Technical limits of comparison of step-sectioning, immunohistochemistry and RT-PCR on breast cancer sentinel nodes: a study on methacarn-fixed tissue

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Received: February 26, 2008; Accepted: July 25, 2008

Abstract

The optimal pathological assessment of sentinel nodes (SLNs) in breast cancer is a matter of debate. Currently, multilevel histological evaluation and immunohistochemistry (IHC) are recommended, but alternative RT-PCR procedures have been developed. To assess the reliability of these different procedures, we devised a step-sectioning protocol at 100 micron-intervals of 74 SLNs using methacarn fixation. mRNA was extracted from sections collected from levels 4 to 5. *Mammaglobin, CEA* and *CK19* were used for RT-PCR. mRNA extraction was successful in 69 SLNs. Of these, 7 showed macrometastases (>2mm), 2 showed micrometastases (<2 mm) and 7 showed isolated tumour cells (ITC) by IHC. RT-PCR was positive for the three markers in 6 of 7 macrometastases and in 1 of 2 micrometastases. In the 2 RT-PCR negative cases, metastases were detected only on sections distant from those analysed by RT-PCR. *CEA* and/or *CK19* were positive by RT-PCR in 3 of 7 ITC and in 23 morphologically negative SLNs. In conclusion, the main goal of our study was to show that the use of alternate sections of the same sample for different procedures is the key reason for the discrepancies between molecular and morphological analyses of SLN. We believe that only prospective studies with quantitative mRNA analysis of specific metastatic markers on the whole lymph node can elucidate the utility of molecular assessments of SLN.

Keywords: breast neoplasm • methacarn fixation • sentinel lymph node • RT-PCR

Introduction

The single most important prognostic indicator in breast cancer patients is the presence of metastatic dissemination to axillary lymph nodes [1]. The sentinel lymph node (SLN) is the lymph node that primarily drains the tumour site and SLN biopsy (SLNB) is considered the standard of care for clinically node-negative disease [2]. However, the role and utility of high throughput evaluation of SLNs and the finding of minimal metastases continue to fuel debate. The pathological assessment of the SLNs with multilevel evaluation and immunohistochemistry (IHC) is common practice [3] and isolated tumour cells (ITCs) have been separated from micrometastases in international staging manuals for breast cancer [4]. However, recent studies with up to 6 years of follow-up have demonstrated that both patients with negative SLN and patients with SLN micrometastases had low recurrence of axillary metastases and they report no statistical differences in overall survival between patients with negative SLN and SLN micrometastases [5, 6]. The upstaging potential of SLNB and the workload imposed by a systematic search for low-volume SLN involvement has stimulated pathologists to move toward establishing alternative procedures such as RT-PCR to detect minimal amounts of mRNAs of specific breast cancer markers [7–13]. The majority of the studies [7, 10, 12, 13] have compared RT-PCR analysis of fresh nodal tissues with H&E and IHC analysis of formalin-fixed paraffin embedded SLNs. Few studies [14, 15] have directly compared RT-PCR and step-sectioning and IHC results on frozen sections. Most mRNA

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doi:10.1111/j.1582-4934.2008.00449.x

extracted for RT-PCR is obtained from fresh SLN tissues for the purpose of determining SLN involvement and of guiding an immediate formal axillary dissection.

A recent study by Langer *et al.* [16] demonstrated that with intraoperative frozen sections at three levels with a cutting interval of 150 μm, nearly all macrometastases can be detected and as a consequence, the number of second operations for axillary dissection was low. In our Breast Unit, we use ultrasonographically guided fine needle aspiration of suspect lymph nodes as a pre-operatory staging procedure. Using this procedure, the percentage of metastatic SLN is markedly reduced and metastatic SLNs are confined to micrometastases and ITCs [17]. Therefore, we use a protocol for management of patients that includes day surgery for breast cancer and SLN excision (without any examination of intraoperatory frozen sections), followed by step-sectioning of SLN on formalin-fixed tissue. However, formalin, a cross-linking agent, induces RNA chemical changes and fragmentation, impairing quantification of gene expression [18]. Recently, new tissue fixatives providing preservation of both tissue morphology and RNA/DNA/proteins have been proposed [19–21]. Methacarn is a non-cross-linking organic solvent consisting of a solution of methanol, chloroform and acetic acid. It not only preserves optimal morphology and antigenicity for IHC, but it also allows for the analysis of mRNA and protein expression levels and the mutational analysis of target genes from microdissected tissue samples of paraffin embedded tissues, with yields close to those achievable using unfixed frozen tissues [22].

