The impact of RNA standardization and heterogeneous gene expression on the results of cDNA array of human breast carcinoma

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Abstract. cDNA microarray is an established technique. However, difficulties such as handling tissue samples under RNase-free conditions, the heterogeneous tumor composition, i.e. non-malignant versus malignant cells and different pathologic types of malignant cells, and lack of appropriate reference may limit the potentially benefit of this method in clinical use. In this study, we examined how standardization of gene expression to total mg RNA or mg tissue and tumor heterogeneity affect the final results. We found that the gene expression of human breast tumors was ~9 times higher in malignant tissue as compared to the non-malignant tissue when expressed per total mg RNA, but ~40 times higher when expressed per mg tissue. Genes that were expected to act as housekeeping genes (PUC18, RPL and ß-actin) varied between different parts of the tumor and also between non-malignant and malignant tissues, excluding them as reference genes. We also found that the gene expression differed in various parts of the breast tumor, probably due to a mixture of different types of cells, i.e. non-malignant and malignant cells. To find out if the variations in the gene expression were due to cell heterogeneity we used microdissection to collect malignant cells separately. We found that the gene expression was markedly different in the isolated malignant cells as compared to the gene expression of the bulk tumor tissue. Thus, to be able to evaluate results from cDNA array gene expression experiments it is, to our opinion, necessary to work with pure tumor cell populations, until solid information is available on the impact of stromal component. Housekeeping genes should be handling with care and mg tissue may be preferred instead of μg RNA for standardization.

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Introduction

Breast cancer is a heterogenic disease, both histopathologically and clinically. Many potential prognostic and predictive factors of breast cancer have been suggested and some of them are used to estimate the level of risk and thereby suggesting adequate treatment. Useful risk factors are patient's age, tumor size, lymph node status, estrogen and progesterone status, Her-2 neu receptor positivity and proliferation rate.

Advances in molecular biological research have led to better understanding of tumor biology and to identification of prognostic and predictive biological factors and signatures. The use of cDNA microarrays for studying gene expression of cancer has increased during the last decade. Gene expression profiles may identify specific phenotypes (diagnosis), establish a patient's expected outcome (prognosis), and indicate the likelihood of a beneficial effect of a specific therapy (prediction) (1-3). cDNA microarray allows the comparison of gene expression profiles from two or more tissues or the same tissue in different biological states (4).

Molecular analysis of tumor requires methods that allow rapid and reproducible detection of alteration in gene expression. The question is how the quality of mRNA changes when using different treatment of tissues samples and isolation techniques. There are still few studies on preparation of RNA for cDNA microarray in malignant tissues. An optimized protocol regarding optimal tissue acquisition, processing, and analysis procedures for exploring the gene expression should be available when using this technology. cDNA microarrays are performed on chips, glass slides, or filters. There is no compelling evidence of major differences in the accuracy or reproducibility of the various microarray platforms (5-7). Only fresh or appropriately frozen tissues provide the necessary quality of RNA for microarray. However, one concern with frozen tissue banks is the frequent lack of a standardized approach for tissue acquisition and processing. Furthermore,

oxygen deprivation occurring upon surgery can trigger a hypoxic response, characterized by an altered expression of specific genes (8-11). Several of these genes are transcription factors that further affect the expression of their target genes (9,12). Thus, factors influencing the molecular quality of tissues are the type of specimen, pre-excision hypoxia, preservation treatment of the tissue, extraction method, type and length of storage and freeze and thaw. Acellular and pancellular tissues are also less labile than densely cellular tissue (13).

In this study, we investigated how tumor heterogeneity, RNA standardization and housekeeping genes affect the evaluation of gene expression determined by cDNA micro-array. We also showed the possibility to overcome these problems by using microdissection by Laser Capture Microscope (LCM).

