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Predictive Value of Intraoperative Sentinel Lymph Node Imprint Cytology Analysis for Metastasis in Patients with Melanoma

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ABSTRACT Since there are no standardized protocols regarding the detection of microscopic melanoma deposits in sentinel lymph nodes (SLN), the aim of this study was to present our experience with intraoperative cytological evaluation of SLN in patients with melanoma. The study included 475 SLN biopsies (SLNB) from 201 patients with primary cutaneous melanoma of intermediate thickness. Each lymph node was cut in half; touch imprint cytology (TIC) preparations of all cut surfaces were performed and stained according to a modified May-Grünwald-Giemsa method. The results were compared to definitive postoperative histology. Twenty of 25 SLNB positive on TIC proved to be metastatic when compared to definitive histology. Most of 32 SLN that were suspicious but not diagnostic on TIC were proven negative (23/32, 71.8%), while 7 nodes had metastases (one micrometastasis and one with isolated tumor cells only). The majority (94%) of SLNBs negative on TIC remained negative on final histology, while 6% or 25 nodes were positive, mostly with micrometastases or isolated tumor cells (17/25). In frozen sections performed in cases of suspicious or positive SLN cytology, metastasis was confirmed in 80% of positive and in 21.9% of suspicious TIC. Altogether, 49% (27/55) of positive SLNB were identified intraoperatively in 57% (24/42) of patients, and in those cases a complete regional lymph node dissection was performed in the first step. TIC assessment of SLNB with 99% specificity and 57% sensitivity for intraoperative identification of metastasis is useful and beneficial for avoiding a second operative procedure.

KEY WORDS: sentinel lymph node biopsy, touch imprint cytology, melanoma, intraoperative

INTRODUCTION

Melanoma accounts for only 4% of all malignant neoplasms, but it is responsible for more than 77% of skin cancer deaths (1). According to the American

Joint Committee on Cancer (AJCC) (2009), metastases to regional lymph nodes are the most important prognostic factor in patients with early-stage mela-

noma (2). Sentinel lymph node biopsy (SLNB) for patients with primary cutaneous melanoma was first introduced in the early 1992 (3) and since then it has been established as a reliable indicator of the presence of micrometastases in the nodal basin and an accurate prognostic factor in primary melanoma (4).

The concept of SLNB is based on the principle that all lymphatic fluid from a certain body part is drained to regional lymph nodes. The first of nodes draining a specific site (thus named "sentinel node"), can be detected, removed, and evaluated for the presence of malignant cells. Intraoperative SLNB examination has been demonstrated to be accurate staging procedure in patients with melanoma that helps avoid complete regional lymph node dissection in patients with clinically negative nodes (5-11). For this reason, an elaborate histopathologic assessment of the SLN that drains primary cutaneous melanoma is essential for an individualized prediction of clinical outcome, and for recognizing the need for immediate complete regional lymph node dissection (CLND). Despite the use of intraoperative SLNB for more than 16 years, there is still no consensus on the "correct" way to sample the SLN (12,13).

SLNB was developed using intraoperative frozen sections because CLND could be performed immediately if the SLN contained tumor cells. When an SLNB sample is prepared for frozen section analysis, part of the lymph node is sacrificed until a reliable cut surface is obtained. This loss of tissue may lead to false negative results in small lymph node metastases. This could be the reason for discouraging the use of frozen section analysis of SLN.

The aim of the present study was to report our experience in handling SLNB in patients with cutaneous melanoma of Breslow thickness >1 mm and <4 mm, with a special goal of comparing the results of intraoperative cytological evaluation of nodal cut surfaces with histological analysis. Since a standard protocol for intraoperative detection of microscopic melanoma deposits in SLNB is still lacking according to literature data, the aim was to present our experience and to discuss and compare our results with literature data.

