Semi-quantitative RT-PCR assessment of molecular markers in breast large-core needle biopsies*

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Large-core needle biopsies are frequently used for the preoperative evaluation of the breast lesions. In addition to initial diagnostic information, they can show the status of molecular markers with predictive and prognostic value and further contribute to an optimal selection of treatment strategy. So far, the potential use of large-core needle biopsies in assessment of marker profile of the breast lesions was studied using the immunostaining approaches. In this work, we sought to determine whether analysis of the large-core needle biopsy by semi-quantitative reverse-transcription polymerase chain reaction reveals molecular data that correspond with the marker status of the subsequently removed tumor. Five molecular markers including *ER*, *PR*, *c-erbB-2/HER-2/neu*, *c-erbB-3/HER-3*, and *CD44* were assessed on mRNA isolated from 23 large-core needle biopsies and corresponding surgical breast cancer specimens using β2-microglobulin as an internal standard. Significant or highly significant correlation between core biopsies and excised tumors was observed for each marker when the mRNA expression status was scored as positive or negative, with the concordance of the data ranging from 73.9% to 86%. Using the dichotomous scoring, majority of the biopsies (75%) displayed molecular profile that was either identical to the profile of the related tumor specimen, or with the difference in one marker. However, no significant correlation was found when the levels of the markers were expressed as continuous variables, possibly due to intratumoral tissue heterogeneity. These results suggest potential usefulness and reliability of semi-quantitative RT PCR in the evaluation of large-core needle biopsies with regard to marker positivity or negativity. On the other hand, the marker-related data expressed as continuous variables cannot be accurately assessed on the large-core needle specimens using this approach, indicating the need for methodological improvements.

Key words: large-core needle biopsy, semi-quantitative reverse transcription PCR, breast cancer, molecular markers

Incidence of breast cancer and related mortality show increasing tendency and shift to the lower age categories. This fact is prompting improvements in diagnostics and management of this complex disease. In recent years, large-core needle biopsies (LCNB) have been increasingly used for the preoperative histological evaluation of the breast lesions. They can be performed promptly, with minimum scarring, and at low costs. Diagnostic accuracy of the LCNB under stereotactic or ultrasound guidance, with high sensitivity and specificity, was proven in many studies [13, 19, 20, 22, 24–26].

In addition to technical improvements, better understanding of molecular mechanisms that underlie oncogenesis and influence the tumor behavior has opened new ways leading to rational management of breast cancer patients. Many molecular markers were identified as products of genes aberrantly expressed in cancer cells and rapid molecular protocols for their detection were elaborated.

Among the currently used molecular diagnostic methods, reverse-transcription polymerase chain reaction (RT PCR) possesses a special position due to its capacity to detect expression of numerous cancer-related genes in a diminished volume of tissue. Therefore, it is especially suitable for situations in which only very small specimens are available [9]. This is particularly the case of LCNB that

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yield tissue cylinders of a minute size what is in contrast to demands for assessment of increasing number of parameters.

This study was undertaken to evaluate reliability of the LCNB technique in conjunction with RT PCR-based molecular diagnostics measured against corresponding surgical specimens. To this end, we examined the use of semi-quantitative (SQ) RT PCR versus standard immunohistochemistry (IHC), and performed SQ RT PCR analysis of the breast specimens for expression of genes encoding five molecular markers followed by comparison of the molecular data obtained from the large-core needle biopsies versus surgical specimens.

Our analysis included the genes coding for estrogen receptor (ER), progesteron receptor (PR), c-ErbB-2/HER-2/Neu, c-ErbB-3/HER-3, and CD44. Steroid hormone receptors (ER, PR) are nuclear proteins that serve as prognostic factors and guides to endocrine therapy [12]. 50–85% of breast cancers contain measurable amounts of ER. PR is expressed following transcriptional activation by functional ER-estrogen complex. Simultaneous presence of ER and PR in a primary tumor indicates a 68% chance for success of hormonal treatment [6]. C-ErbB-2/HER-2/Neu is a transmembrane receptor tyrosine kinase over-expressed in 20–30% of breast tumors. It is associated with poor prognosis and may predict poor response to anti-estrogens in ER-positive tumors [17]. Catalytically inactive homologue c-ErbB-3/HER-3 binds a range of ligands and possesses capacity to enhance activity of c-ErbB-2/HER-2/Neu via heterodimerization [23]. Increased c-ErbB-3/HER-3 expression in breast tumors appears to be associated with the prognostically favorable ER phenotype [10]. CD44 is a cell surface glycoprotein occurring in several isoforms. It appears to be correlated with advanced stages of tumor growth and might serve as a useful prognostic indicator of metastatic potential [7].

