Author's response to reviews

Title: Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis

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Version: 3 Date: 12 February 2007

Author’s response to reviews: see over
Dear Editor and Reviewers,

Thanks very much for the careful review of our manuscript. We have revised it according to your comments and we indeed feel that the manuscript has been improved. Enclosed please see our responses to your comments:

**Title:** Novel markers for differentiation of lobular and ductal invasive breast carcinomas by laser microdissection and microarray analysis

**Reviewer:** Rémi Houlgate

**General**

The authors have made major revisions making their objectives more clear. From the actual version, The positive points are: 1) There is a lot of work in that paper: laser microdissection, Affy microarrays, TMA, In situ hybridization (asporin). 2) Even if the initial clustering shows noisy data (absence of correlated expression in figure 1), the method used by the authors (Pairwise comparaison and Rank products) allowed them to find differentially expressed genes in Normal/Tumors and Ductal/Lobular comparaisons, and those genes were validated by TMA.

**Answer:** Thank you. Major revisions have indeed been done and manuscript seems to be improved. We do appreciate your suggestions.

The negative point is: 3) the discussion and conclusion focus too much on TGFβ and Wnt signaling. The results presented in this paper does not really support that hypothesis. One remark: 4) It should be more interesting to discuss the distinction between Ductal and Lobular samples. No antibody is able to distinguish perfectly these 2 kinds of samples (Table 7). Is the combination of antibodies tested able to do that distinction ? That is, Is that distinction clear? This point could be discussed, and would improve the paper.

**Answer:** The discussion and conclusion have been revised.
Reviewer: José Palacios

General

Major Compulsory Revisions

The main limitation of this study is the low number of cases analyzed, which difficult statistical comparisons and preclude general conclusions. Regarding the tumours studied, it is not clear why the authors have selected 2 ILC (out of 5) that are ER-negative, where most ILC usually are ER-positive. In addition, IDCs are very heterogeneous and it is difficult to accept that 5 cases are representative of ductal cancer heterogeneity. It is probable that results were different if other 10 tumours were selected.

Answer: Thanks very much for your comments. Obviously, the mechanisms of invasion and metastasis of invasive ductal and lobular breast cancers are not completely understood, no studies have been addressed to gene expression profiling of microdissected tumor and normal mammary cells to find differentially expressed probe sets. Despite analyzing only thirty samples from ten patients, our study is the first to use full-genome Affymetrix arrays for microdissected tumor and normal cells from the breast. Affymetrix arrays are among the best validated microarray platforms and we also used high quality Arcturus laser microdissection system. Also, our data are in good accordance with literature (Zhu et al. 2003, Sarrio et al. 2004, Korkola et al. 2003, Nishidate et al. 2004, Tang et al. 2006). Regarding the tumours studied, as we were unable to study hundreds of patients and sort them according to ER/PR status, we selected patients according to the histological subtype of cancer, disregarding IHC data and microdissected normal epithelial cells from surrounding normal tissue in order to detect normal mammary epithelium- and cancer-specific genes.

Taken into account that few breast cancers were analyzed, microarray experiment should have been done in duplicate. The authors should have analyzed some up- and down-regulated genes by RT-PCR to validate microarray data in this series of tumours.

Answer: We have been able to examine 30 samples from 10 patients due to the limited funding available for the project. We are aware of several papers on laser microdissection and microarray analysis examining only a few patients, such as 6 cases (Mimori et al. Clin Exp Metastasis 2005;22:59-67), 5 patients (Thelen et al. Int J Oncol 2004;24:1085-1092), 28 samples (Yang et al. Oncogene 2006;25:1413-1419), 38 samples (Adeyinka et al. Clin Cancer Res 2002;8:3788-3795), etc.
With respect to the limited funding our samples were not analyzed in duplicate. On the other hand, the samples from particular cell types (normal ductal, normal lobular, tumor ductal, tumor lobular - 10, 10, 5, 5 samples, respectively) were considered as biological replicates and genes differentially expressed across patients were considered (the experiment design has been uploaded and approved by Gene Expression Omnibus, our GEO Series accession number is GSE5764, LOGIN: bouchal_rev_1, PASSWORD: affymetrix). In support to microarray findings, we have validated 7 genes (CDH1, EMP1, DDR1, DVL1, KRT5, KRT6, KRT17) by immunohistochemistry at the protein level, and ASPN by in situ hybridization. We have also analysed three differentially expressed genes (ASPN, CTHRC1, COL3A1) by PCR and results are now included in the manuscript. As both RNA and cDNA were fully used for amplification, we used PCR amplification products for validation by another PCR with specific primers (please see novel paragraph in Materials and methods).

Validation study on TMA should have included other markers instead of E-cadherin, CK17 and CK5/6. It is obvious that ILC do not express E-cadherin, and that most lobular and ductal carcinomas are of "luminal type" and do not express basal cytokeratins, that are markers of normal myoepithelial cells, and some normal non-myoeplathelial cells.

