From Lab to Clinic

Partially Degraded RNA from Bladder Washing is a Suitable Sample for Studying Gene Expression Profiles in Bladder Cancer

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Abstract

Objectives: To determine the impact of different levels of RNA degradation on gene expression measurements and to ascertain if the gene expression profile obtained from bladder washing (BW) correlates to that obtained from the related bladder tumour (BT).

Methods: BT and BW RNAs from the same patient were heat shocked to obtain three RNA degradation states, which were compared with intact RNAs from healthy bladders by using complementary DNA (cDNA) microarrays. All samples were amplified by means of a T3N9-based transcription method. In addition, four of the differentially expressed genes in microarrays related to bladder cancer (KRT20, IGF2, GSN, and CCL2) were analyzed in 36 tumoural and 14 control BW samples by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR).

Results: A high percentage of overlapping differentially expressed genes were detected between BT arrays (85–91%) and between BW arrays (78–93%). Furthermore, the similarity between BW and BT arrays was relatively high and independent of the RNA degradation state (52–60%). Finally, expression differences for the four selected genes were confirmed in the vast majority of extended BW samples tested by qRT-PCR.

Conclusions: Our results showed that partially degraded RNA samples analyzed by cDNA microarrays yielded gene expression profiles comparable to those obtained using intact RNA. Moreover, BW RNA exhibited gene expression patterns similar to those identified in the BT, indicating that BW is an appropriate sample for studying gene expression profiles of BT using cDNA microarrays. In addition, qRT-PCR results further support the suitability of BW for gene expression profiling and its potential use for routine diagnostics.

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1. **Introduction**

Optimal diagnosis of bladder cancer currently relies on cystoscopy, which is an invasive method. Urinary cytology supplements but cannot replace endoscopic evaluation [1].

As a noninvasive alternative for diagnosing bladder cancer, gene expression levels of several molecular tumour markers have been recently studied in voided urine or bladder washing (BW) [2–6]. Unfortunately, these studies have been performed with a small number of genes, and further confirmation is needed before their clinical application.

The development of DNA microarray technologies is likely to facilitate the generation of novel diagnostic tools for bladder cancer detection. Thus, the study of bladder fluids by this technology could provide a noninvasive diagnostic approach as well as improve the reliability of current bladder cancer diagnostic methods. However, application of microarray technology is limited by the requirement of large amounts of high quality RNA. This requisite is especially difficult to achieve in some human samples because of the limited amounts available and their inherent low RNA quality.

Protocols based on the linear T7dT-based in vitro transcription method described by Van Gelder and Eberwine [7,8] can amplify small amounts of mRNA without significantly distorting the information content of the sample [9], but they can be sensitive to RNA degradation. Xiang and colleagues [10] developed a new procedure that is able to amplify degraded RNA [10]. This alternative is based on the use of random nonamer primers modified by the addition of an upstream T3 promoter sequence (T3N9) to prime the initial round of reverse transcription.

In this report, we have used the T3N9-based in vitro transcription method to analyze the suitability of partially degraded RNA from BW as a sample for bladder cancer diagnostics with the use of cDNA microarrays, and we have validated the differential expression detected by microarrays for four bladder cancer–related genes on an extended set of BW samples by quantitative RT-PCR (qRT-PCR).

2. **Methods**

2.1. **Samples and RNA preparation**

All samples used in this study were collected between June 2003 and January 2005. Individual tumour tissue and bladder washing samples (T0 and BW0, respectively) were obtained from a patient with bladder cancer pathologically diagnosed as pT2 high-grade (HG) transitional cell carcinoma [11,12] during therapeutical surgery. Four urothelium specimens from individuals without a history of urothelial cancer were obtained intrasurgery as control tissue. Tissue samples (tumour and controls) were immediately frozen after collection in liquid nitrogen and stored at −80 °C until processed. Fifty BW, 36 from bladder cancer patients (8 pTa low grade [LG], 5 pTa HG, 3 pT1 LG, 5 pT1 HG, 4 pTis, 9 pT2 HG and 2 pT4 HG) [11,12] and 14 from patients without a history of bladder cancer were collected. None of the bladder cancer patients were treated with BCG therapy prior to sample collection.

The hospital ethics committee approved this study and the patient and controls provided their informed consent before participating in the study.