So far, there were only several studies published on the immunohistochemical determination of the tumor marker status in large-core needle biopsies. This is the first report on the RT PCR in LCNB as an approach principally allowing for assessment of broader spectrum of markers and within a shorter time period than IHC. We demonstrated that SQ RT PCR analysis of LCNB specimens is a feasible and promising approach. Based on the detection of ER, we found significant relationship between the data obtained by RT PCR and standard immunohistochemistry, when they were expressed in terms of positivity/negativity. We have also observed significant relationship between the dichotomously scored RT PCR data from LCNB and corresponding surgical specimens and found that the expression profile of the markers is identical or highly similar in 75% of the LCN biopsies when compared to the related surgical specimens. However, because of the lack of reliability of the data expressed as continuous variables, routine application of RT PCR-based molecular diagnostics of LCNB requires further refinement of the conditions mainly regarding the elimination of the problems related to tumor tissue heterogeneity.

Material and methods

Human breast tissues. Breast cancer specimens were collected from 31 patients with mean age of 53.6+4.0 years (ranging from 33 to 82) at the Department of Gynecology and Obstetrics II, Comenius University School of Medicine, Bratislava. Patients did not received anti-neoplastic treatment before surgery. Both palpable and non-palpable breast lesions were examined with ultrasound (ATL, linear high resolution scan). All lesions with ultrasound findings were preoperatively evaluated by ultrasound-guided LCNB. The biopsy was excised using a MAGNUM* Core High Speed biopsy gun, 13 gauge coaxial system and 14 gauge core-cut needle (all equipment from C.R.BARD GmbH, Karlsruhe, Germany). Up to five biopsy specimens were obtained from each tumor. Three to four specimens were subjected to histological evaluation (1–2 out of them with frozen-section technique), and 1–2 biopsy specimens were frozen at –80 °C for RT PCR analysis. Part of each tissue specimen obtained at definitive surgery was processed for histological diagnosis and immunohistochemistry while another part was frozen in liquid nitrogen for RNA isolation and consequent RT PCR.

RNA extraction. Total RNA was extracted from frozen breast tumor tissue by disrupting specimens in Trizol solution (GIBCO, Life technologies). RNA was precipitated by ethanol and pellet was dissolved in water treated with diethylpyrocarbonate (Sigma).

Reverse transcriptase cDNA synthesis. Total RNA from each tumor sample was reverse transcribed with oligo$(dT)_{12\text{-}18}$ (500 μg/ml). 5 μl RNase-free sterile water containing 1 μg total RNA from each sample was added to a reaction mixture composed of 0.5 mM M dNTPs (Pharmacia) of each, reverse transcriptase buffer 6 mM $MgCl_2$, 40 mM KCl, 1 mM DTT, 0.1 mg/ml BSA, 50 mM Tris-HCl pH 8.3 in 20 μl final volume. The mixture was heated 10 min at 70 °C, and cooled quickly on ice, supplemented with 200 U of reverse transcriptase SuperScriptII (GIBCO Life Technologies), incubated for 1 h at 42 °C, finally heated for 15 min at 70 °C and stored at –80 °C until used.

PCR amplification. Semi-quantification of *ER*, *PR*, *c-erbB-2/HER-2/neu*, *c-erbB3/HER-3* and *CD44* expression were performed following co-amplification and normalization with β2-microglobulin (β2-m) as an internal control. The nucleotide sequences of the cDNA-derived primers for individual markers, with relevant positions and database references in parentheses, were as follows:
estrogen receptor (M12674):