Materials and methods

Patients. Samples from breast tumors of six women who were referred at the Department of Endocrine Surgery, Karolinska University Hospital, Huddinge, were collected, and prepared for the cDNA array analysis as describe below. Routine breast surgery procedures were used, and to avoid RNA degradation, the tissues samples were put into RNAse free tubes containing RNA Later buffer (Qiagen GmbH, Germany) within 10-15 min after operation, analyzed directly for cDNA array or stored at -70˚C. Specimens consisted of invasive ductal breast cancer (IDC) and ductal breast cancer *in situ* (DCIS). Normal breast tissues were obtained from three patients (no. 1, 2 and 6) who underwent surgery for their breast cancer. The gene expression of tumor and normal tissues is based of patients no. 1, 2 and 6. The samples from the other patients could not be used for gene expression because of problem to fully evaluate the results. The samples supplied to this study were not tissues removed specially for research but were excess tissue not essential for routine diagnosis and histophatological staging. Patients were required to give consent.

Laser capture microscope. Individual normal (non-malignant) and malignant cells were collected from the tissue section of human breast carcinoma (patient no. 6) by Laser Capture Microscope (LCM) technique (Arcturus Ltd., UK). A skilled pathologist judged the type of cells, i.e. malignant versus non-malignant. The cells were collected into RNAse-free tubes and immediately prepared for RNA amplification and extraction according manufacturer's protocol (Arcturus Ltd.). The RNA extracts were stored at -70˚C before the cDNA array analysis.

RNA extraction. Total RNA was isolated using an RNA-Bee isolation kit (BioSite, Sweden). Samples of 50 mg tissue were homogenized in 1 ml RNA-Bee, initially using a pair of RNA-free scissors and then the samples were homogenized. Chloroform (0.2 ml) was added to the homogenized sample. The sample was stored on ice for 5 min and then centrifuged at 12,000 x g for 15 min. The colorless aqueous phase was collected and 0.5 ml of isopropanol was added to the sample (aqueous phase) and then the sample was stored at room temperature for 10 min before it was centrifuged at 12,000 x g for 5 min. The RNA pellet was washed twice with 75% ethanol and then the sample was dried for 10 min. The total amount of RNA was measured at 260 and 280 nm in an UV spectro-photometer.

Quality of total RNA. The integrity of the total RNA was analyzed on a denatured 1% agarose/formaldehyde gel according to the manufacturer's standard protocol (Qiagen GmbH).

cDNA array. Human Signal Transduction Pathway Finder Gene of 87 genes (the GEArray Q Series Human Signal Transduction Pathway Finder Gene Array: HS-008, Super Array Inc., Maryland, Bethesda, MD, USA), corresponding to 18 signal transduction pathways, was used for this experiment. PUC18 and RPL were used as negative controls and ß-actin was used as positive control.

Total RNA (5 μg) were used for each labelling. The total RNA was used as a template for the biotinylated probe synthesis. Probe preparation and hybridization of the membranes were done according to the manufacturer's manual as described by SuperArray Inc. The only modification of the protocol was a change of the washing temperature and time from 60˚C for 15 min to 68˚C for 20 min for washing solution no. 1 and 2. Quantitative data were obtained using AGFA Curix 60 photographic film (AGFA, Sweden). The results were obtained from two experiments (two determinations per each experiment). The deviation in the determination was <10%.

Results

Standardization of gene expression. In this part of the study, we examined how gene expression values may change when normalizing to total mg RNA or mg tissue. The mg tissue corresponds approximately to the number of cells in a tissue. In this study, we used malignant and the non-malignant tissues from the same patient. We found that the total RNA concentration in the malignant tissue was ~12 times higher (0.5 μg/mg tissue) as compared to the non-malignant tissue (0.04 μg/mg tissue) (Fig. 1A), which should be expected in tissues containing growing cells. The gene expression was generally higher in the malignant tissue as compared to the non-malignant tissue independent of normalizing to total RNA (Fig. 1B) or mg tissue (Fig. 1C). However, the gene expression was ~9 times higher in the malignant tissue as compared to the non-malignant tissue when normalizing to total RNA, but ~40 times higher when normalizing to mg tissue. Since the non-malignant cells are in G_0 stage of the cell cycle, while the malignant cells are in G_1, S- and G_2 stages, the non-malignant and the malignant cells are not comparable from growth related compounds point of view. Thus, it should be more accurate to normalize to number of cells than to total RNA, when comparing non-malignant tissue to malignant tissue. It is not possible to count cells in a tissue, therefore in this study we used mg tissue, which approximately corresponds to number of cells.