PATIENTS AND METHODS

Patients and operative procedure

Between 2006 and 2014, 201 consecutive patients diagnosed with cutaneous melanoma of Breslow thickness >1 mm and <4 mm undergoing surgery at University Department of Surgery, Rijeka University Hospital Centre for SLN biopsies were included in the study. Patients gave oral informed consent. The study

was performed with the approval of Ethics Committee of Clinical Hospital Center Rijeka and the Faculty of Medicine, University of Rijeka. Sentinel lymph node detection was performed with ^{99m}Tc labelled human serum albumin colloid (Nanocoll*, GE Healthcare, Italy), with 95% of particles with a diameter ≤80 nm. Four injections surrounding the scar after initial surgical procedure for melanomas located on the trunk and two injections for the lesions located on the extremities were applied intradermally within 1 cm of lesion margins. Total activity administered was 30-40 MBq, split into 2 or 4 aliquots of 0.1-0.2 mL volume per aliquot, each followed with 0.2 mL air bubble.

Imaging was performed with a two-headed gamma camera (Siemens E-CAM or Siemens Symbia T, Germany) immediately after the application of the radiopharmaceutical, starting with 20 minutes of dynamic acquisition over the region of interest (matrix 128×128, 20 frames, 60 s/frame). Then, static acquisitions of the same region in different projections were performed depending on the localization of the lesion (matrix 256×256, 300 s per projection). Single photon emission computerized tomography (SPECT, matrix 64×64, 32 views, 30s/view, detectors, non-circular orbit, step and shoot) and additional hybrid SPECT/CT imaging (SPECT combined with "low dose" CT) was performed when appropriate, usually in melanoma of the trunk, in difficult locations ("in transit" nodes), or equivocal situations. "Low dose" CT imaging started immediately after completed SPECT acquisition, following determination of cranial and caudal limits of the scanned region in the CT protocol (30 mA, 130 kV, slice 5 mm, Acq 2×2.5 mm, PITCH: 1.5). After the CT session, reconstructions were performed with specific software applications (B08s SPECT AC; B30s; B70s).

The imaging ended with whole body planar scanning in anterior and posterior projections at a scan speed of 18 cm/min. Focal, persistent activity in the regional lymph node basin with associated lymphatic path leading from the injection site was considered the first draining node and defined as a sentinel lymph node (SLN). It was marked on the skin with a water resistant pen and on the image as SLN. When more than one lymph node was visualized, they were marked on the skin and on the image with numbers assigned according to the order of appearance. If more than one region contained lymph node activity, the first node (SLN) was marked in each basin.

The patients were then transferred to the operating room, where the radioactive SLN (or SLNs) was detected with a hand-held gamma probe (Neoprobe 2000, Ireland). The signal was considered significant when the number of counts detected in the node

exceeded ten times the background activity of the body. After the removal of the suspected SLN, the activity in the removed nodes was confirmed with a gamma probe, as well as residual (diminished) activity in the extraction site. In cases where the remaining activity was still present in the operating field, further nodes were searched for and removed. The extracted nodes were sent for intraoperative analysis.

Sentinel lymph node examination

A total of 475 SLNB from 201 patients with primary cutaneous melanoma were assessed. Depending on the node size, each node was longitudinally cut into 2-3 mm sections. Care was taken to obtain complete cross sections of the maximum diameter, preferably including the hilum and the marginal sinus. For each lymph node half, a pair of imprints were made by gently touching the cut surface of the SLNB to glass slides that were subsequently processed with modified May-Grünwald-Giemsa staining. Touch imprint cytology (TIC) of every section was assessed intraoperatively by two cytologists. Diagnostic categories included positive, negative, and suspicious but not diagnostic findings. Suspicious TIC results were considered negative. In case of a negative cytology report, the node was fixed in neutral buffered formalin and embedded in paraffin, according to standard procedures (Table 1). In case of positive or suspicious cytological findings, the selected sections were frozen and 5 serial sections (6 µm thick) were taken. The sections were immediately fixed with cold acetone for 2-3 minutes and then stained. Positive histological results prompted CLND.

