Analysis of differential gene expression in human melanocytic tumour lesions by custom made oligonucleotide arrays

Nijmegen, The Netherlands; E-mail: g.vanmuijen@pathol.umcn.nl

Radboud University Nijmegen Medical Centre, PO Box 9101, 6500 HB Nijmegen, The Netherlands; *Correspondence: Dr GNP van Muijen, Department of Pathology, Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands; 1Department of Biochemistry, Radboud University Nijmegen, Nijmegen, The Netherlands; 2Roche Diagnostics GmbH, Pharma Research, Penzberg, Germany

Melanoma is one of the most aggressive types of cancer and resection of the tumour prior to dissemination of tumour cells is still the most effective treatment. Therefore, early diagnosis of melanocytic lesions is important and identification of novel (molecular) markers would be helpful to improve diagnosis. Moreover, better understanding of molecular targets involved in melanocytic tumorigenesis could possibly lead to development of novel interventions. In this study, we used a custom made oligonucleotide array containing 298 genes that were previously found to be differentially expressed in human melanoma cell lines 1F6 (rarely metastasising) and Mel57 (frequently metastasising). We determined differential gene expression in human common nevocellular nevus and melanoma metastasis lesions. By performing nine dye-swap array experiments, using individual as well as pooled melanocytic lesions, a constant differential expression could be detected for 25 genes in eight out of nine or nine out of nine array analyses. For at least nine of these genes, namely THBD, FABP7, HZAF1, RRAGD, MYADM, HR, CKS2, NCK2 and GDF15, the differential expression found by array analyses could be verified by semiquantitative and/or real-time quantitative RT–PCR. The genes that we identified to be differentially expressed during melanoma progression could be potent targets for diagnostic, prognostic and/or therapeutic interventions.

British Journal of Cancer (2005) 92, 2249–2261. doi:10.1038/sj.bjc.6602612 www.bjcancer.com

Published online 17 May 2005

© 2005 Cancer Research UK

Keywords: melanoma; differential expression; tumour progression; oligonucleotide array; real-time PCR

Melanoma is a very aggressive type of tumour, as it metastasises early in tumour progression. Due to its relative insensitivity to systemic therapies, such as chemotherapy and radiation, the most effective cure for melanoma patients nowadays remains surgical excision of the tumour before onset of the metastatic growth phase. This means that early diagnosis of melanocytic tumour lesions is essential.

The molecular mechanisms underlying malignant transformation of melanocytes and melanoma tumour progression are not very complete yet. Extensive analysis of molecular changes that occur during tumour development may not only provide better insight in melanocytic tumorigenesis, but additionally yield valuable tools for clinical applications. Novel diagnostic and/or prognostic markers and targets for (immuno-)therapy could be identified by determining the differential gene expression in different stages of melanoma progression.

For most microarray studies related to melanocytic tumorigenesis, cell lines or fresh tumour cells that were cultured for some passages were used to examine differential gene expression (Baldi et al, 2003a; Dooley et al, 2003; Rumpler et al, 2003; Hoek et al, 2004). However, usage of these cells is not ideal, as culturing conditions can influence the genetic expression. In a previous study, we also determined differential gene expression in two human melanoma cell lines, 1F6 and Mel57, showing distinct metastatic behaviour after subcutaneous inoculation into nude mice, by using high-density oligonucleotide array analyses (Affymetrix) (Westphal et al, 1997; de Wit et al, 2002). We found an up- or downregulation of 298 genes/ESTs among the more than 40 000 genes that were analysed. In this study, we now used fresh human melanocytic tumour lesions of different progression stages to determine whether the previously identified genes remained differentially expressed in the in vivo situation. This would probably be more informative regarding involvement of the genes in melanoma progression. Custom oligonucleotide arrays were designed representing the 298 genes/ESTs and hybridisation was performed using target probes derived from common nevocellular nevus (NN) and melanoma metastasis (MM) samples. After array analyses, differential gene expression was verified by semiquantitative and real-time quantitative reverse transcriptase (RT)–PCR. The reliability of our custom array analyses is discussed, next to the putative involvement of the differentially expressed genes in melanoma tumour progression and their potential significance as new diagnostic/prognostic markers and/or targets for (immuno-)therapy.