Thus, in the present study, we fixed a series of SLNs in methacarn with the aim to evaluate and compare the reliability of conventional histopathology or IHC *versus* RT-PCR to detect nodal metastatic foci on the same SLN.

Materials and methods

Sentinel lymph node gross slicing and histological processing

As a pre-operatory staging procedure, all patients with invasive breast cancer are submitted to ultrasound (US) examination of the axilla followed by ultrasonographically guided fine needle aspiration (US-FNA) of suspect lymph nodes. Patients with negative US or negative FNA of axillary lymph node proceed to SLN biopsy and cancer excision in day surgery without any intraoperatory frozen section examination. The criterion for SLN biopsy was early-stage (T1–2 N0 M0) invasive breast cancer [23] (Table 1). Using the pre-operatory staging procedure and the SLN biopsy criterion, from March 2006 to December 2006, a total of 74 SLN biopsies were performed in 62 patients out of 137 breast cancer cases treated in our institution.

During the study period, all SLNs were immediately fixed in methacarn (60% methanol, 30% chloroform and 10% glacial acetic acid) [24]. Human tissue samples were used according to the guidelines of the local Ethics Committee. The SLN was processed using the regional guideline of Piemonte, Italy [25]. This regional guideline was developed to help ensure that none of the macro or micrometastases are missed during tissue processing, since in the local surgical protocol, an axillary dissection is performed only in the presence of metastases larger than 2 mm. However, each patient's management was discussed at multidisciplinary meetings and therapeutic decisions could be modified depending on other clinical and pathological parameters such as presence or absence of lymphovascular invasion.

After 6 hrs of fixation, SLNs were grossly sectioned at 2 mm intervals perpendicular to the larger axis. Gross sections were placed in one or two blocks depending on the size of the SLN and kept in their correct position with the aid of a sponge. During embedding in paraffin wax, care was taken to preserve the original sequence of slices; the top cut-surface of a gross section and the bottom surface of the next section represented contiguous areas of the SLN (Fig. 1A).

Each SLN was step-sectioned at 100 μm intervals with a standard microtome until the extinction of the wax blocks. Twelve to twenty-two levels were obtained from each block. The first two consecutive sections (4 μm thickness) of each level were used for H&E staining and for IHC analysis. Six sections (7 μm thickness) were cut from between levels 4 and 5 and were collected in an RNAse-free tube for molecular analysis. To reduce the risk of contamination, new blades were used for SLN cutting during both gross and microscopic sectioning.

Similar to the SLNs, the non-SLNs were isolated from the fat tissue of the axillary dissection specimen and grossly sliced at 2 mm intervals if larger than 0.5 cm. Two H&E stained sections were produced from each block.

Immunohistochemistry and examination of the sentinel nodes

If the SLN was found to be negative on H&E staining, the sections collected for IHC analysis were stained using an automated immunostainer (BenchMark AutoStainer, Ventana Medical Systems, Tucson, AZ, USA) using AE1/AE3 cytokeratin antibodies (clones AE1/AE3 & PCK26, pre-diluted, Ventana-Diapath, Tucson, AZ, USA). Metastatic deposits were measured in two dimensions and categorized according to the new American Joint Committee on Cancer [4] staging system as isolated tumour cells/clusters (ITCs; pN0(i+)) when they were 0.2 mm or less in the larger dimension and as micrometastases (pN1mi) when they measured more than 0.2 mm, but not greater than 2.0 mm; SLN metastases larger than 2 mm were categorized as having pN1a category nodal involvement.