**Answer:** Validation study on TMA slides also included other markers such as EMP1, DDR1, DVL1 as well as asporin CISH on frozen sections. CK17 and CK5/6 were differentially expressed between tumour and normal tissues (due to the higher number of myoepithelial cells in normal tissue). We performed IHC staining for all those markers as a validation of microarray data. No other luminal cytokeratins were differentially expressed between normal and tumor cells or tumor cells types.

In addition, some data TMA are surprising and should be discussed. For example, absence of E-cadherin usually does not occur in IDC, even in those cases that are high grade. CK5/6 expression is frequent in medullary carcinomas, since these tumors have a basal-like phenotype, but the authors only comment about CK5/6 expression in a papillary carcinoma.

**Answer:** a) Absence of E-cadherin has been shown in IDC, from 4% (Acs et al. Am J Clin Pathol 2001;115:85-98) to 28% (Brinck et al. Anticancer Res 2004;24:2237-42) of cases. A number of studies have reported loss or reduction of E-cadherin using IHC but predominantly in higher-grade ductal carcinomas (Gamallo et al. Am J Pathol 1993; 142: 987-993, Gillet et al. J Pathol 2001; 193: 433-441). Genetic studies have revealed a high frequency of allelic loss in ductal carcinomas in the
region of CDH1 (Dorion-Bonnet et al. Genes Chromosomes Cancer 1995; 14: 171-181, Skirnisdottir et al.. Int J Cancer 1995). Grade I tumours have the highest frequency of 16q loss, compared with the other ductal subtypes (Roylance et al. Cancer Res 1999; 59: 1433-1436). Roylance et al. (2003) hypothesized that E-cadherin is also involved in low-grade ductal tumourigenesis, with perhaps a different spectrum of mutations giving rise to the ductal phenotype, however, they could not confirm this hypothesis.

b) We had 3 cases of medullary carcinomas (2 atypical, 1 typical) and 2 of them, indeed, expressed basal cytokeratins, however, these results were omitted in the manuscript. The text has now been revised.

Minor Essential Revisions

The discussion is very general and speculative. The authors do not mechanistically explore any of the suggested pathways discussed (for example TGF-beta or WNT).

**Answer:** The discussion has been revised.

Although E-cadherin loss is present in cells that undergo EMT, absence of E-cadherin is not sufficient to induce EMT. The authors do not discuss or study the expression of other luminal keratins, or other luminal markers, that are always expressed in ILC.

**Answer:** a) Lost, non-polar or cytoplasmic expression of E-cadherin protein and/or transcriptional repression of its mRNA are believed to be hallmarks of EMT in cancer progression (Thiery 2002, 2003). It has been shown that several proteins such as fibronectin and integrin αvβ6 (Lee et al. 1990), Ets, TGFβ, FGF-1,-2,-8, α-SMA, collagen type I, III and thrombospondins increase in abundance during EMT (Kalluri and Neilson 2003). Not only E-cadherin but also other genes such as collagen type I and III as well as fibronectin, Ets domain transcription factor were upregulated in both types of our tumor cells, whereas thrombospondin 4 was upregulated only in lobular cancer cells. Thus we proposed that the EMT plays a role in both tumor types but appears to be more important in lobular carcinoma.

b) We only studied (validated) genes which were differentially expressed between tumour and normal cells or between the two tumour types. No luminal markers have been found to be differentially expressed between our samples.
Reviewer: Edgar Dahl

General
This study by Turashvili et al. used a combination of laser-microdissection and DNA array based expression profiling to identify genes that are specifically expressed in lobular and ductal invasive cancer. Furthermore, the authors compared expression between normal ductal and normal lobular cells and third, gene expression between breast tumor and normal breast tissue in general. This is an interesting piece of work. Though the number of tumors (10) and samples (30) analyzed is rather small the data appear useful since quite a number of well known breast tumor markers were confirmed in this study. The study is suitable for publication in BMC Cancer if the following items are clarified.

Major Compulsory Revisions
The study somewhat suffers from having included too many comparisons and too many descriptions of deregulated genes. The authors should first focus on the comparison of lobular invasive tumor and ductal invasive tumor, which has not been carefully analyzed (if at all) by other groups. Secondly, the comparison of normal lobular and normal ductal tissues is interesting and should be discussed, however one should keep in mind that these "normal breast tissues" were derived from postmenopausal women, which breast tissues are rather inactive in metabolism.

Answer: We agree that there are too many comparisons and many descriptions of deregulated genes, however, all these comparisons and descriptions have been suggested by one of the reviewers from the first submission. Comparison of lobular invasive tumour and ductal invasive tumour is discussed in detail. We have included comment on the comparison of normal lobular and normal ductal tissues in the discussion.

Abstract
The abstract should give a clear understanding on the number and types of analyzed tissues (10+10+5+5). Results should name the major finding (those differential expressed genes, that were validated by in situ hybridization or immunohistochemistry).

Answer: The abstract has been revised.