MATERIALS AND METHODS

Human tissue samples and cell lines

Human melanocytic tumour samples were obtained by resection of the lesions at University Medical Centre (UMC) St Radboud
Nijmegen (The Netherlands). This was all performed according to local ethical guidelines and approved by the local regulatory committee. After resection, all tissue samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

Human melanoma cell lines, 1F6 and Mel57, were grown in Dulbecco’s modified Eagle’s medium (DMEM) as described previously (de Vries et al., 1996; Westphal et al., 1997).

Design and printing of custom oligonucleotide arrays

In a previous study, 298 genes/ESTs were identified showing differential gene expression in two human melanoma cell lines, 1F6 and Mel57 (de Wit et al., 2002). For preparation of custom arrays these genes/ESTs were selected, together with four housekeeping genes, namely glyceraldehyde-3-phosphate dehydrogenase (GAPDH), phosphoglycerate kinase 1 (PGK1), porphobilinogen deaminase (PBGD) and β-actin. Based on these genes/ESTs, 70-mer amino-linked oligonucleotides were designed (Operon Technologies Inc., Qiagen, Alameda, CA, USA), complementary to the 3’-side of the corresponding genes/ESTs. These oligonucleotides were dissolved in spotting buffer (3 × SSC, 1.5 M betaine) and spotted in octaplicate onto UltraGAPS slides (Corning, New York, USA) using the Prosys 5510TL arrayer (Genomic Solutions, Huntingdon, Cambridgeshire, UK) (Figure 1). Additionally, various controls, which provided information about the target probe labelling efficiency, blocking, and nonspecific binding of the various controls, which provided information about the target probe labelling efficiency, blocking, and nonspecific binding of the arrays, were spotted onto the array. These controls included oligonucleotides of various non-human species, Cot-1 repetitive sequences, polyA sequences (SpotReport, Stratagene, La Jolla, CA, USA), tRNA and spots containing only spotting buffer.

For total RNA extraction from tissue samples, at least 10 frozen slices of 20-μm thickness were collected in 1 ml TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). After the TRIzol method, total RNA was subjected to an additional RNeasy (Qiagen, Hilden, Germany) cleaning step. Concerning the cell lines, total RNA was isolated from 10⁶ cultured cells using the RNeasy mini kit (Qiagen). All methods were performed conform the manufacturer’s protocol.

Target labelling, hybridisation and array analysis

Both direct and indirect labelling methods were used for labelling of target probes. Using the CyScribe first-strand cDNA labelling kit (Amersham Biosciences, Freiburg, Germany), cDNA was directly labelled by incorporation of Cy3- and Cy5-DUTPs during a RT reaction. We first isolated mRNA from 50 μg of total RNA using the Oligotex mRNA mini kit (Qiagen). The subsequent labelling reaction was performed according to the manufacturer’s protocol. For indirect labelling, 2 μg of total RNA (ratio 28S/18S RNAs > 1) were subjected to linear RNA amplification using the Amino Allyl MessageAmp aRNA Kit (Ambion, Cambridgeshire, UK). During this procedure, 5-(3-aminoallyl)-UTP was incorporated into the amplified antisense RNA (aRNA). N-hydroxysuccinimidy (NHS) ester-derivatised reactive Cy3 and Cy5 dyes (Fluorolink Cy3/Cy5 Monofunctional Dye S-Pack, Amersham Biosciences) were then chemically coupled to 3 μg of amino allyl aRNA. RNA amplification as well as chemical labelling was performed according to the instruction manual of the Amino Allyl MessageAmp aRNA Kit. Cy3 and Cy5 dye incorporation was measured by spectrophotometric analyses at 550 and 650 nm, respectively.