ER1 5' ACTCGCTACTGTGCAGTGTGCAATG 3' (776-800)
ER2 5' CCTCTTCGGTCTTTTCGTATCCCAC 3' (1014-990)
progesteron receptor (M15716.1):
PR1 5' GGGATGAAGCATCAGGCTGTCATT 3' (1887-1911)
PR2 5' TCATTTGGAACGCCCACTGGCT 3' (2170-2148)
c-erbB-2/HER-2/neu (M11730):
EB2-1 5' AGTTTCCAGATGAGGAGGGCGCATGCC 3' (1994-2020)
EB2-2 5' TTCTCCCCATCAGGGATCCAGATGCCC 3' (2384-2358)
c-erbB-3/HER-3 (M34309):
EB3-1 5'GGTGCTGGGCTTGCTTTTCAGCCTGG 3' (222-247)
EB3-2 5'ACCACGCGGAGGTTGGGCAATGGTAG 3' (512-487)
CD44 (M59040.1):
CD44-1 5' GGGTCCCATACCACTCATGGATCT 3' (725-748)
CD44-2 5' GGGAAAGGTTGGCGATCAGGAATA 3' (1444-1421)
β2-microglobulin (AF072097):
B2M1 5' CATCCAGCGTACTCCAAAGA 3' (860-879)
B2M2 5' GACAAGTCTGAATGCTCCAC 3' (1024-1005).

PCR was performed with an automatic DNA thermal cycler (BIORAD). Reaction mixture was composed of 1/40 of cDNA template obtained from 1 μg of RNA, 15 pmol of each upstream and downstream primer for both marker and β2m amplification, 0.2 mM of each dNTP, 1 U of Taq DNA polymerase (Boehringer Mannheim), 1X PCR buffer with Mg^{2+} (Boehringer Mannheim) for amplifications in a final volume of 25 μl. Following an initial denaturation at 95 °C for 1 min, the step cycle program was set as follows: denaturation at 95 °C for 20 sec, annealing at 65 °C for 30 sec, and extension at 72 °C during 40 sec for a total of 30 cycles, and finally 5 min at 72 °C. In these conditions, amplified products were obtained in the exponential phase for the two sets of primers, and the yield of PCR products obtained following co-amplification was similar to those obtained following individual amplification in separate tubes. In addition to tumor samples, negative and positive controls were systematically processed in parallel.

Quantification of PCR products. Twenty μl of PCR products were run on a 1.8% agarose gel. Digital video images of ethidium bromide stained gels were analyzed using Scion Image software. Amount of marker-specific PCR product was expressed in a value corresponding to percentage of amount of internal control. Threshold for positivity was determined at 5% of β2m.

Immunohistochemistry. Five micron sections of formalin-fixed and paraffin-embedded tissues were deparaffinized and immunostained by avidin-biotin complex method according to instructions of manufacturer (ABC kit, Vector Laboratories, Burlingame, CA). Detection of ER was performed using specific anti-ER monoclonal antibody (clone ER1D5 from Immunotech, Marseille, France) at a dilution 1:50, followed by the secondary biotinylated horse anti-mouse IgG (dilution 1:300) and incubation with avidin-biotin-peroxidase complexes. Diaminobenzidine tetrahydrochloride (DAB) was used as chromogene (Sigma, St. Louis, MO). After treatment, the sections were counterstained with hematoxylin and mounted with permount. Nuclear staining of neoplastic cells was scored as positive. The number of positive cells in tumor area per tissue section was determined and total proportion (%) of neoplastic cells that were stained positively at any intensity was scored. The threshold for positivity was 5%.

Statistics. No ($p>0.05$), significant ($p\leq0.05$) and highly significant ($p<0.01$) relationship in positivity/negativity determination was tested by the Chi-square test. Quantitative correlations were determined only for non-zero samples using Pearson's test.

Results and discussion

Histological evaluation of large-core needle biopsies versus surgical specimens. The breast tissues examined in this study involved 31 surgical specimens containing 3 ductal carcinomas *in situ*, 24 invasive ductal carcinomas, 2 invasive lobular carcinomas, 1 mucinous carcinoma and 1 tubular carcinoma. In addition, large-core needle biopsies corresponding to 23 surgical specimens were analyzed. Histological diagnoses of LCNBs including stage and grade fully corresponded with the data obtained from matched surgical specimens in all cases. This fact indicated that LCNB was an appropriate alternative to surgical biopsy with the accurate tissue sampling and reliable preoperative histological assessment. Thus the LCNB specimens fulfilled the inevitable prerequisite for their use in assessment of molecular status.