Gene expression in various part of the tumor. Herein we investigated the degree of variation in gene expression in

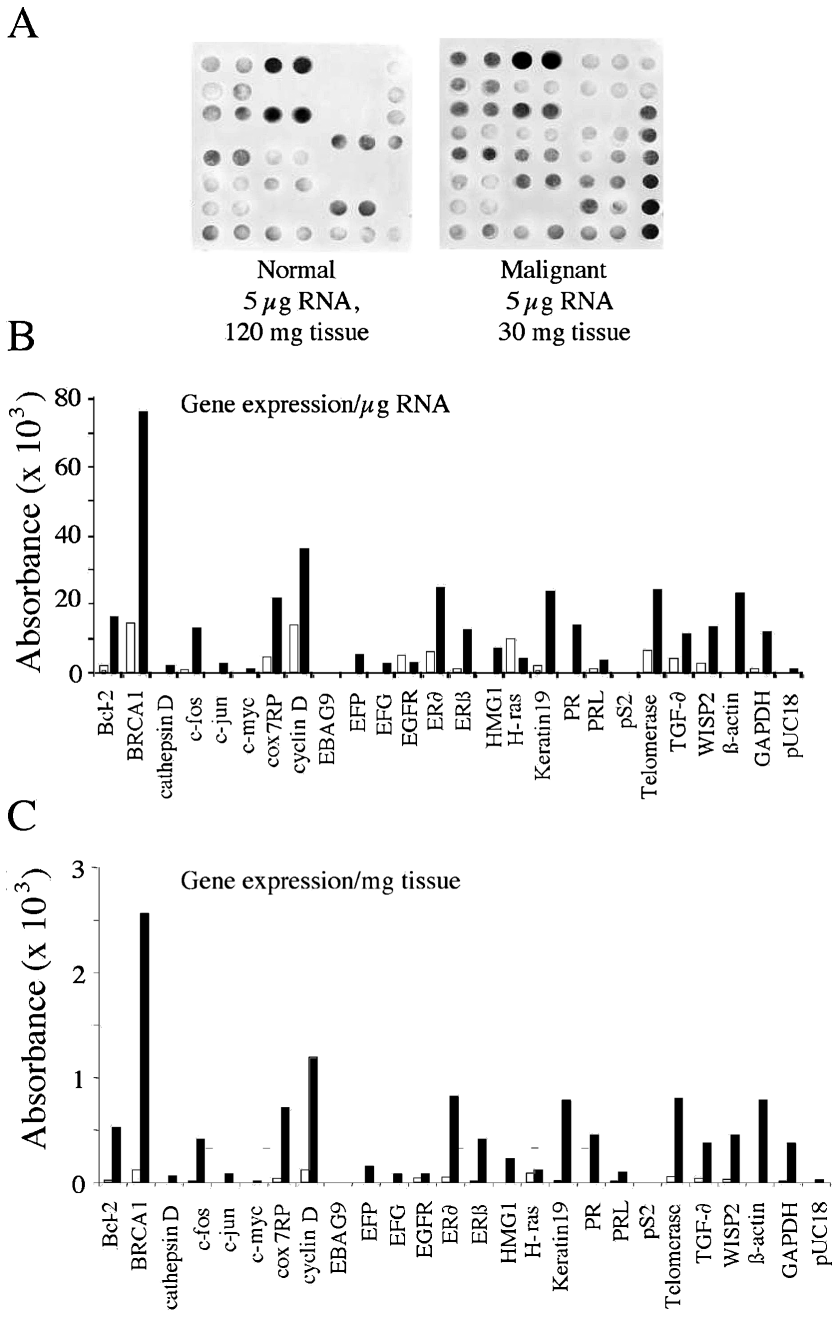

Figure 1. Gene expression by means of cDNA array of invasive ductual breast carcinoma of one patient (patient no. 1). (A) Example of cDNA array of normal non-malignant and malignant tissue of 23 genes and 3 housekeeping genes; (B and C) are results from part A expressed as per μg RNA (B) and per mg tissue (C). (□) normal tissue; (■) malignant tissue.

different parts of the human breast tumor. We cut the breast tumor (invasive ductal breast cancer, patient no. 2) in 5 pieces and measured the gene expression in each of them by means of cDNA-array. Total RNA (<0.5 μg) was enough for the cDNA array analysis, corresponding to a fine needle biopsy. The gene expression between the various pieces differed 2-4 times (Fig. 2). We also found that the expression of housekeeping genes deviated markedly between malignant and non-malignant tissues, and thus were not useful as reference genes (Fig. 2).