Prior to hybridisation, Cy3 and Cy5 labelled cDNA/aRNA samples were mixed (1:1), together with 7 μg Cot-1 DNA (Roche Diagnostics) and 3 μg polyA (Amersham Biosciences). After precipitation, the sample was dissolved in 130 μl of a hybridisation solution containing 50% formamide, 10% dextran sulphate, 2 × SSC, 0.1% sodium dodecyl sulphate (SDS) and 10 μg/ml yeast tRNA (Invitrogen). Hybridisation to our custom arrays and posthybridisation washing procedures were performed using a GeneTAC Hybridization Station (Genomic Solutions), according to the manufacturer’s protocols. In short, a 16 h hybridisation with active circulation of the probe was followed by five posthybridisation wash cycles in 50% formamide/2 × SSC at 45°C and five wash cycles in PBS at 20°C. The slides were briefly washed in water and dried by centrifugation. In dye-swap experiments, two replicate arrays were hybridised with similar cDNA/aRNA samples, but with swapped dyes.

Arrays were scanned and imaged on an Affymetrix 428 scanner (Affymetrix, Santa Clara, CA, USA) using the Affymetrix 428 scanner software package (version 1.0). Generally, the Cy5 dye showed a higher fluorescence intensity than the Cy3 dye. Therefore, scanning intensities used to image the individual dyes were adjusted to obtain a Cy5/Cy3 ratio = 1 for the housekeeping genes GAPDH and β-actin. To determine differential gene expression, the acquired array images were first analysed visually (red or green spots), but additionally GenePix Pro 4.0 software (Axon Instruments, Union City, CA, USA) was used. In the latter, differential gene expression was determined by comparing the median of the pixel intensities minus the median local background (F-B) of Cy5 with that of Cy3. Spots displaying F-B values smaller than 100 for both Cy dyes were excluded from further analysis. Alternatively, differential gene expression was determined by transforming median of ratios values (GenePix Pro 4.0) by taking the log₂. All octaplicate gene-specific spots were included in the analyses.
Supplementary microarray data can be found in the NCBI Gene Expression Omnibus, GEO (www.ncbi.nlm.nih.gov/geo/).

Semiquantitative RT–PCR
Aliquots of 1 and 0.5 μg of total RNA from cell lines and human melanocytic tissue samples, respectively, were reverse-transcribed using Moloney murine leukaemia virus reverse transcriptase (M-MLV RT) (Promega, Madison, WI, USA). Apart from 200 U of M-MLV RT, the reaction mixture consisted of 250 pmol of random hexadeoxynucleotide primers (Roche Diagnostics GmbH, Penzberg, Germany), 4 μl of RT buffer (250 mM Tris-HCl pH 8.3, 75 mM KCl, 15 mM MgCl₂, 50 mM DTT) and 4 μl of 1 mM dNTPs (Roche Diagnostics GmbH), completed with water to a final volume of 20 μl. This mixture was incubated 10 min at 25 °C, 59 min at 42 °C and 5 min at 95 °C. CDNA samples of human melanocytic lesions were diluted 1 : 1 in water.

Generally, PCR amplification was carried out in a total volume of 25 μl containing 1 μl reverse-transcribed cDNA, 2.5 μl of PCR buffer IV (20 mM (NH₄)₂SO₄, 75 mM Tris/HCl pH 9.0 and 0.1% Tween), 5 pmol of each primer, 0.15 U of Thermoperfectplus DNA polymerase (Amersham Pharmacia Biotech, UK) and appropriate MgCl₂ concentration. After 5 min denaturation at 94 °C, 25/30 and 30/35 cycles of amplification were carried out for cell lines and human melanocytic lesions, respectively: 45 s at 94 °C, 2 min at 59 °C followed by a 1-min elongation step at 72 °C. Housekeeping genes GAPDH and β-actin that were used for normalisation were amplified in 25/30 cycles independent of cDNA origin. The primer sequences and MgCl₂ concentrations used for each single PCR reaction are shown in Table 1.