For washings, the patient 100 ml of physiologic NaCl solution was flushed through the bladder about five times via a urethral catheter with a syringe. Ice-cooled BW samples were mixed with 1/25 volumes of 0.5 mol/l EDTA, pH 8.0, and were centrifuged at 1000 × g for 10 min. The cell pellets were resuspended in 1 ml of TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and frozen at −80 °C until RNA extraction. Tissue and BW RNAs were extracted with the use of TRIzol reagent according to the manufacturer’s instructions. Total RNA was quantified by spectrophotometric analysis at 260 nm.

Equal amounts of intact RNA from T0 and BW0 were incubated for 15 (T1 and BW1), 30 (T2 and BW2) and 60 (T3 and BW3) minutes at 80 °C as described [10], with the exception that RNAse-free water was used instead of base buffer (Table 1). The four RNAs obtained from the healthy urothelium specimens were pooled in an equimolar proportion (C0). One microliter of each intact and partially degraded RNA sample was analyzed with the use of the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) [13] to assess the degree of degradation (Fig. 1).

2.2. **T3N9-based in vitro amplification and direct labelling method**

Five micrograms of RNA templates were used for RNA amplification as previously described by Xiang et al. [10,14]. Probes were synthesized by a direct labelling method [15] and were purified with QIAquick PCR purification columns (Qiagen, Valencia, CA, USA). Labelling efficiency was calculated by quantification with NanoDrop (NanoDrop Technologies, Wilmington, DE, USA), obtaining between 40–50 pmol/μl of Cy3- or Cy5-labelled probes.

2.3. **Array processing and data analysis**

Two-colour microarray hybridizations were performed, confronting the four progressively degraded RNAs from each of the two types of cancer-derived samples against the intact pool of control bladder RNA, used as common reference in all hybridizations (Table 1).

Onchip version 2 glass human cDNA microarrays produced at the Centro Nacional de Investigaciones Oncológicas (http://grupos.cnio.es/ing/) were used. Fluorescent images were obtained with the use of the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and TIFF images were quantified with the use of the Spot program (http://experimental.act.cmis.csiro.au/Spot/index.php) under
### Table 1 – Summary of RNA sample preparation and processing

<table>
<thead>
<tr>
<th>RNA source</th>
<th>Bladder carcinogenesis</th>
<th>RNA degradation</th>
<th>RNA yield (ng)</th>
<th>Log 2 ratio limits of the 100 most differentially expressed genes of each array</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control bladder</td>
<td>-</td>
<td>-</td>
<td>8.4</td>
<td>-</td>
</tr>
<tr>
<td>Bladder tumour</td>
<td>-</td>
<td>-</td>
<td>27</td>
<td>-</td>
</tr>
<tr>
<td>Cancerous BW</td>
<td>-</td>
<td>-</td>
<td>15.2</td>
<td>-</td>
</tr>
<tr>
<td>T0</td>
<td>-</td>
<td>-</td>
<td>23.44</td>
<td>-</td>
</tr>
<tr>
<td>T1</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>-</td>
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<tr>
<td>T2</td>
<td>-</td>
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<td>30</td>
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<tr>
<td>T3</td>
<td>-</td>
<td>-</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>BW0</td>
<td>-</td>
<td>-</td>
<td>1.5</td>
<td>-</td>
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<tr>
<td>BW1</td>
<td>-</td>
<td>-</td>
<td>15</td>
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<td>BW2</td>
<td>-</td>
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<td>-</td>
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<tr>
<td>BW3</td>
<td>-</td>
<td>-</td>
<td>15</td>
<td>-</td>
</tr>
</tbody>
</table>

**Notes:**
- CO refers to the intact pool of RNA obtained from four healthy bladder tissues (control sample).
- T0 and BW0 are the intact RNAs isolated from BT and BW samples, respectively, from the same bladder carcinoma patient.
- 28S/18S rRNA ratios, % of total 28S and 18S area, and RNA integrity numbers (RINs) are used to quantify the state of RNA degradation.
- Each specific RNA comparison was performed in duplicate, with dye swapping for a total of 16 hybridization experiments (as a matter of convenience, when we refer to the array name shown in the table, we consider the mean values of the array and its dye swap duplicate).
- Log 2 ratio limits of the 100 most differentially expressed genes of each array.
- N/A: not available.