Lymphadenectomy completion was at the surgeon's discretion when only the TIC result was positive and the frozen section negative. After intraoperative interpretation, the SLNB sample was fixed in 10% buffered formalin, processed in the usual manner, and

embedded in paraffin. For each SLN, an initial section was cut and stained with hematoxylin-eosin (HE). Following histopathologic examination, the SLNB that were initially negative for tumor on HE were further examined according to the accepted protocol (14-16). The pathologist recorded the extent, size, and extracapsular extension of the tumor. For statistical purposes, the metastases were classified according to the largest nodal tumor deposit as a metastasis (>2 mm), micrometastasis (>0.2 to <2 mm), and isolated tumor cells (<0.2 mm). TIC results were compared with definitive postoperative histopathology results.

Statistics

Sensitivity was defined as the percentage of positive TIC results among those with a positive definitive histology. Suspicious TIC results were considered negative in statistical analysis. Specificity was defined as the percentage of negative TIC results among those with a negative definitive histology. The false-negative rate was defined as the number of false-negative intraoperative TIC results divided by the sum of false negative and false-positive results. For the purpose of this study, the level of statistical significance was set at $P < 0.05$.

RESULTS

The study cohort comprised 201 patients with cutaneous melanoma whose 475 SLNB were analyzed. The mean number of analyzed lymph nodes per patient was 2.4 (range, 1 to 7). Patient age ranged from 30 to 88, mean age 59 years. There were 94 (45%) female and 114 (55%) male patients. Out of 201 patients undergoing SLNB analysis, positive nodes were found in 42 (20%) patients.

TIC was performed intraoperatively on all 475 SLNB and the results were compared with the final histology findings. Of 475 TIC specimens, 418 (88%) were negative, 32 (6.7%) suspicious but not diagnostic, and 25 (5.3%) were positive, while the corresponding distribution of histopathologic diagnosis was 420 (88.5%) negative and 55 (11.5%) positive. Of 55 positive SLNB on final histological analysis, 35 (64%) were metastases (>2 mm), 16 (29%) micrometastases (0.2-2 mm), and 4 (7%) isolated tumor cells (<0.2 mm).

The concordance of TIC and final histology results is summarized in Table 2. Out of 25 SLNB positive on TIC, 20 (80%) proved to be metastases and one to be a micrometastasis on final histology as compared with definitive histology. Four out of 25 SLN were proven negative on final histology. Most of 32 SLNB that were suspicious but not diagnostic on TIC, were negative on definite histology ($n=23$, 72%), while 7

Table 1. Sentinel lymph node biopsy (SLNB) intraoperative evaluation with a decision on further steps using the the cutaneous melanoma protocol for SLNB

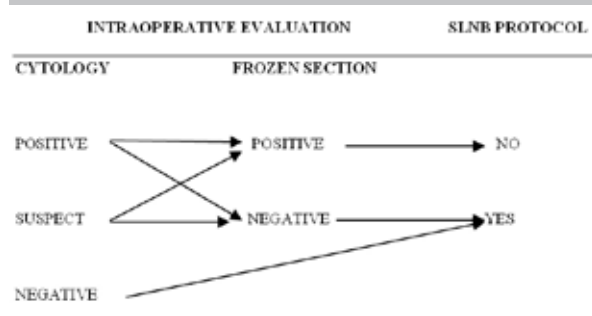


Table 2. Comparison between intraoperative touch imprint cytology (TIC) and final histology diagnosis

| | | TIC (N=475) | | |
|-----------------|-------------------------|-------------|------------|-------------|
| | | Positive | Suspect | Negative |
| Final histology | Metastasis (>2 mm) | 20 (80.0%) | 7 (22.0%) | 8 (1.9%) |
| | Micrometastasis (<2 mm) | 1 (4.0%) | 1 (3.0%) | 14 (3.3%) |
| | Isolated tumor cells | 0 (-) | 1 (3.0%) | 3 (0.7%) |
| | Negative | 4 (16.0%) | 23 (72.0%) | 393 (94.0%) |
| | Total | 25 | 32 | 418 |

(22%) had metastases, one micrometastasis, and one isolated tumor cells. SLN that were negative on TIC were confirmed to be negative on final histology in 94% (393/418) of cases, while 6% (25/418) were positive, mostly presenting as micrometastases and isolated tumor cells (17/25).