RNA extraction and RT-PCR

mRNA was extracted from the six sections collected from between step-sectioning levels 4 and 5. In one case, the tissue for RT-PCR was also obtained from seven 4-μm-thick sections previously collected for IHC. Sections were deparaffinized in xylene and washed in ethanol. RNA was isolated using the Masterpure Purification kit (Epicentre, Madison, WI, USA); a DNAse I treatment step was included. RNA concentration was measured with a spectrophotometer (BioPhotomer Eppendorf AG, Hamburg, Germany). One microgram of each sample RNA was reverse transcribed to cDNA with SuperScript II enzyme (Invitrogen Corporation, Carlsbad, CA, USA) using oligo-dT according to the manufacturer's instructions. cDNA samples were subsequently amplified for the target sequences by using published primers for *CEA* (CEACAM5, NM_004363.2) [26] and *Mammaglobin* (SCGB2A2, NM_002411.2) [8] and semi-nested and single step PCR, respectively. To optimize *CK19* (KRT19, NM_002276.4)

mRNA detection in methacarn-fixed tissue, we developed a semi-nested RT-PCR with a first round PCR using primers previously reported [27], followed by a second PCR using an inner primer designed with the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) from the sequence of the human *CK19* gene. mRNA for *beta-2 microglobulin* (B2M, NM_004048.2) was amplified for each sample for quality control of RNA integrity and absence of DNA polymerase inhibitors. The *CEA* gene was amplified with a touchdown (TD) PCR program [28]. In selected cases, RT-PCR for *Mammaglobin* was performed on RNA extracted from six sections (7-μm thick) of the corresponding primary tumour, using the same extraction protocol and the same quality and quantity controls reported above. In this case, the histological tissues were formalin fixed and paraffin embedded (FFPE); therefore, we optimized *Mammaglobin* molecular analysis by targeting a smaller amplicon with primers designed with the Primer 3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) on the same human *Mammaglobin* gene sequence. Forward and reverse primer sequences, the size of the corresponding amplicon and the annealing temperature are reported in Table 2. PCR products were amplified in a 50 μl final volume. Every reaction included 5 μl cDNA (corresponding to 100 ng), 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 1 U of Taq DNA polymerase, 5 pmoles of each primer and PCR buffer 1x. For *Mammaglobin* expression analysis on cDNA from FFPE primary tumours, 25 pmoles of each primer and 300 ng of cDNA were used. Nested and semi-nested PCRs were performed with 5 μl of the first step PCR product in 50 μl of final volume with the same starting conditions. Sentinel nodes of six patients with cutaneous melanoma who underwent lymphadenectomy (reactive lymph nodes by histology without evidence of malignancy) were used as negative controls. Both mRNA extracted from a pool of MCF-7 and SKBR3 breast cancer cell lines and from a primary breast cancer fixed in methacarn and embedded in paraffin served as positive controls. All PCR products were visualized by electrophoresis on 2% agarose gels, containing 0.5 μg/ml ethidium bromide.

To reduce the risk of contamination from previously amplified products, separate areas were used for RNA isolation, amplification and electrophoresis.

Table 1 Characteristics of the 58 breast cancer patients

	N = 58
Age	
<50	6
≥50	52
Tumour size	
pT1b	15
pT1c	31
pT2	12
Sentinel node	
pN0(i−)	42
pN0(i+)	7
pNmi	2
pN1a	7
Axillary node dissection*	
Total	13
Non-SLN Negative	6
Non-SLN Positive	7
Histologic type	
Invasive ductal carcinoma	41
Invasive lobular carcinoma	10
Tubular carcinoma	4
Other	3
Histologic grade	
I	18
II	29
III	11
Vascular invasion	
Absent	41
Present	17

*9 cases with macro- and micro-metastases in SLN, 2 cases with ITC and in 2 cases with negative SLN (pN0(i−)).