Real-time quantitative RT–PCR
To determine differential gene expression of fatty acid binding protein 7 (FABP7, UniGeneID Hs.26770) and growth differentiation factor 15 (GDF15, UniGene ID Hs.296638) by real-time quantitative RT–PCR (qPCR), we used Assays-on-Demand Gene Expression Assays (Applied Biosystems, Cheshire, UK); assay ID Hs00361426_m1 and Hs00171132_m1, respectively. These assays consisted of a mix of unlabelled PCR primers and TaqMan MGB probe (FAM dye-labelled). Additionally, for normalisation, Assays-on-Demand Gene Expression Assays (Applied Biosystems, Cheshire, UK); assay ID Mm00475809_m1 and Mm00478085_m1, respectively, were used for normalisation.

Sequence analyses

Sequence analyses

The specificity of PCR products was validated by sequence analyses. Sequence reactions were performed with about 25 ng PCR product in addition of 10 μM specific forward or reverse primer, using the ABI PRISM 3700 DNA Analyser (Perkin-Elmer, Applied Biosystems, Forster City, CA, USA).

Immunohistochemistry

Cell suspensions of human melanoma cell lines 1F6 and Mel57 were processed into AgarCyto’s as previously described (Kerstens et al, 2000). 4 μm sections of paraffin-embedded AgarCyto’s and melanocytic tumour lesions were mounted on Superfrost microscope slides. These sections were dewaxed in xylene and rehydrated in a series of graded alcohols. To block endogenous peroxidase activity, slides were incubated with 3% H₂O₂ for 20 min. In contrast to immunohistochemical detection of thrombomodulin (THBD), for GDF15 and FABP7 antigen retrieval was essential; rehydrated slides were placed in citrate buffer (pH 6.0) and heated in a microwave oven to 97°C at 850 W for 5 min. This temperature was maintained with an additional 10 min heating at 350 W. After cooling down to room temperature, the sections were briefly washed with PBS. Next to AgarCyto sections,
Differential gene expression in melanocytic tumour lesions

NJW de Wit et al.

RESULTS

Differential gene expression in human melanoma cell lines 1F6 and Mel57

The genes/ESTs that were spotted on our custom oligonucleotide array, were previously found to be differentially expressed in human melanoma cell lines 1F6 and Mel57 by using Affymetrix Hu6800 and Hu35K arrays (de Wit et al, 2002). To determine whether previous Affymetrix results could be reproduced using our custom made oligonucleotide arrays, we first performed a dye-swap experiment using Cy3 and Cy5 labelled cDNA of cell lines 1F6 and Mel57. Differential gene expression in these genes could also be detected in human melanocytic tumour lesions, performed custom array analyses using target probes derived from NN and MM tissue samples. These NN and MM lesions contained a minimal tumour percentage of 50 and 60%, respectively. After Cy3 and Cy5 labelling of the probes using the indirect labelling method, individual NN and MM samples were hybridised in eight independent dye-swap experiments. Additionally, pooled samples of these NN (n = 6) and MM lesions (n = 6) were used to perform a dye-swap experiment. Compared to hybridisation experiments using cell line material, overall in the dye-swap experiments using melanocytic lesions, a lower percentage of the spots could be analysed, namely 15 – 40% (n = 45 – 119). This can be explained by the considerably lower quality of total RNA of the lesions, influencing the labelling efficiency. In first instance, we determined differential expression only by visual analysis and by comparing the F-B values of the Cy dyes (GenePix Pro 4.0 software). In total, 42 genes/ESTs showed a highly reproducible differential gene expression, meaning similar results in more than five out of nine experiments. Although all of these genes might be candidate players in tumour progression, in this study we further focused on the 25 genes that showed comparable results in eight out of nine or even nine out of nine experiments (Table 2). For genes with similar differential expression in eight out of nine dye-swaps, we mostly found no detectable differential expression in the remaining experiment (no fluorescence signal or equal intensities for Cy3 and Cy5) and occasionally a swift in differential expression as was previously found by Affymetrix array analyses. For genes that showed a similar expression pattern and only one of the differences, no detectable differential expression was found by Affymetrix arrays. Although our results demonstrated that a reduced number of spots could be analysed for differential gene expression using the indirect labelling method, the reproducibility of previous Affymetrix array results remained very high.