Background
The author mention that IDC and ILC are similar in many respects, but then refer to references. The state of the art knowledge on this important point should presented in more detail. On the other hand, the study of Korkola et al. is discussed in detail with gene names in the Background/Introduction. These concrete data should rather be discussed in the actual discussion in comparison to the genes found by Turashvili et al.

**Answer:** The introduction has been revised. More detailed discussion of the study of Korkola et al. was suggested by one of the reviewers from the first submission. However, we feel that it would be better to include it in the discussion, so discussion has also been revised.

**METHODS:**
The first sentence: Should mean: Altogether ten surgical specimens with either invasive ductal carcinoma (n=5) or invasive lobular carcinoma (n=5) were investigated.

**Answer:** This has been revised.

What does the lysis buffer for the microdissected cells from Qiagen contain?

**Answer:** According to the manufacturer (Qiagen), the lysis buffer contains guanidine isothiocyanate which not only lyses the cells, but also denatures almost all of the RNases. Further detailed information on the lysis buffer is not provided.

RNA amplification: The authors should state how many cycles (21-33) they actually did for their experiments. They should provide a reference that such high level amplification of Target RNA will not let to a reduction in RNA complexity (=loss of non-abundantly expressed genes). Why did they not use linear amplification e.g. by two round of in vitro transcription?

**Answer:** Only 27 (samples 3, 4, 8, 9, 10) and 29 (samples 1, 2, 5, 6, 7) PCR cycles were used for amplification. All three populations from the particular patient were amplified by the same number of PCR cycles. If higher PCR cycling was needed for any cell type, new microdissection, RNA isolation, cDNA synthesis and PCR amplification were performed until the same PCR cycling was possible. In support to the PCR amplification of Target RNA against linear amplification, we can provide the following references: a) Random PCR amplification was shown to be more reproducible, requires smaller RNA input, and generates cRNA of higher quality than linear amplification (Klur S, Toy K, Williams MP, Certa U. Genomics 2004;83:508-17); b) Combined T7 promoter ligation and
PCR amplification in one step was suggested to be an effective method for gene expression analysis from minute biological materials (Ji et al. Anal Biochem 2004;331:329-39); c) It is an effective method for high-fidelity global mRNA amplification for in vivo gene expression profiling of as few as 100 cells obtained by laser-captured microdissection (Aoyagi et al. Biochem Biophys Res Commun 2003;300:915-20). These references are included in the manuscript now. The combined system of PCR and IVT for amplification was chosen because of our good experience with Roche products but two rounds of linear amplification could, of course, have been used as well.

RESULTS:
Genes differentially expressed between normal ductal and lobular cells: In the text USP25, TMPRSS3, ACACB ..... until MAP4K5 are described as upregulated in normal lobular cells (please mention! compared to normal ductal cells) however in Table 2 this genes have a arrow down, meaning according to the legend of Table 2: downregulated in lobular cells. What is correct? The authors should verify once more all descriptions on up- and downregulated genes on consistency.

**Answer:** Thanks very much for this note. The table is correct and the mistake in the text has been corrected. Other comparisons and descriptions have also been carefully checked.

Table 3 starts with Tumor vs Normal cells and continues with IDC vs normal before ILC vs Normal is shown. In the text first ILC vs Normal is discussed. It would be nicer to have a continous order (with a focus on the two most important comparisons mentioned above).

**Answer:** This has been revised.

The SFRP1 gene is mentioned as downregulated in the paragraph "comparison between lobular carcinoma vs. normal cells", however, in Table 3 it is only found in the list tumor vs. Normal. In the same context DVL1 is mentioned as being differentially expressed between lobular and ductal carcinoma. This makes things complicated. Within the results sections the author should precisely stick to the genes of the actual comparison. In the discussion one could mention that Wnt pathway genes have been found in several comparisons.

**Answer:** This has been revised.
DISCUSSION:
The discussion is written quite nicely. However, I cannot follow the conclusion that Wnt signalling is not activated in ductal carcinoma cells because SFRP1 and MMP7 are downregulated. SFRP1 is a potent inhibitor of Wnt signalling (e.g. Suzuki et al.; Nat Genet. 2004 36:417-22), its downregulation in breast cancer is associated with poor prognosis (Veeck et al.; Oncogene. 2006 25:3479-88) that may be caused by aberrant Wnt signalling via β-catenin (Lin et al., PNAS, 97:4262-6.). As the authors state MMP7 expression in different tissue may be regulated by different pathways, this may hold true even for normal and malignant breast tissue.

Answer: The conclusion has been revised.

Minor Essential Revisions

Page 13
was downregulated in our tumor cells, rather: was downregulated in the tumor specimens analyzed in this study.

Answer: This has been revised.

Page 15
p=0.000000 is rather uncommon, better use p<0.0001

Answer: This has been revised.

We do hope that we have addressed each of the comments and answered all criticisms in a satisfactory manner.

Thanks very much
With best regards

Jan Bouchal, PhD
Gulisa Turashvili, MD
and on behalf of all co-authors
February 12, 2007