Only one of 16 cases with micrometastases was positive and another one was suspicious on TIC. Finally, TIC yielded a suspicious result in only one of 4 SLNB with isolated tumor cells.

Intraoperative TIC identified positive SLNB in 4 (16%) cases, while metastases were not detected on HE and immunohistochemistry.

Simultaneous frozen section analysis was indicated in 57 cases with positive and suspicious TIC results. Concordance between intraoperative TIC and frozen section in cases of suspicious or positive SLNB cytology is shown in Table 3. In frozen sections, metastases were confirmed in 20/25 (80.0%) positive and 7/32 (21.9%) suspicious TIC. Altogether, 27/55 (49.0%) positive SLNB were identified intraoperatively.

However, the number of positive SLNB increased to 31/55 (56.3%) on final examination. More precisely, 1 SLNB from the group of positive TIC (metastasis greater than 2 mm) and another 3 from suspicious TIC, but negative on frozen section, were positive on final histology (two micrometastases and one isolated tumor cells). However, the majority of suspicious TIC findings (72%) were negative on final analysis. During surgery, suspicious but not diagnostic TIC results were considered negative. Thus, CLND was performed in the first step in 18 (43%) of 42 SLNB positive cases.

The accuracy of intraoperative TIC according to the size of tumor infiltration in SLNB is shown in Table

4. Specificity was 99%, while sensitivity was lower, i.e. 38%. However, the sensitivity increased to 57% in cases with lymph node tumor cell infiltration greater than 2 mm.

DISCUSSION

It is well accepted that lymph node status is an important prognostic indicator in patients with cutaneous melanoma. Since lymph node dissection is associated with significant morbidity and SLN proved representative of the remaining nodal basin status, the SLNB technique has been increasingly used.

SLNB was developed using intraoperative frozen sections because CLND could be performed immediately rather than later if the SLN contained a tumor, thus obviating the need for two surgical procedures (17,18). However, experience has shown that frozen sections are not reliable enough and the whole procedure is associated with several problems. Preparation of a full-face frozen section often requires discarding a substantial amount of nodal tissue during frozen block cutting. Thus, there is often a significant tissue loss, potentially interfering with subsequent more detailed pathological examination of paraffin-embedded specimens. The procedure is time-consuming, and some artefacts may be introduced during the process of specimen freezing and thawing, thus additionally hampering identification of melanoma cells, which are generally more difficult to recognize in frozen tissue sections than in slides from well-fixed tissues. Consequently, some consensus groups have discouraged frozen section examination of SLNB. According to their opinion, the melanoma-draining SLNB should be evaluated as sections from well-

Table 3. Concordance between intraoperative touch imprint cytology (TIC) and frozen section in cases of suspect or positive cytology (N=57)

| TIC | Total | Frozen section | | Final histology | |
|----------|------------|----------------|------------|-----------------|------------|
| | | Positive | Negative | Positive | Negative |
| Positive | 25 (47.5%) | 20 (80.0%) | 5 (20.0%) | 21 (84.0%) | 4 (16.0%) |
| Suspect | 32 (52.5%) | 7 (21.9%) | 25 (78.1%) | 10 (31.2%) | 22 (68.7%) |

Table 4. Accuracy of intraoperative imprint cytology according to the size of nodal tumor infiltration

| | Specificity | Sensitivity | PPV | NPV |
|-------------------------|-----------------|-------------------|-------------------|-------------------|
| Total* | 99% (0.97-0.99) | 38,2% (0.25-0.52) | 84% (0.64-0.95) | 92% (0.89-0.94) |
| Macrometastasis (>2 mm) | 99% (0.97-0.99) | 57,1% (0.39-0.74) | 83,3% (0.62-0.95) | 96,5% (0.94-0.98) |