Optimization of the RT PCR procedure for semi-quantitative analysis. First of all, we performed preliminary experiments to fully optimize the RT PCR conditions for our purposes determining that the most effective cycle number was 30. This cycle number was within the linear range of amplification for all analyzed mRNA species. Optimum time and temperature were determined in order to receive a balanced co-amplification of marker-specific and internal control genes. Furthermore, RT PCR performed repeatedly (4–5 times) in a small subgroup of tumor samples for each analyzed marker was found to be highly reproducible, with a mean densitometry variance of only 5% (data not illustrated).

Initial RT PCR amplifications were done using surgical

specimens as a source of mRNA. The next step was to confirm that LCNBs that are of diminished size allow for isolation of sufficient amount of template mRNA to perform simultaneous semi-quantification of multiple molecular markers. From one LCNB cylinder of approximate size 20 x 1 mm, we were able to obtain intact RNA template sufficient to produce reverse transcribed cDNA for 30 to 50 independent PCR reactions. This allows for simultaneous assessment of relatively broad spectrum of molecular markers.

Evaluation of RT PCR versus immunohistochemistry. Although high sensitivity and specificity of RT PCR was proven before in numerous studies, we wanted to know to what extent the information obtainable by this method is in accordance with that revealed by standard immunohistochemistry (IHC). Therefore, we performed a comparative analysis of estrogen receptor (ER) protein versus *ER* mRNA in 31 surgical specimens of malignant lesions partly processed for routine IHC and partly frozen for RT PCR.

The tissues were first immunostained for expression of ER protein with the MAb ER1D5 used in the routine practice. Percentage of the tumor cells that showed distinct nuclear staining signal was recorded for each specimen. The specimens were categorized as negative (-) when less than 5% of neoplastic cells stained for ER and as positive (+) with the percentage of stained cells equal to or higher than 5%.

In RT PCR, semi-quantitative level of *ER* mRNA in each specimen was expressed in percentage calculated from the ratio between *ER* and β2-m internal standard amplicons. Similarly as in IHC, the specimens with equal to or higher than 1:20 ratio (i.e. 5%) of *ER*-specific amplification product to β2-*m* were scored as positive.

First, we compared the results of IHC and RT PCR using data scored as negative and positive. These data showed significant relationship (p=0.0303) with a concordance of 70.9% suggesting that RT PCR can provide results principally similar to IHC. However, analysis of samples with the value of expression above zero, that were given as continuous variables, did not show any significant correlation between IHC and RT PCR determinations of ER level (Pearson's correlation coefficient R=0.0899, Fig. 1a).

The lack of full concordance between IHC and RT-PCR in negativity/positivity evaluations and the absence of significant relationship between semi-quantitatively determined values of ER expression may have similar reasons. First, it could be due to use of different portions of the tissues for each type of assessment. Indeed, the intratumoral differences and related regional heterogeneity of marker expression is frequently underestimated in similar studies, but may account for many discrepancies in results. In this respect, IHC possesses some advantage over RT PCR, because it allows the recognition of tumor cells based on morphological criteria, estimation of the tumor area extent and elimination of the marker expression in normal cells, while RT PCR detects the marker in the whole tissue specimen, including those areas that may contain more stromal and less tumor cell components. Additional important factors may be the differences in the methods and the fact that they deal with either mRNA or protein as the products of the gene whose expression may be differentially affected by control mechanisms and processing. This can play a major role in cases, where differently spliced mRNA variants produce defective proteins, as observed for ER [3, 4]. In this respect, RT PCR may be of advantage over IHC as it can disclose expression of such mRNA variants when using appropriately designed primers. The discrepancy between IHC and RT PCR may also stem from the differences in the rate of the degradation and stability of mRNA versus corresponding protein, as demonstrated for ER, PR and c-erbB-2/HER-2/Neu, whose autoregulation is affected by