Microdissection. To overcome the contribution of non-malignant cells to the gene expression of the malignant cells we used microdissection by means of a Laser Capture Microscope. Thirty malignant individual cells were collected from a section of the breast tumor (patient no. 6) by a pathologist; the RNA was extracted and then amplified by means of an amplification kit provided by the company (Arcturus Ltd.). The gene expression was determined by cDNA array. In parallel we cut a piece of the same tumor (~5 mm in diameter), extracted the RNA and then determined the gene expression in the same manner as for the single malignant cells. Of the genes we detected, there was an extensive difference in the expression between individual malignant cells and the expression seen in the piece of the tumor tissue. The results are shown in the Fig. 3.

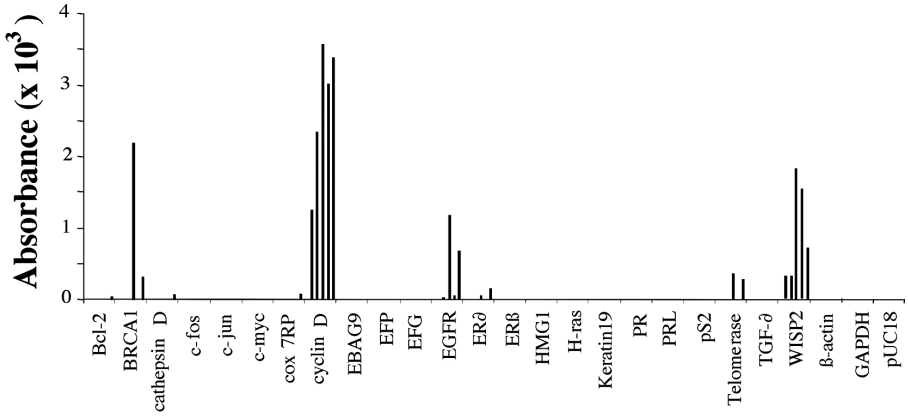

Figure 2. Gene expression of invasive ductual breast carcinoma of one patient (patient no. 2). The tumor was cut into five pieces and the gene expression was determined by means of cDNA array. Each bar represents one piece of the tumor.

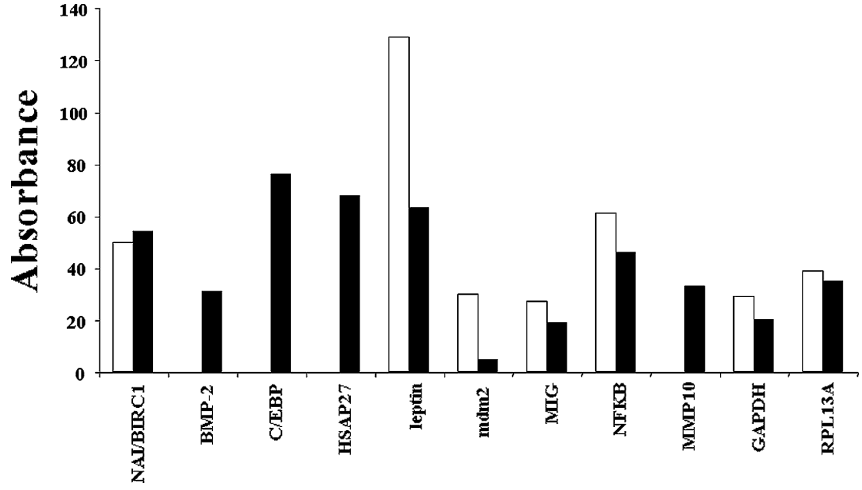

Figure 3. Gene expression of invasive ductual breast carcinoma of one patient (patient no. 6). A smear of part of the tumor was applied to glass and fixed. By means of a microdissection (see Materials and methods), single malignant cells were isolated, RNA extracted and genes expressions was determined by cDNA array (□). Gene expression of a piece of the tumor, including both non-malignant and malignant cells, was also determined by means of cDNA array (■).