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To validate gene expression data from microarrays (technical validation of microarray data), 10 of the most differentially expressed genes (ANXA1, CD74, CDH11, EGR1, HIST1H2AC, IGF2, ITGA5, KRT20, PPAP2B, and VAV3) were tested by qRT-PCR on intact and partially degraded RNA obtained from BT and BW. On the other hand, to demonstrate that the differences between tumoural and control microarrays were mainly due to the presence of a bladder carcinoma (biologic validation of the microarray results), we selected four differentially expressed genes related to bladder cancer according to the literature (KRT20, IGF2, GSN, and CCL2) [17–20] to be tested in 50 BW (36 cancerous and 14 controls). Two of the selected genes for the technical validation (KRT20 and IGF2) were also chosen for the biologic validation since they are involved in bladder carcinogenesis.

cDNA was synthesized from 1 μg of total RNA with the use of the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions, except that the final volume of the reaction was 50 μl. The gene GUSB was used as endogenous control. PCRs were performed with the use of Assays-on-Demand gene expression products in an ABI Prism 7000 SDS (Applied Biosystems) according to the manufacturer’s recommendations, except that the final volume of the reaction was 20 μl. All samples were amplified in duplicate and the CT mean was obtained for further calculations. Duplicates with SD ≥ 0.38 were excluded, and these samples were reamplified. The ΔΔCt method (ABI Prism 7700 Sequence Detection System User Bulletin #2: relative quantification of gene expression P/N comparisons, except that the final volume of the reaction was 20 μl. All samples were amplified in duplicate and the CT mean was obtained for further calculations. Duplicates with SD ≥ 0.38 were excluded, and these samples were reamplified. The ΔΔCt method (ABI Prism 7700 Sequence Detection System User Bulletin #2: relative quantification of gene expression P/N comparisons, except that the final volume of the reaction was 20 μl. All samples were amplified in duplicate and the CT mean was obtained for further calculations. Duplicates with SD ≥ 0.38 were excluded, and these samples were reamplified. The ΔΔCt method (ABI Prism 7700 Sequence Detection System User Bulletin #2: relative quantification of gene expression P/N
Fig. 1 – Agilent 2100 bioanalyzer electropherograms from intact and partially degraded RNA samples from bladder tumour (BT), bladder washing (BW), and control samples. Intact RNAs from tumour and bladder washing samples (T0 and BW0) were heat shocked at 80 °C for different time periods to obtain three degradation states. The numbers 0, 1, 2, and 3 were assigned to samples in order of increasing degradation; the same number was chosen for samples with comparable RNA quality.
was used to quantify the relative amount of mRNA in each tumoural sample in comparison with the C0 sample (technical validation) or to the mean of the 14 control samples (biologic validation). Next, $2^{-\Delta\Delta Ct}$ of each gene was transformed into logarithmic scale (log 2 ratio), and comparisons to the microarray results were performed by means of linear regression. The analysis of variance test was used to determine if the expression levels measured by qRT-PCR of intact and partially degraded RNA were significantly different. The SPSS software version 12.0 (SPSS Inc, Chicago, IL, USA) was used for all statistical calculations.

3. Results

3.1. Effect of degraded RNA on microarray profiling

Intact RNA from tumour (T0) and bladder washing (BW0) samples were heat shocked to obtain three controlled degradation states (Fig. 1 and Table 1). We cohybridized the four progressively degraded RNA aliquots from BT and BW with the intact control bladder RNA. A high percentage of overlapping differentially expressed genes were detected between BT arrays (85–91%) and between BW arrays (78–93%) (Fig. 2A). Interestingly, the percentage of genes in common identified between the two arrays hybridized with different RNA degradation states (e.g., BW0 and BW1) was, sometimes, higher than the percentage between dye swap duplicates of the same array (e.g., BW0 vs BW0-DS; Fig. 2B).

When we analyzed the suitability of BW for diagnosing bladder cancer using cDNA microarrays, we found that the similarity between BW and BT was relatively high and independent of the RNA degradation state (52–60%; Fig. 2A). Among the differentially expressed genes, we detected 48 genes in common between BW and BT samples (see Materials and Methods for details). These genes showed enrichment in “growth” and “differentiation” go categories. On the other hand, we detected 33 and 35 differentially expressed genes exclusive to BW and BT, respectively. The BW-specific genes were enriched in “immune response” and “catabolism,” whereas tumour-related genes were, once again, enriched in “growth.”
Fig. 3 – Comparison of microarray and quantitative RT-PCR (qRT-PCR) expression data of 10 selected differentially expressed genes. Positive values indicate overexpression in BT or BW, compared with a pool of healthy bladder tissue (C0). The down arrows on BW indicate that these samples do not contain enough CDH11 cDNA for an accurate comparison with the control bladder tissue. Therefore, the stated expression level should be interpreted as the minimum value of differential expression for BW samples. The numbers 0, 1, 2, and 3 correspond to the RNA degradation states indicated in Table 1.