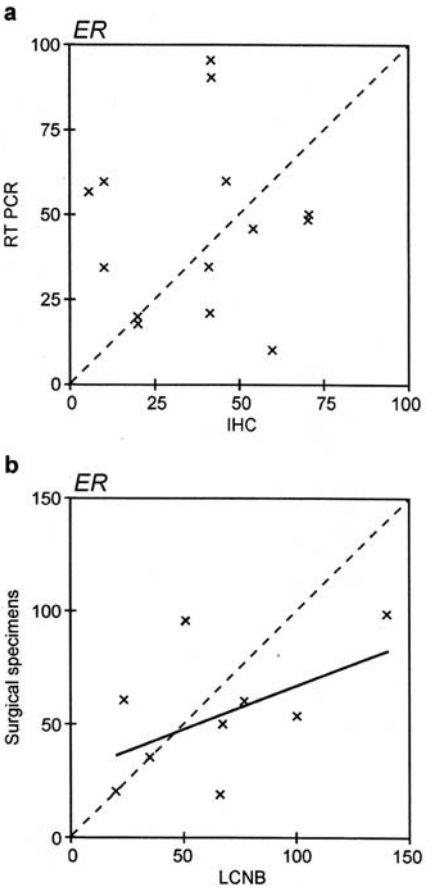

Figure 1. Graphic illustrations of the relationship between non-zero *ER*-related data obtained (a) from surgical specimens analyzed by IHC versus RT PCR and (b) from paired LCNB versus surgical specimens both analyzed by RT PCR. Values represent expression of ER protein given as percentage of ER-positive tumor cells in tissue section (IHC) and relative level of *ER* mRNA expressed as per cent of β2-m internal control (RT PCR). No correlation was observed by Pearson's test.

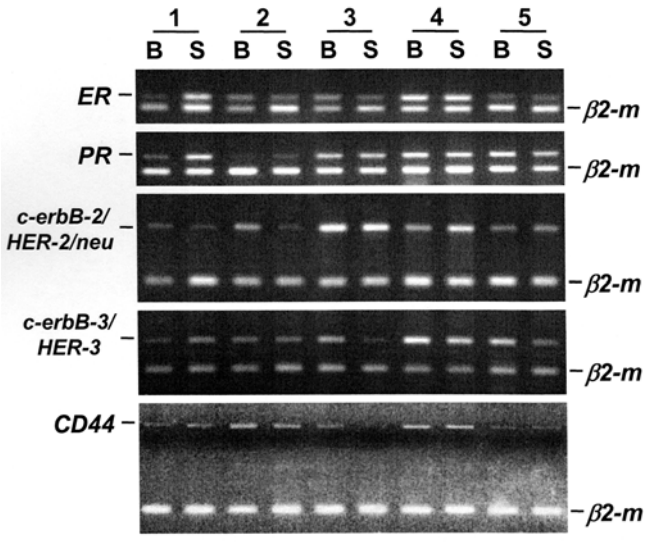

Figure 2. RT PCR analysis of breast LCNB (B) compared to corresponding surgical specimens (S). Five paired samples are shown for each marker. Gene-specific amplifications were performed simultaneously with β2-microglobulin that served as an internal standard for semiquatitation of resulting products.

both transcriptional and posttranscriptional mechanisms [14–16, 18].

RT PCR analysis of ER in large-core needle biopsies versus surgical specimens. Next we asked whether results of RT PCR assessment of *ER* mRNA in LCNB biopsies correspond to those from RT PCR in matched surgical specimens (Fig. 2). The specimens were evaluated as described above. Relationship between LCNB and surgical specimens is shown in Table 1 and Figure 1b. Again, we found lack of correlation between RT PCR data from surgical specimens and from LCN biopsies when these were expressed as continuous variables (R=0.3312), while the dichotomously scored *ER* expression (i.e. positive versus negative) revealed significant relationship (p=0.0191) with the 73.9% concordance. Out of 23 LCN biopsies, there were 4 biopsies scored as positive, but found negative in the corresponding surgical specimens. In addition, there was an overall tendency to obtain higher ER values from LCN biopsies than from excised tumors. Also DOUGLAS-JONES et al [2] observed higher ER score in IHC staining of LCNB compared to excised tumor. They found that ER expression was higher at the periphery of tumor than at the center and deduced that higher expression in LCNB may reflect the higher chance of sampling the peripheral part of a tumor. In our study, LCN biopsies corresponding to *ER*-negative lesions showed a false-positivity of 17% (4 cases). On the other hand, two LCN biopsies were negative while their surgical counterparts expressed low level of *ER* mRNA. These 2 cases account for 9% false negativity of LCN biopsies with respect to *ER* expression.