Discussion

The study of human cancer has mainly been limited to surgical and histophatological description of the malignancy up to almost a decade ago. After completion of the Human Genome Project attention has been turned to functional genomics and proteomics, and thus increasing number of studies have been done on human malignant tissues using cDNA array, for example leukemia, lymphoma, adenocarcinoma of lung, breast, melanoma, and prostate. Despite the concern about the mRNA integrity, due to inaccurate sampling procedures, the gene expression based on cDNA array seems to have diagnostic, prognostic and predictive values, as well as being able to modify the way that tumors are treated. However, recent studies show that a number of factors during the cDNA array procedure can affect the final results of gene expression. For example, the way tumor tissues are collected and preserved influence the mRNA integrity, use of inaccurate 'housekeeping genes' and tumor heterogeneity affects the gene expression levels estimated (14). Such uncertainty in the cDNA methods may explains the lack of compliance between some clinical studies.

Gene expression of cells is determined by measuring the mRNA concentration. cDNA microarray measures the steady-state levels of mRNA. Different genes are expressed in various degrees in different tumors, and in the same tumor at different times. Also, the concentration of mRNA measured depends on where, when and how the samples have been collected. The development of invasive tumors is a dynamic process including several different stages also involving activation and deactivation of different genes. Furthermore, a growing tumor demands more oxygen and nutrition which could result in an uneven tumor content regarding malignant and normal cells, probably also appearance of necrosis in the centre of the tumor with well feed cells at the tumor margin.

Zhu *at al* showed in a study on breast ductal carcinoma *in situ* and on invasive carcinoma that gene expression profiles obtained from cells collected from different location in the same tumor were different (15). A malign tumor that develops

in a particular organ has its own composition. The host organ may or may not have a homogeneous structure, for instance lung and liver are homogeneous while breast is a heterogeneous organ. Thus, in the case of breast carcinoma the content of a sample can vary depending on the characteristics of the tumor (ducal or lobular) and patient (age, pre-postmenopausal, parity status).

In this study, we found that normalization of gene expression per mg tissue gave larger differences between non-malignant and malignant tissues, than normalization to μg total RNA. The amount of RNA was low in non-growing cells, but increased when the cells became proliferating. Since the non-malignant tissue represents almost non-growing cells, while malignant tissues proliferate, from gene expression point of view they are not exactly comparable.

We also studied the variation in gene expression within the same tumor, and differences in gene expression profile between normal breast tissue and breast malignancy. We found a different expression profile between normal breast tissue and breast malignancy, which should be expected, but also within the same tumor. As expected in growing cells, our results showed higher levels of cell cycle related genes in the breast malignant cells. Very high level of cyclin D has been reported in human breast tumor cells. In MCF-7 Tam-resistant cells of breast origin, of 127 genes studied, only cyclin D1 was found to be overexpressed (16). We also found in this study that Bcl-2 and BRAC1 were overexpressed in the breast tumor as compared to normal breast tissue. Bcl-2 is supposed to inhibit apoptosis, and thus higher level of Bcl-2 expression may be promoting tumor growth (17-19). In contrast to our result, other groups have reported low level of BRCA1 in invasive breast cancer (20,21). Similarly BRCA1 is up-regulated in proliferating cells and high level of BRCA1 mRNA has been reported in growing cells (22,23). Thus, caution must be used in interpreting studies in which this gene is overexpressed (20).

Our results showed that gene expression is not homogeneous in breast ductal carcinoma cells as well as in normal breast tissue. This heterogeneity in expression cannot be explained by the way we have processed the RNA, because we followed a standard procedure for all samples. However, it is possible that pre- and peri-operative conditions such as hypoxia influence the level of mRNA. This heterogeneous gene expression is in accordance with the histopathological heterogeneity found in breast cancer tumors.