Results

The mRNA extraction from fixed tissue was successful only in 93% of cases, corresponding to 69 SLNs from 58 patients. The total RNA extracted from each SLN varied from 4 to 140 μg (see Fig. 2). Clinical-pathological characteristics of these 58 patients are detailed in Table 1

By H&E, breast cancer metastases were detected in 12/69 nodes (17%). Seven of these cases were classified as macrometastases and two as micrometastases. In one SLN, a macrometastasis of 3.3 mm infiltrating the capsule and the pericapsular fat tissue was observed only in the sections cut from the last seven consecutive levels (Fig. 1B). In seven cases, ITCs were detected by IHC staining and in three of these, the results were confirmed by retrospective examination of H&E stained slides.

Cell line mRNA and/or mRNA from the primary methacarn-fixed breast cancer used as positive controls showed strong combined expression of *CEA*, *CK19* and *Mammaglobin*. None of the SLNs from the 6 melanoma patients used as negative controls were amplified by RT-PCR using the 3 breast cancer mRNA markers. Overall, 33 out of 69 (47.8%) SLNs were positive for at least one gene with RT-PCR performed on sections obtained from between levels 4 and 5 (Table 3). Specifically, 6 out of 7 cases positive for metastases and 1 out of 2 micrometastases were RT-PCR positive with all three markers. The specificity of this kind of positivity was indirectly shown by the negative results of RT-PCR performed on sections obtained in early levels of the SLN affected by macrometastases in the last 7 levels of sectioning (Table 4, case 10, Fig. 1C). The sections collected for IHC analysis of these last levels were thus used for RT-PCR and this time the tissue was positive for all 3 markers (Fig. 1D). Analogously, RT-PCR

Table 2 Forward and reverse primer sequences, corresponding amplicons and annealing temperature of *CEA, CK19, Mammaglobin* and *β2 microglobulin*

GENE (size)	PCR (size)	PRIMERS	ANNEALING TEMPERATURE
β2 Microglobulin	Single step (118 bp)	F 5'-ACCCCCACTGAAAAAGATGA-3'	55°C
		R 5'-ATCTTCAAACCTCCATGATG-3'	
Mammaglobin*	Single step (329 bp)	F 5'-AGCACTGCTACGCAGGCTCT-3'	58°C
		R 5'-ATAAGAAAGAGAAGGTGTGG-3'	
CK19	Seminested		
	1st step (321 bp)	F 5'-ATTCCGCTCCGGGCACC-3'	1st step
		R 5'- CGCTGATCAGCGCCTG-3'	72°C
	2nd step (219 bp)	F 5'-ATTCCGCTCCGGGCACC-3'	2nd step
		R 5'-GCAGCTCAATCTCAAGACCC-3'	55°C
CEA	Seminested		
	1st step (160 bp)	F 5'-TCTGGAACTTCTCCTGGTCTCTCAGCTGG-3'	TD PCR
		R 5'-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3'	60°C to 50°C
	2nd step (131 bp)	F 5'-GGGCCACTGTCGGCATCATGATTGG-3'.	TD PCR
		R 5'-TGTAGCTGTTGCAAATGCTTTAAGGAAGAAGC-3'	60°C to 50°C
Mammaglobin*	Single step (134 bp)	F 5'-GCACTGCTACGCAGGCTCT -3'	57°C
		R 5'-GCATTTGTAGTGGCATTGTCG-3'	

*For *Mammaglobin* RT-PCR analysis using RNA from methacarn-fixed paraffin embedded tissues.
#For *Mammaglobin* RT-PCR analysis using RNA from formalin-fixed paraffin embedded tissues.