*All positive lymph nodes regardless of the size of nodal tumor infiltration; PPV: positive predictive value; NPV: negative predictive value; 95% confidence interval in parentheses

fixed paraffin-embedded tissues (18) or with rapid immunohistochemistry for HMB45, S-100, and the melanoma cocktail that may help detect melanoma cells in SLNB intraoperatively (19). Contrary to “common knowledge”, performing frozen section analysis of SLNB in patients with breast cancer, according to some authors, does not impair the probability of detecting lymph node metastases (20).

Generally, frequently reported methods of intraoperative assessment are frozen section histology and imprint or touch-preparation cytology (14). Touch imprint cytology has been demonstrated to be a very efficient tool for intraoperative assessment of SLNB and, according to literature data, comparable in accuracy to frozen sectioning. In our department, TIC has been included in the protocol for intraoperative analysis of SLNB in association with frozen section in case of positive or suspicious TIC. According to the results obtained, the specificity of this method was 99%, while the sensitivity was lower, ranging from 38% to 57% depending on the size of SLNB metastasis. In a previous report by Messina *et al.*, the specificity and sensitivity of TIC in patients with melanoma was 100% and 62%, respectively (21). However, the

results referred to a small number of cases analyzed by this technique, i.e. 23 lymph nodes from 13 of 357 patients. The authors conclude that a thorough pathologic evaluation of SLN in patients with melanoma requires complete submission of the whole tissue, routine use of immunohistochemistry, and TIC in selected cases (21). In breast cancer, the positive predictive value of intraoperative TIC analysis of SLNB for axillary metastasis was 100%, sensitivity was 48%, and specificity 100% (22). In lobular carcinoma, where the evaluation of SLNB is frequently challenging, the sensitivity was 52%, specificity 100%, accuracy 82%, and negative predictive value 78% (23).

A relatively high number of suspicious TIC results in our study were probably associated with the findings of pigmentophages or cell aggregates that were difficult to analyze; thus, most of these cases were negative on definitive histology analysis. The similarity between histiocytes and melanocytes in cytological smear is well known (Figure 1, A) and therefore great effort was invested in some studies to identify the best protocol for assessment of SLN using histology, immunohistochemistry, and even RT-PCR (15,24).