Differential gene expression in human melanocytic lesions

As human melanoma cell lines 1F6 and Mel57 display different metastatic behaviour after subcutaneous inoculation into nude mice (Westphal et al, 1997), we reasoned that the genes that were found to be differentially expressed in these cell lines could well be involved in melanocytic tumour progression, especially in metastatic processes. To determine whether differential expression of these genes could also be detected in human melanocytic tumour lesions, we performed custom array analyses using target probes derived from NN and MM tissue samples. These NN and MM lesions contained a minimal tumour percentage of 50 and 60%, respectively. After Cy3 and Cy5 labelling of the probes using the indirect labelling method, individual NN and MM samples were hybridised in eight independent dye-swap experiments. Additionally, pooled samples of these NN (n = 6) and MM lesions (n = 6) were used to perform a dye-swap experiment. Compared to hybridisation experiments using cell line material, overall in the dye-swap experiments using melanocytic lesions, a lower percentage of the spots could be analysed, namely 15 – 40% (n = 45 – 119). This can be explained by the considerably lower quality of total RNA of the lesions, influencing the labelling efficiency. In first instance, we determined differential expression only by visual analysis and by comparing the F-B values of the Cy dyes (GenePix Pro 4.0 software). In total, 42 genes/ESTs showed a highly reproducible differential gene expression, meaning similar results in more than five out of nine experiments. Although all of these genes might be candidate players in tumour progression, in this study we further focused on the 25 genes that showed comparable results in eight out of nine or even nine out of nine experiments (Table 2). For genes with similar differential expression in eight out of nine dye-swaps, we mostly found no detectable differential expression in the remaining experiment (no fluorescence signal or equal intensities for Cy3 and Cy5) and occasionally a swift in differential expression in NN and MM lesions (i.e. THBD, D4S234E, CTSL and GDF15).

To obtain an indication of the fold change of the differential gene expression, we also calculated the log2 of the median of ratios for the dye-swap experiments. Table 3 shows an example of these values for the dye-swap experiment using pooled NN and MM samples. For most of the genes, comparable fold changes could be determined within one dye-swap experiment, indicating the high reproducibility of the dye-swap arrays. However, log2 values were not always in accordance with data demonstrated in Table 2, as for instance is seen for NCK2 and ITGB5 genes in Table 3. Moreover, comparing all dye-swap experiments, we saw that although the differential expression in NN and MM was highly consistent for the genes, the fold changes were quite variable. This suggests that
the extent of differential gene expression is not necessarily the same when comparing different individual NN and MM lesions.

To determine whether among the 298 genes that were present on the array, differential gene expression also could be analysed in melanocytic tumour lesions of similar progression stages, we performed a dye-swap experiment using two different NN samples. Only eight genes showed differential expression, of which TYRP1 was one of them. The other seven genes did not belong to the group that showed differential gene expression comparing NN and MM in more than five dye-swap experiments. These eight genes most probably represent patient-specific and/or pigment-related differences in gene expression.