Table 3 Results of RT-PCR markers in RT-PCR positive SLNs

RT-PCR positive SLNs	*CEA* + *CK19*+ *MMG*+	*CEA*+ *CK19*+ *MMG*–	*CEA*+ *CK19*– *MMG*–	*CEA*– *CK19*+ *MMG*–	Total
SLNs with metastases	6 (100%)	0	0	0	6
SLNs with micrometastases	1 (100%)	0	0	0	1
SLNs with ITC	0	2 (66%)	1 (34%)	0	3
H&E/IHC negative SLNs	0	8 (35%)	8 (35%)	7 (30%)	23
Total	7 (21,2%)	10 (30,3%)	9 (27,3%)	7 (21,2%)	33

performed on tissue obtained between levels 4 and 5 was negative for 3 markers in one SLN affected by a micrometastases detected by H&E staining of sections from levels 1 through 4 (Table 4, case 9). In this same case, ITCs were detected by IHC of sections from levels 7 to 16 only. None of the SLNs affected by ITC were positive for *Mammaglobin* by RT-PCR. In the set of ITCs, one SLN was positive for *CEA* alone (Table 4, case 4) and two were positive for *CK19* and *CEA* (Table 4, case 5 and 7). Of the 23 SLNs negative by both H&E and IHC, 8 were both *CK19*- and *CEA*-positive; 8 were only *CEA*-positive and 7 were *CK19*-positive only. None of them were positive for *Mammaglobin*.

To assess the sensitivity of the procedure, we selected the 8 cases with SLNs affected by ITC or micrometastases that resulted negative for *Mammaglobin* in RT-PCR and performed the RT-PCR study of *Mammaglobin* in the FFPE primary tumour. RNA extraction was adequate in 7 out of 8 FFPE samples. *Mammaglobin* was expressed in 6 cases (Fig. 3).

Axillary dissection was completed in the 9 cases of SLNs with macro and micro-metastases and in 2 cases with SLNs with ITC.