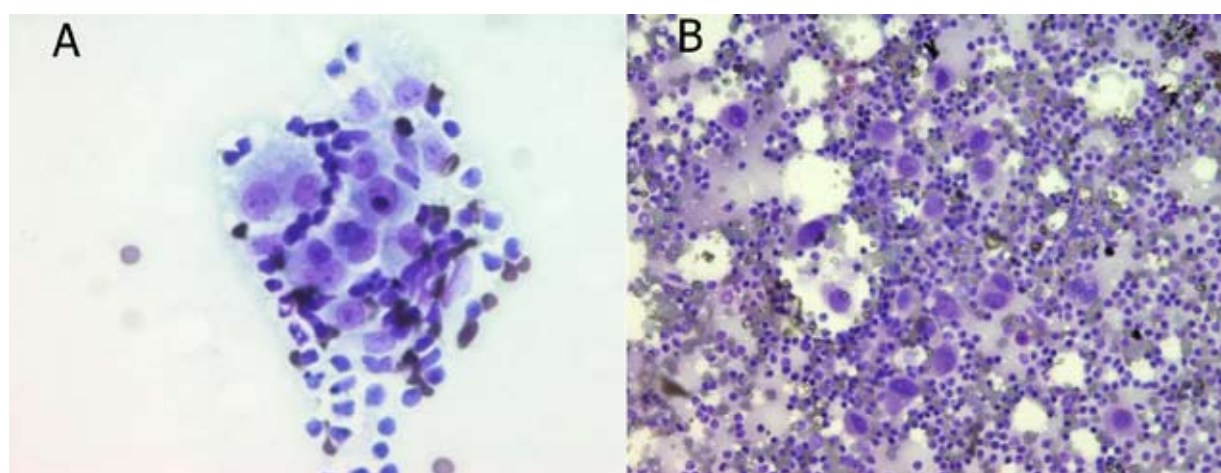


Figure 1. A: Imprint preparation of the sentinel lymph node biopsy (SLNB) cut surface (modified May-Gruenwald-Giemsa -MGG staining technique, magnification $\times 200$): among numerous lymphocytes, there are many single large melanoma cells with large nucleoli, some of them binuclear and with abundant cytoplasm. Cytologic diagnosis on TIC was positive for melanoma cells while final histology analysis was negative. **B:** Imprint preparation of the SLNB cut surface (modified May-Gruenwald-Giemsa -MGG staining technique, magnification $\times 200$): a cluster of large cells with large nucleoli, some binuclear cells, with abundant cytoplasm and dark pigment in one cell resembling pigmentophages or melanocytes. Cytologic diagnosis on TIC was suspicious of melanoma cells while final histopathology was negative.

In case of "false" positive results, TIC was revised and malignant cells were confirmed in all cases (Figure 1, B). A possible explanation for the likely "false" positive TIC could be a very small amount of tumor cells or that tumor cells were lost during frozen section preparation. On the other hand, the "false" negative results could probably be explained by the fact that only a touch imprint of cut surfaces was analyzed intraoperatively, while the tumor cells were probably located in a deeper part of the lymph node. Most "false" negative results were associated with micrometastases (<2 mm) and isolated tumor cells.

The joint committee of the Society of Surgical Oncology and the American Society of Clinical Oncology has recently issued an evidence-based guideline recommending SLN biopsy in patients with intermediate-thickness melanoma and considering the procedure in patients with thick melanomas. The final long-term follow up data from a randomized international clinical trial of SLN biopsy versus observation reinforces the evidence for the use of SLN biopsy in these patients (25). Even in thick (>4 mm) melanomas, SLNB is recognized as an important prognostic factor for overall survival and disease-free survival (26); on the other hand, relatively few patients with thin melanomas have positive SLN, thus alternative strategies to identify patients at risk of nodal disease are needed (27).

The prognostic significance of small foci of melanoma in SLN has been reevaluated over time. According to an Australian report, nearly 20% of patients with micrometastases <0.1 mm died from metastatic melanoma (28). Moreover, patients with only a single metastatic melanoma cell identified in the SLN may develop widely disseminated metastatic disease (29-31). In the American Joint Committee staging scheme, the identification of solitary melanoma cells in SLN upstages the patient and leads to a shift in clinical management from observation (if the lymph node is negative) to CLND and often adjuvant therapy. In contrast, some authors report that CLND did not significantly influence disease-specific survival in patients with SLN-positive melanoma (32). In addition, the most recent studies do not recommend CLND in patients with melanoma with lymph node micrometastases of at least a diameter of 1 mm or smaller (33).

Regardless of the clinical significance of SLN micrometastases, there is still interest and progress in the development of protocols for the analysis of melanoma SLNB. A standardized protocol could have many potential benefits, including improvement in cross-institution reproducibility of diagnostic and

clinical trial enrolment criteria. We have accepted the protocol for SLNB in melanoma patients, which includes intraoperative TIC assessment, since we believe that specificity of 98% and sensitivity of up to 71% for intraoperative identification of metastases is useful and beneficial for avoiding a second operative procedure. The method can be improved by raising the quality of tissue used for imprint, the quality of imprint smears, and the quality of staining. In addition, the experience of cytologists and simultaneous intraoperative TIC analysis by two cytologists, although time consuming and involving more staff members, is likely to make this protocol even more accurate and reliable.

CONCLUSION

In our experience, touch imprint cytology assessment of sentinel lymph nodes had 99% specificity and 57% sensitivity for intraoperative identification of melanoma metastasis, making it a useful and beneficial tool for avoiding a second operative procedure.

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