In this study, we found that the reference genes we used were expressed at different levels in normal and breast malignant tissues. This is in accordance with earlier results, where 'Housekeeping' genes were expressed at different levels in normal and tumor cells (24). Several studies have demonstrated that the gene expression profiles of many commonly used internal controls may vary depending of tissue type, experimental conditions or pathological state (25-27). Lee and coworker analyzed a group of large mammalian microarray datasets including the NCI60 cancer cell line panel, a leukemia tumor panel, and a phorbol ester induction time course as well as human and mouse tissue panels. They found that 12 housekeeping genes commonly used showed considerable variability of expression both within and across microarray datasets (28). Such variability in gene expression of controls genes makes it difficult when evaluating tumor gene expression in relation to normal cells and can lead to incorrect conclusions. One method is to determine gene expression in individual tumor cells and normal cells by using the Laser Capture Microscope technique. This technique makes it possible to collect determinable numbers of well-defined cells and its combination with methods such as real-time quantitative RT-PCR will allow a more precise determination of cell specific gene expression (29-31). However, it has been reported that LCM technique may induce degradation of RNA during the procedure of laser capture microdissection (32), making the conclusion less reliable. Despite this, the feasibility of combination of LCM and cDNA microarray hybridization has been demonstrated by Luo *et al*, who showed reproducible differences in gene expression between large and small neurons isolated from rat dorsal root ganglia (33).

A similar approach combining LCM, cDNA arrays and real-time quantitative PCR, was used showing altered gene expression patterns at various stages of breast cancer progression (31). Comparing gene expression profiles of carcinoma cells obtained using macrodissection or microdissection, led to the conclusion that stroma cells disturb tumor gene expression profiles (34). However, De Bruin *et al* found in rectal carcinoma a minor influence of stroma cells on tumor cell gene expression profiles and concluded that macrodissection can be adequately used to obtain reliable data (35).

Yang *et al* showed different gene expression profiles in node-negative breast tumor cells, in which 17 patients with ER-α positive tumor were compared with 11 patients who had ER-α negative tumors. Gene expression profiles were analyzed in both bulk tumors and laser capture microdissection (36). In another study by Makino *et al* on carcinoma of gastric and on colon using PCR after collecting cells by LCM, different gene expression profiles were found in the malignant cells as compared to the stroma cells (37). By using LCM technique in combination with complementary cDNA microarrays, Nakamura and coworker found a novel panel of candidate marker genes for pancreatic cancer, due to the high purity of the cells obtained by LCM (38). Furthermore, in many other studies on different types of malignancies such as ovarian (39,40), breast (31), prostate (41), gastric (42), pancreas (38), lung adenocarcinoma (43) and non-small cell lung cancer (44) combination of LCM and cDNA microarray, resulted in the discovery of new cancer markers.

The amount of tissues we used in this study for cDNA array analysis corresponded to the amount of tumor material obtained with fine needle biopsy (FNAB). This amount of tissues is enough for cDNA array analysis. FNAB and/or core needle biopsy (CBX) have been used to collect samples from primary and metastatic tumors for many decades. FNAB is safe, simple, and inexpensive and already commonly used with routine investigation of tumors. Cancer cells have poor attachment and it is more probable to be grabbed by fine needle aspiration then normal cells. It could also be used together with sonography or X-ray to reach tumor sites deep in the body. mRNA yield by the mean of fine needle biopsy is enough to perform microarray analyses and obtain gene expression profiles.

Furthermore, it is possible to take more than one FNAB from the tumor and thus get an average of the gene expression in the tumor. Symmans *et al* found in 68 patients with breast cancer that both FNAB and CBX yield a similar quality and quantity of total RNA and were suitable for cDNA microarray analyses in 70-75% of single-pass samples (45). Furthermore, Sotiriou *et al* reported on the suitability of FNAB-derived cDNA microarray in breast cancer (46). However, in another study, <15% of FNABs were reported to be sufficient for microarray analyses (47). Though, inconsistent results in relation to suitability of FNAB are reported. The present study showed gene expression analyses by cDNA array technology is applicable to samples from FNAB.

We conclude that gene expression study of breast carcinoma using cDNA microarray should be done with caution because of tumor heterogeneity. It is also important to consider appropriate housekeeping genes and standardization of mRNA. We must stress that this study was based on few tumor samples and that the conclusion, which can be made are limited.

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