Fig. 4 – Differential expression of four bladder cancer-related genes between tumoral and control BW by qRT-PCR. Positive values indicate overexpression in tumoral BW compared with the control BW samples. For the control reference the mean of the 14 BW controls tested was obtained. Samples are arranged by tumoral stage and grade; low grade (LG) superficial tumours (8 pTa LG and 3 pT1 LG), high grade (HG) superficial tumours (5 pTa HG, 5 pT1 HG, and 4 pTis), and muscle invasive tumours (9 pT2 and 2 pT4).
3.2. Validation of microarray data using qRT-PCR

We were able to verify the 10 tested genes in BW samples (100%). In the tumour samples, we could validate 90% (9 of 10) of tested genes (except HIST1H2AC) (Fig. 3). Perhaps cross-hybridization processes that are much more common in microarrays than in qRT-PCR could account for these differences. The comparison of microarray and qRT-PCR results for the 10 selected genes showed a strong correlation between the two methods (r = 0.84, p < 0.0005), although differences in the magnitude of change between microarrays and qRT-PCR were found; 78% of samples had greater gene expression in qRT-PCR than in microarrays. Otherwise, qRT-PCR analysis showed no significant differences in relative differential expression levels between intact and partially degraded RNA samples (p = 0.99).

Gene expression validation of KRT20, GSN, IGF2 and CCL2 by means of qRT-PCR in 36 individual BWs from bladder cancer patients is shown in Fig. 4. The percentage of BW samples that confirmed microarray results was 81% for KRT20, 89% for GSN, 64% for IGF2, and 89% for CCL2.

4. Discussion

In this study, cDNA microarrays were used to determine if gene expression profiles derived from low-quality RNAs obtained from a cancerous BW sample correlated with those obtained from the related BT. 

Using a controlled RNA degradation procedure, we obtained different RNA degradation states that could represent the quality of the vast majority of the RNA that we generally isolate from BW samples. Subsequently, we selected the linear T3N9-based in vitro transcription method to amplify the limiting RNA quantities usually isolated from bladder fluids. We found that the quality of the starting RNA was directly related to the quantity and quality of the RNA produced by the T3N9-based transcription method (Table 1). Nevertheless, microarray expression data did not seem to be practically affected by the initial RNA quality. Thus, we were able to identify a high percentage of differentially expressed genes in common between the different degraded RNA states of both BT (85–91%) and BW (78–93%) samples (Fig. 2A). However, even though the RNA degradation patterns of our RNAs are very similar to those obtained by other authors who used more physiologic RNA degradation methods, such as endogenous tissue ribonucleases [21] or RNAse A [22], it remains to be shown whether physiologic RNAse degradation would have an effect on the reproducibility of measurements on equivalent samples.

Therefore, our data suggest that small amounts of partially degraded RNA amplified with the T3N9-based method may be used in cDNA microarray studies without a great reduction in the ability to detect differentially expressed genes. Moreover, it is also of note that relative gene expression levels measured by qRT-PCR do not seem to be affected by RNA degradation either, as previously described [22]. This observation opens up the possibility of performing qRT-PCR studies on inherently degraded RNA samples such as those extracted from urine, archival paraffin-embedded specimens, faeces, and so forth.

In the present study, we also sought to determine whether gene expression profiles found in BW reflected those obtained in the related tumour. The comparison of microarrays hybridized with both intact RNA samples (T0 vs BW0) yielded an important percentage of overlapping differentially expressed genes (59%), indicating the suitability of the BW sample to reflect tumour gene expression profiles. It is remarkable that the percentage of differentially expressed genes in common among all the microarrays hybridized with different RNA degradation states is practically constant (52–60%).

On the other hand, although our aim was not to identify differentially expressed genes in bladder cancers, we determined that the common differentially expressed genes between BT and BW were mainly related to cancer (the GO functional annotations were growth and differentiation). It is important to emphasize that some of the genes identified (e.g., UPK1A, KRT20, IGF2, GSN, ANXA1, CCL2, NRAS, etc) had been previously described as bladder cancer-related genes [17–20,23–26] and, interestingly, some of them had already been suggested as bladder cancer markers in urine or BW [18,27,28]. To confirm some differentially expressed genes detected in our microarrays in a larger set of BW, we evaluated KRT20, IGF2, GSN, and CCL2 gene expressions in 36 individual BW samples by qRT-PCR. The concordance of microarray data of a single patient and qRT-PCR results in an extended series of BW strongly suggest that our findings were not due to the particular gene expression profile of the analyzed patient.