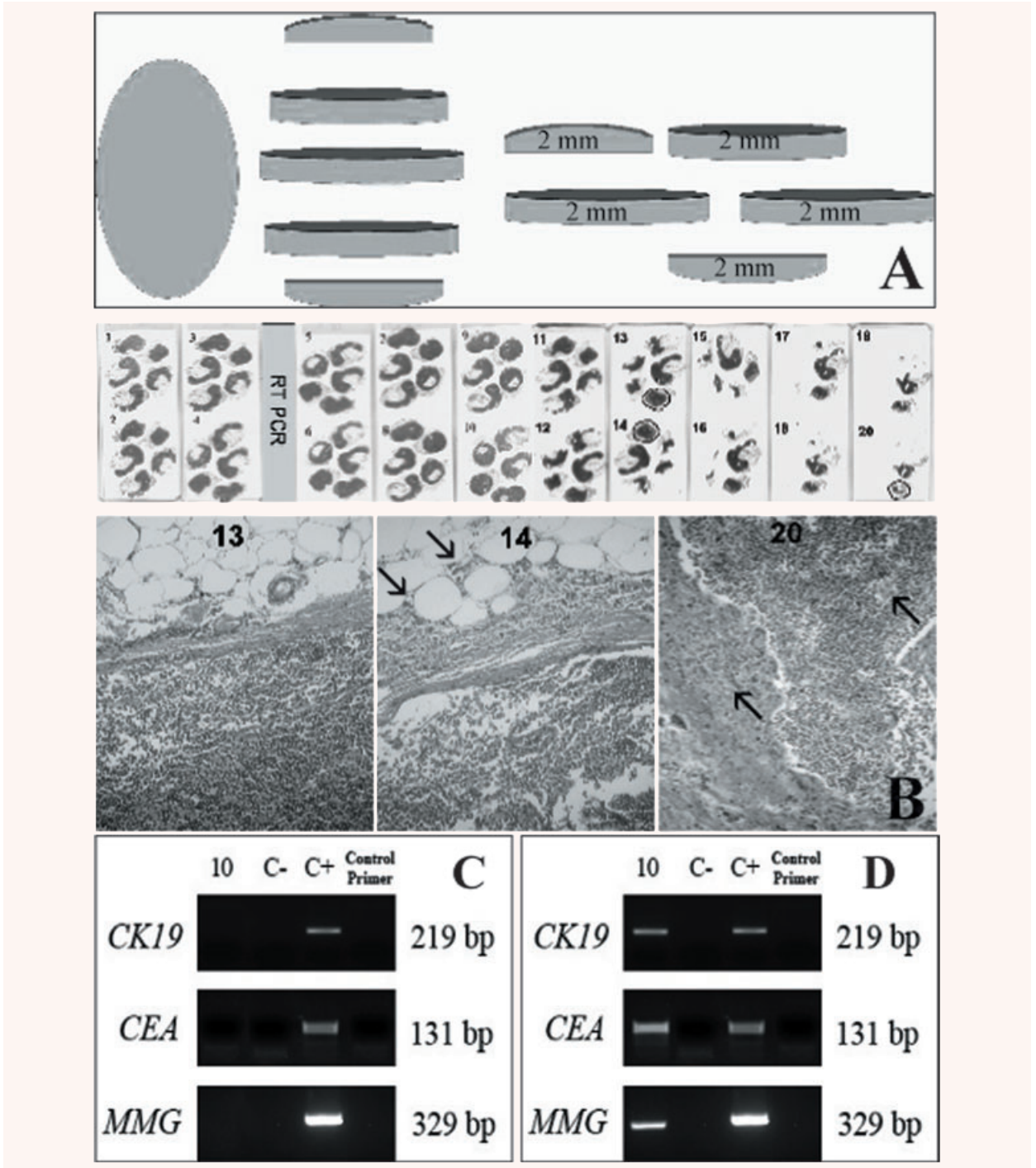

Fig. 1 (**A**) Sentinel nodes gross sectioning at 2 mm and (**B**) sentinel node Haematoxylin and Eosin stained slides of case 10. Sections of level 13 without metastases, 14 and 20 with metastases (circled) are shown at higher magnification (20×) (arrows); (**C**) RT-PCR: mRNA for *CK19, CEA* and *Mammaglobin (MMG)* not detected on sections from levels 4 to 5 and (**D**) detected on sections from levels 17 to 20 corresponding to the site of metastases.

Table 4 Mapping of the results obtained by H&E/IHC and RT-PCR in consecutive levels of 16 SLN with isolated tumour cells (ITC) (≤0.2 mm), micrometastases (>0.2 ≤2mm) and macrometastases (<2mm). RT-PCR was performed on sections obtained from levels 4 to 5

	ITC (H&E/IHC)											RT-PCR		
SLN	**levels 1&2**	**levels 3&4**		**levels 5&6**	**levels 7&8**	**levels 9&10**	**levels 11&12**	**levels 13&14**	**levels 15&16**	**levels 17&18**	**levels 19&20**	**levels 21&22**	***CK19***	***CEA***	***Mamm-aglobin***
1	−/−	−/+	RT-PCR	−/−	−/−	−/−	−/−						−	−	−
2	−/−	−/−		−/−	−/−	−/−	−/−	−/−	−/+				−	−	−
3	−/−	−/−		−/−	−/+	−/−							−	−	−
4	−/−	−/−		−/+	+/−	−/−	−/+	−/−	−/−				−	+	−
5	−/−	−/−		−/+	−/−	−/−	−/−						+	+	−
6	−/−	−/−		−/−	−/−	+/+	−/−	−/−					−	−	−
7	+/+	+/−		−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	−/−	+	+	−
	MICROMETASTASES (H&E)														
8	+/+	+/+	RT-PCR	+/+	−/+								+	+	+
9	+/+	+/+		−/−	+/−	+/+	+/+	+/−	+/−				−	−	−
	METASTASES (H&E)														
10	−/−	−/−	RT-PCR	−/−	−/−	−/−	−/−	+/+	+/+	+/+	+/+		−	−	−
11	+/+	+/+		+/+	+/+	+/+	+/+	+/+	+/+				+	+	+
12	+/+	+/+		+/+	+/+	+/+	+/+	−/−					+	+	+
13	+/+	+/+		+/+	+/+	+/+							+	+	+
14	+/+	+/+		+/+	+/+	+/+							+	+	+
15	+/+	+/+		+/+	+/+	+/+							+	+	+
16	+/+	+/+		+/+	+/+	−/−							+	+	+