Table 1. Relationship between data obtained by semiquantitative RT PCR for individual markers in paired LCNB versus surgical specimens

Marker	χ^2 test (p value)[a]	Concordance (%)[a]	Pearson's correlation (R value)[b]
ER	0.0191	73.9	0.3312
PR	0.0273	78.0	0.1985
c-erbB-2/HER-2/neu	0.0099	78.0	0.2597
c-erbB-3/HER-3	0.0164	78.0	0.4750
CD-44	0.0085	82.0	0.4053

[a]Specimens were categorized as negative or positive for the particular marker. [b]Pearson's correlation test was performed using non-zero data expressed as continuous variables.

RT PCR analysis of other molecular markers in LCN biopsies versus surgical specimens. Correlation of RT PCR data from LCNB versus surgical specimens was determined also for additional four molecular markers examined in this study (Fig. 2). Non-zero data scored as continuous variables did not display significant correlation for any of the markers, as shown by the values of the relevant correlation coefficients (Fig. 3, Tab. 1).

Analysis of dichotomously scored data with values equal to or above 5% of specific product level measured against β2-m revealed values of concordance and corresponding correlation coefficients as summarized in Table 1. Significant correlations were found for *PR* and *c-erbB-3/HER-3*, while *c-erbB-2/HER-2/neu* and *CD44* showed highly significant positivity/negativity correlations between LCNB and surgical specimens. False positivity of LCN biopsies for *PR*, *c-erbB-2/HER-2/neu*, *c-erbB-3/HER-3* and *CD44* was observed in 26% (6 cases), 17.4% (4 cases), 13% (3 cases), and 13% (3 cases) respectively, while each marker showed less than 5% false negativity corresponding individually to 1 case.

These data confirmed the finding described above for *ER*, that the results of RT PCR assessment of expression levels of markers as continuous variables are not reliable in evaluation of LCN biopsies. JACOBS et al [5] encountered similar problem in their IHC study of prognostic markers. Immunostaining of LCNBs and corresponding excisions gave significant correlations for the markers scored as positive or negative. However, microvessel density, which was scored as a continuous variable, could not be reliably assessed on the LCNB specimens. The authors argue with a wide variation in microvascular density in the same tumor, not only between different tissue blocks, but also between different areas on the same section, what was also documented in another study [1]. Our finding with RT PCR data may have the same background. This problem could be probably solved by performing RT PCR on mRNA isolated directly from the frozen sections of LCNB cylinder used for evaluation of histological parameters. Such attitude would facilitate more precise interpretation of the results especially