Finally, we focused on gene expression differences between BT and BW. These gene expression variations may reflect differences in tissue composition, since BW represent primarily urothelium-derived cells, whereas the tumour sample may contain other cell types such as smooth muscle, mesenchymal fibroblasts, and endothelial cells [29].
We found the growth biologic process category overrepresented within the list of tumour-specific genes. Specifically, we identified many genes involved in nucleic acid metabolism such as EGR1, ERF, HIST1H1C, or ENTPD5. This observation could be due to the enrichment of cancer cells in the BT, compared with the BW sample, which must be composed by a mix of normal and cancerous cells producing a dilution effect. When we analyzed genes expressed exclusively in BW, we found that functional annotations for immune response and catabolism were overrepresented. For example, we have identified genes such as HLA-G, IGLL1, or IFITM1, which are strongly related with immunity and defence processes, and genes such as MALT1, TIMP3, MMP2, or TIMP2, which are involved mainly in proteolysis and protein metabolism. A possible explanation for these findings could be the need for a constant defence of the urothelial mucosa against external agents, which would be reflected in the BW. In addition, catabolism processes could be occurring in the damaged cells present in the bladder.

5. Conclusions

In this study, we demonstrated that a small amount of partially degraded RNA isolated from BW, processed with the T3N9-based in vitro amplification method, is suitable for inferring gene expression profiles in the related BT by microarray expression analysis. Our results provide information that could lead to encouraging the development of a noninvasive method to diagnose and provide a prognosis in BT, which is based on gene expression patterns derived from bladder fluid cellular fractions. This alternative diagnostic method could become available in the future once appropriate molecular bladder cancer biomarkers are selected. The next step should be to test if similar results can be obtained with the use of RNA isolated from urine samples.

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References

Bladder cancer is the fourth most common malignancy among men in the western world. The majority of these tumors are of the superficial phenotype and most patients (>50%) develop more than one carcinoma in their lifetime [1]. Approximately 90% of bladder transitional cell carcinomas are superficial (pTa, pT1, pTis) at initial diagnosis. The rate of tumor recurrence ranges 50–80% in the couple of years following tumor resection and progression to invasive disease accounts for 15–23% of recurrent cases [1]. Because tumor recurrence, if not detected, may progress and invade the musculature, early detection of tumor recurrence is of primary importance and requires close surveillance for successful treatment. Follow-up is essentially based on cystoscopy: the gold-standard method, despite being invasive and uncomfortable [1]. Non-invasive ancillary methods, less expensive, with high sensitivity and sensibility would be of great help in the diagnosis and follow-up procedures in the daily urological practice. It is widely accepted that human neoplasms including bladder cancer arise from the accumulation of multiple genetic events leading to activation of proto-oncogenes or inactivation of tumor suppressor genes. New noninvasive methods for the diagnosis and surveillance of urothelial carcinomas are required to replace cystoscopy. Analysis of the DNA extracted from exfoliated cells can be used for noninvasive tumour detection. A non-invasive diagnostic molecular method based on the detection of loss of heterozygosity (LOH) or microsatellite instability (MSI) in cells exfoliated in urine has been reported previously [2]. However, most molecular biomarkers analysed to date lack sufficient specificity or sensitivity for their widespread use and require extensive genetic analysis preventing their routine clinical use [1,2]. The present article from Mengual et al. is a study about technical feasibility and ability of bladder washing to detect or identify early recurrence in bladder cancer. Although the aim of the study is not novel, it is quite important to determine a suitable way to detect urothelial tumors regarding these new biological tools. The current results are very interesting because this
The team has decided to focus on exclusive RNA material. The effect of RNA degradation is apparently less important than the type of sample that is being analyzed. Moreover, gene expression analysis by microarrays using small amounts of RNA is becoming more and more popular against the background of advances and increasing importance of small-sample acquisition methods like laser microdissection techniques [3]. The quality of RNA preparations from such samples constitutes a frequent issue in this context [3]. If the results from the next step of the study (i.e., RNA isolated from urine samples) are conclusive, then this should be one of the methods of choice for these analyses (instead of DNA). One important issue is to make basic research more understandable for clinicians and urologists and to involve deeply our community in the ability to use new, accurate and reliable methods of diagnosis. These results are a good example and will probably be of clinical relevance in the daily urological practice for the nearly future.

References