Table 5 Correlation of H&E/IHC diagnosis of SLN with NON-SLN status in 13 cases with axillary dissection (AD). Effect of RT-PCR SLN results and lymphovascular invasion (LVI) status

	AD	Non-SLN positive	Non-SLN negative
SLN H&E/IHC	N° of cases	SLN RT-PCR+* LVI present	SLN RT-PCR −# LVI absent
pN0 (i−)	2/42	1	1
pN0 (i+)	2/7	0	1
pNmi	2/2	1	1
pN1a	7/7	6	1

* RT-PCR+ for at least one marker.
# RT-PCR− for all markers.

Axillary dissection was performed also in 2 cases with negative SLN due to the high-grade of the primary tumour in one case and to the presence of lymphovascular invasion in the other. The histological analysis of the axillary dissection suggested a correlation of non-SLN metastases with the presence of lymphovascular invasion; the positive results of the RT-PCR are shown in Table 5.

Discussion

It is commonly reported that the discrepancy between the results of RT-PCR and immuno-morphological analysis in the study of SLN from breast cancer depends on the gross sampling procedure [15]. The majority of protocols use half of the unfixed lymph node for molecular analysis and the other half as fixed samples for traditional immuno-morphology [12, 13, 26]. Using methacarn for fixation to preserve both morphology and high quality mRNA [21, 22], we demonstrated that the step-sectioning procedures may

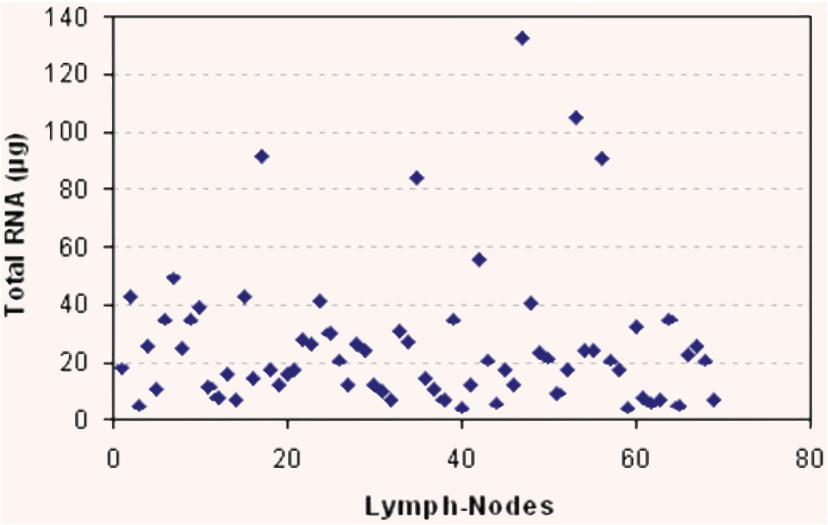

Fig. 2 Distribution of the amount of total RNA (µg) extracted from the 69 SLNs studied by RT-PCR.

Fig. 3 RT-PCR analysis of *Mammaglobin* in FFPE primary tumours corresponding to SLNs with ITC (cases 1, 2, 3, 4, 6, 7, 8) and with micrometastases (case 5) not detected by *Mammaglobin* in RT-PCR.