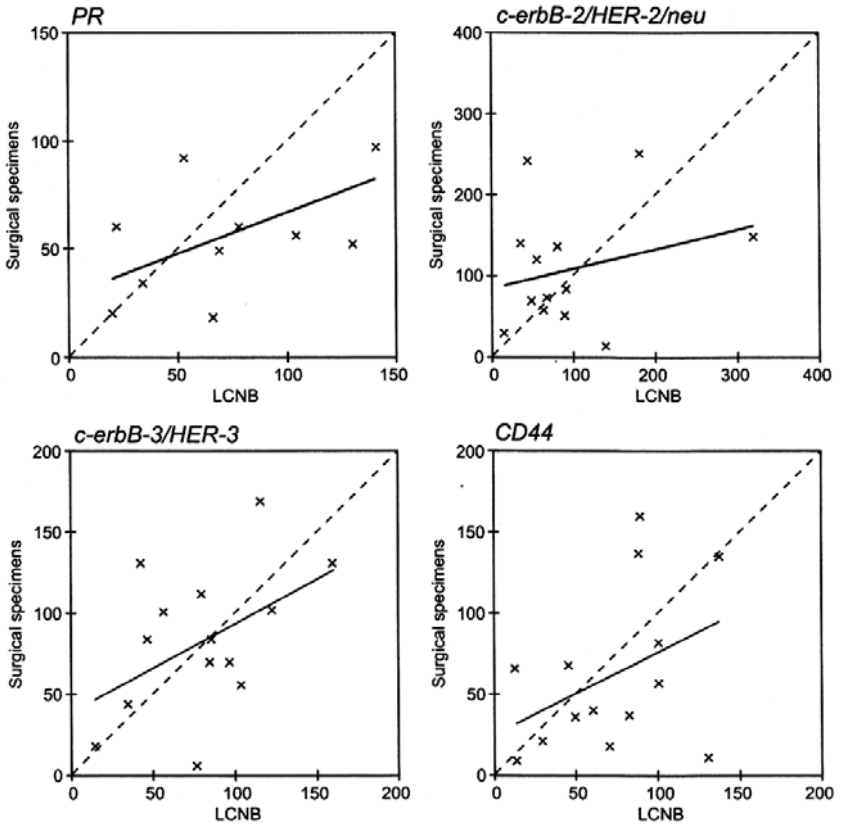

Figure 3. Pearson's correlation of non-zero values for *PR*, *c-erbB-2/HER-2/neu*, *c-erbB-3/HER-3* and *CD44* markers obtained by semi-quantitative RT PCR from LCNB and corresponding surgical specimens. Values represent relative levels of marker mRNAs expressed as per cent of β2-m internal control. Any of the data series did not show correlation when analyzed as continuous variables.

with respect to the presence and proportion of morphologically abnormal tumor cells. Another possibility is to use a relational RT PCR [21] based on detection of the cell-specific gene such as the gene coding for cytokeratin 19 that is expressed only in breast epithelial cells but not in tumor stroma. However, the intratumoral heterogeneity will still remain an important problem that has to be taken in account not only in LCNB evaluation, but also in classical studies of molecular markers using surgical specimens.

Comparison of the molecular marker profiles obtained from LCNB versus surgical specimens. Availability and relative reliability of dichotomously scored RT PCR data on several markers obtained from a single LCNB specimen could be finally used to built a miniature molecular profile of the biopsy that can become very useful for better preoperative characterization of the tumor and planning the type and radicality of surgical treatment with patient's consent. This would only be possible providing that this molecular profile reflects the molecular status of the tumor determined after surgery. Therefore, we compared the molecular profiles of the five markers evaluated here, using positivity/negativity criterion. The comparison has shown that the majority of LCNB displayed identical or highly similar profile as their surgical counterparts, with fully corresponding results in 56% of paired specimens, while 75% of specimens would satisfy the requirement for at most one-marker difference and 94% of specimens for maximum two-markers difference. This favorable finding supports potential usefulness of semi-quantitative RT PCR application in LCNB, but also indicates the need for improvements in methodology and for increase in the number of analyzed specimens. Indeed, it cannot be excluded that evaluation of larger collection of specimens might allow to obtain significant relationships also between the continuously expressed data. Moreover, it is highly probable that evaluation of increased number of markers on a single LCNB specimen could further improve confidence limits for the obtained results.

Conclusion

LCNB gains significant importance as a time- and cost-saving approach to preoperative characteristics of breast tumors including selection for neoadjuvant regimens. They do not require general anesthesia, leave minimal scaring, potentially spare patients with benign lesions from unnecessary surgery and reduce the number of surgical procedures required for complete surgical treatment of non-palpable breast cancer. In addition to histological parameters, they may become important source of the pretreatment information on the tumor molecular status and potentially allow faster and more precise surgical treatment planning [8, 13, 25, 27]. These benefits depend on the correlation between information obtained from LCN biopsies and from tumor as definitely assessed postoperatively. There is sound amount of data showing that the diagnostic accuracy of LCN biopsies is sufficiently reliable. There are also several studies describing immunohistochemical determination of the molecular markers including c-ErbB-2/HER-2/Neu, ER, bcl-2, and p53 [2, 5, 11]. Here we provide the first evaluation of the semi-quantitative RT PCR assessment of molecular markers in LCNB. We show that this approach may be potentially useful for fast small molecular profiling of the tumor, reliably offering the basic information on individual marker positivity or negativity. However, in the present settings, the semi-quantitative data cannot be accurately assessed on the LCNB

specimens. Based on this experience, further development of the SQ RT PCR towards its more reliable use in molecular evaluation of LCN biopsies is definitely desirable.

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