasin (1.5%) [21] and the OSNA system (1.4%).[22] The reproducibility of Metasin for CK19, MGB and PBGD is excellent for a RT-qPCR assay, where variation in Cq values should generally be <1.5.[23]

In the operating theatre the SLNB is performed first and the breast cancer resection is performed while the node is being analysed. The number of nodes received is the biggest single factor influencing TATs for the Metasin assay. TATs improved as we gained more experience with the method and could be improved further by the adoption of whole node analysis as recommended for the OSNA system [18]. Overall Metasin intraoperative analysis does not greatly alter the time to perform a wide local excision and SLN biopsy.[7] In comparison the OSNA system takes <30 – 39.6 minutes to process 1 node and this time is increased by 5 - 10 minutes for each additional node analysed.[18]

The sensitivity of routine paraffin block Histology is less than 100%. With additional testing the sensitivity can be raised [24] but these methods still fall short of sensitivity offered by molecular techniques.[23] Our 250 patients had a node positive rate of 30%, compared to rates of 33%, 26% and 28% reported in previous studies. [2,5,7] In this analysis micrometastases were considered positive as at the time axillary clearance was performed for micrometastases in a proportion of cases.

6 patients (2.4%) were singularly positive for MGB compared to 1.63% in a previous study.[21] These patients are significant because their metastases would not be detected by the OSNA system which only detects CK19.

In our evaluation, Metasin, based on patient results, was 89.2% concordant with Histology. Based on individual lymph nodes, the concordance was 92.8%. These figures improved to 92.4 % and 93.8% respectively after further microscopical examination of deeper sections. A further study has reported a 93.5% concordance, based on a case analysis. The concordance improved to 96.5% after examination of deeper sections, immunostaining with MNF116, a pancytokeratin marker and using Genesearch.[11]. A multicentre study reported a 95.6% concordance with Histology, based on a case analysis and examination of deeper sections and immunostaining with MNF116.[21] Snook *et al* [22] gave the overall concordance between OSNA and Histology as 96%. The NICE Guidelines do not give concordance figures for the OSNA system and Histology. In their opinion the assessment was hindered by TAB and the inconsistent Histology reference standards.[18]

Before adjustment for TAB our sensitivity compared to routine Histology is 91.4% and the specificity is 88.5%. After adjustment for TAB the figures are 92.2% and 91.4% respectively. Two previous Metasin studies, after adjustment for TAB, have reported the sensitivity of Metasin compared to Histology, as 92-95% and the specificity as 97%.[11,21]

When comparing the OSNA system to Histology, before tissue adjustment for TAB, the sensitivity is 84.5% and the specificity 91.8%. After adjust for TAB the figures are 91.3% and 94.2% respectively.[18]

It is difficult to give an accurate and meaningful comparisons between individual studies or between methods in the presence of the variable TAB and differing confirmatory methodologies. Concordance, sensitivity and specificity can only be considered to be indicative of a tests overall performance and on that basis we believe Metasin compares well to previous data and to the OSNA system.

Metasin has been used study to classify metastases in macro and micro groups and our results are 90% concordant with routine Histology, when compared on the basis of individual nodes. Whilst most of the discordant results can be explained by TAB, we have a significant group of 26 nodes (4.9%) which were classified as having a micrometastases by Metasin which were negative by Histology. Only 4 of these discrepancies were resolved by further histologically examination, leading to the conclusion that Metasin is more sensitive than routine Histology. This factor should be taken into consideration when making clinical decisions based on Metasin. Despite the high sensitivity of the Metasin assay, we found 9 nodes (1.7%) to be positive by Histology (2 macro and 7 micro) but negative by Metasin. This is not an unexpected result given the potential level of TAB in this evaluation. It is important that the possibility of such discordant results is discussed with patients prior to their consenting to surgery.[7]

We chose not to use whole node analysis because Histology gives us a backup in case of Metasin failure, the ability to size the metastasis and a visual evaluation of any extracapsular spread of the metastasis. When implementing the Metasin assay an informed judgement has to be made as to which tissue allocation protocol to follow.

There is an on-going debate as to the efficacy of axillary clearance after a positive SLNB [25] and the finding that only 32.8 – 44.1% of histologically positive SLNBs had positive axillary clearance lymph nodes (ACLN), only adds to this debate.[26,27,28] Patients with macrometastasis in their SLN biopsies (41.5-50.3%) had a greater incidence of axillary node positivity than those with a micrometastasis (17.9-26.8%).[26,27,28] Using the OSNA system, Barber et al [29] reported that 26.7% of OSNA positive SLNB had positive ACLNs. 38% of patients with macrometastasis and 15% with micrometastasis had positive ACLNs.

Our limited Metasin data, shows that 50.9% of patients with positive SLNBs had positive ACLNs and that those with macrometastasis (60%) had a greater incidence of ACLN positivity than those with micrometastasis (29.4%). These figures need substantiating over a larger series of patients.

Metasin has proven to be an accurate, reproducible and reliable assay, comparing well to paraffin block Histology in terms of concordance, sensitivity and specificity. Metasin delivers timely results allowing clinical decisions to be made intraoperatively and delivers tangible benefits to patients.

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**Competing Interest**

None declared

**Conflicts of interest**

The intellectual property rights for the Metasin assay rest with the Princess Alexandra Hospital, NHS Trust, Harlow, Essex. The authors do not have any conflicts of interest.

**Author contributions**

G Smith, R Cutress, H Markham, S Zhang and E Hodges all contributed to the writing, editing and proof reading of this manuscript. G Smith and S Zhang validated and implemented the Metasin assay. H Markham was the lead pathologist and performed extended analysis of the lymph nodes. E Hodges and R Cutress led respectively, the service and surgical implementation, of the intraoperative assay.

**Collaborators**

The Molecular Pathology staff who performed the Metasin analysis:- A Afonso, Dr S J Doherty, Dr R H Ganderton, C V Lee, N Meakin. The breast surgeons:- Mr D A Rew, Mr G T Royle, Miss T G Simoes, Miss C M Summerhayes. The breast pathologists: - Dr V Bhargava, Dr A. C Bateman, Dr H Roche, Dr J, M Theaker, Dr C Tilley.

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**KEY MESSAGES**

When used to analyse intra operative sentinel lymph nodes during breast cancer surgery:

1. Metasin is a reliable, reproducible and sensitive laboratory test.

2. Metasin is comparable to routine paraffin block Histology.

3. Metasin can be used to make timely and informed decisions

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