also impair the results of H&E, IHC and RT-PCR to detect metastatic involvement of SLNs. First of all, our data confirmed that the examination of the whole tissue block might be necessary even to detect all metastases measuring >2 mm, using either morphological or molecular procedures. In a previous geometrical model, Cserni showed that incomplete sectioning of the SLNs in extreme situations would almost completely miss metastases >2 mm, which would be identified at best as ITC [29]. A step-sectioning protocol with levels of 250 µm or 200 µm would be adequate for detecting nearly all micrometastases as defined by the current TNM definitions (*i.e.* metastases >0.2 mm). In the present study, using a step-sectioning protocol with intervals of 100 µm, we demonstrated that both IHC and RT-PCR are of limited value to detect all ITCs. In particular, we confirmed that ITCs are randomly distributed in the lymph node and that some of them may be missed regardless, or alternately, that they might be found by chance. These data should be taken into account when dealing with studies considering the predictive and prognostic value of ITCs, such as a recent study [30] that concluded that 'nanometastases' (defined identically to ITCs; *i.e.* as tumour cell deposits ≤0.2 mm) detected by IHC in axillary lymph nodes predict a worse survival than larger micrometastases which do not influence the outcome. In addition, recent data suggest that patients with occult micrometastases in axillary lymph nodes not receiving adjuvant systemic therapy have a prognosis similar to that of patients without micrometastases [31].

Ideally, for comparing different methodologies, sections obtained from all cutting levels should be analysed by RT-PCR, but the labour-intensive nature of the process makes this prohibitive. Using the protocol described herein, we could precisely map the distribution of metastases and indirectly prove that RT-PCR analysis using *Mammaglobin* would be specific enough to find all metastases and micrometastases, but that it is relatively insensitive for submicroscopic disease. The expression of *Mammaglobin* in the primary tumours that gave rise to ITC in *Mammaglobin* negative SLNs reinforces this hypothesis. On the contrary, a recent study that tested both *Mammaglobin* A and B isoforms in SLN using RT-PCR reported a high sensitivity for these markers [32].

On the other hand, the expression by RT-PCR of *CK19* and/or *CEA* in morphologically negative SLN could be more effective just in detecting ITCs considering that the thickness of the tissue analysed by RT-PCR in our study was 0.042 mm (6 sections of 7 microns). However, one case with lympho-vascular invasion and SLN negative at histology, but positive for *CEA* by RT-PCR, had non-SLN metastases, suggesting that sporadic ITC could have been missed in the histological examination but recognized by RT-PCR. The other possibility is to consider this case as one missed SLN.

Previous studies utilizing epithelial mRNA markers to detect breast cancer micrometastases by RT-PCR [7–11, 14, 26] have outlined the risk of false-positive results, whereas tumour mRNA markers are more specific, but are not uniformly expressed in all

cancers causing the risk of false-negative results. Thus, a combination of both epithelial and tumour markers could potentially yield the most reliable results [12].

Recently, new assays for quantitative measurement of *Mammoglobin* and/or *CK19* expression [33–35] have been described and proposed for rapid molecular diagnosis on SLN. In addition, one of these assays indicates specific cut-off values for CK19 mRNA to distinguish micrometastatic from metastatic SLNs and to define pN0 SLN (including ITCs) with an overall concordance rate between the molecular assay and histopathology of 98.2% [33]. However, in the sixth edition of the AJCC Cancer Staging Manual, metastatic lesions identified only by RT-PCR are classified as pN0(mol+) because there are insufficient data to determine whether such lesions are clinically significant. Prospective studies that would analyse the whole SLN using quantitative mRNA measurement and a pool of markers would probably validate molecular procedures in substituting the high-throughput histopathological analysis of the SLN.

In conclusion, the main goal of our study was to show that the sampling procedures of the tissue to be submitted to RT-PCR or conventional histopathological processes are the main reason for discrepancies between molecular and morphological analysis.

Acknowledgements

We acknowledge the financial support received in grants from Associazione Italiana per la Ricerca sul Cancro (AIRC), Compagnia di San Paolo special project 'Oncology', Ministero Istruzione Università e Ricerca (MIUR) Progetti di Ricerca di Interesse Nazionale (PRIN) 2005, Ministero dell'Università della Ricerca Scientifica e Tecnologica (MURST) (ex 60%) and Fondazione Cassa di Risparmio di Torino (CRT). Regione Piemonte Comitato Interministeriale per la Programmazione Economica (CIPE) 2004 also provided valuable financial contributions.

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