Verification of differential gene expression

To validate the differential gene expression we found by custom array analysis, we performed semiquantitative RT–PCR for the 25 genes indicated in Table 2. We first looked at their differential expression found in melanoma cell lines 1F6 and Mel57 (Figure 2). Using GAPDH and β-actin for normalisation, we determined that differential expression could be corroborated for all genes, except for MYL6 and FLJ10349. PGK1, which was initially selected as housekeeping gene, showed indeed no differential expression in the melanoma cell lines. By performing immunohistochemistry (IHC) on AgarCyto’s of 1F6 and Mel57, we wanted to determine whether differential expression could also be detected on the protein level. Unfortunately, antibodies were only limitingly available. Nevertheless, IHC using THBD, GDF15 and FABP7 specific antibodies showed that a similar differential protein expression could be detected as was seen for their mRNA expression by array analyses and semiquantitative RT–PCR (Figure 3). Figure 3C and D shows that THBD was mainly localised to the cell membrane in 1F6 cells, whereas Mel57 cells were completely negative. For GDF15 the difference in protein expression was less distinct; however, an upregulated cytoplasmic expression could be detected in cell line Mel57 compared to 1F6 (Figure 3E and F). FABP7 showed a significant higher cytoplasmic protein expression in 1F6 than could be detected in Mel57 (Figure 3G and H). Next to AgarCyto’s, also slides containing cultured 1F6 and Mel57 cells were stained for THBD, GDF15 and FABP7. These cultured cells showed comparable staining results as were found with IHC on AgarCyto’s. Even though the observed differences in protein expression for FABP7 and especially for GDF15 were a little less profound in the cultured cells (data not shown), THBD again showed a marked expression in 1F6, whereas Mel57 cells remained negative (Figure 3I and J). The differential protein expression of GDF15 could be better visualised by Western blot analysis as is demonstrated in Figure 3K. In this figure, the 35 kDa band represents the pro-form of GDF15 (propeptide + mature protein), whereas the 25 kDa band shows the cleaved GDF15 propeptide. The mature form of GDF15 (10 kDa) could hardly be detected by Western blotting using cell lysates as this protein is secreted by the cells.

Also for human melanocytic tumour lesions, semiquantitative RT–PCR was used to verify differential gene expression. As only a limited amount of cDNA was available of most lesions, a pilot experiment was performed in which differential expression of the 25 genes listed in Table 2 was determined in two samples of NN, atypical nevi (AN), primary melanoma (PM) and MM. This experiment showed that 12 of the 25 genes displayed distinct differential expression during melanoma progression (data not shown). These 12 genes were selected for further expression profiling in a larger series of melanocytic tumour lesions. For all 12 genes, the relative expression to GAPDH was determined as is demonstrated for FABP7 in Figure 4A. Figure 4B shows the graphical representation of the relative expression of these 12 genes during melanoma progression. For MYL6 and C7orf20, no distinct differential expression could be detected in this larger series and CTSL showed an inverted differential expression by semiquantitative RT–PCR than was found by custom array analyses. In accordance with our array data, downregulation of THBD, FABP7, H2AJaF, RRAGD, MYADM and HR and upregulation of CKS2, NCK2 and GDF15 was seen in MM compared to NN lesions. The upregulation of CKS2, NCK2 and GDF15 seemed to follow a somehow gradual course during melanocytic tumour progression, whereas downregulated expression of THBD, FABP7, RRAGD, MYADM, HR and especially H2AJaF was more predominantly restricted to MM lesions. To exclude that differential expression was significantly influenced by the presence of keratinocytes in the NN, AN and MM lesions, we performed semiquantitative RT–PCR on two normal skin (NS) and laser dissected melanocytic lesions. Only for C7or2D and MYADM, we found that the presence of keratinocytes most probably caused the differential expression seen by array analyses and RT–PCR. Overall, it has to be noted that for some differentially expressed genes no PCR product could be detected after 35 cycles, meaning that expression of these genes might even be stronger downregulated in the melanocytic tumour lesions than is demonstrated in Figure 4B.

To strengthen our semiquantitative RT–PCR results and more precisely quantify the differences found in expression, we performed real-time qRT–PCR for two randomly selected genes, namely FABP7 and GDF15. We first determined fold change of differential expression between cell line 1F6 and Mel57. A 24-fold increase of FABP7 expression was found in 1F6 compared to Mel57; for GDF15 a 10-fold decrease was detected. Figure 5 demonstrates the expression of FABP7 and GDF15 in melanocytic tumour lesions relative to 1F6 and Mel57, respectively. qPCR results were highly similar to our data derived from custom array analyses and semiquantitative RT–PCRs. For FABP7 a decreased expression could again be determined when comparing MM lesions to NN lesions. The opposite was true for GDF15. During tumour progression, we found that down- or upregulated expression of FABP7 and GDF15, respectively, could already be detected in PM lesions, but was most prominent in MM. Moreover, NS and laser dissected NN samples (NN#) that were included in this qPCR ensured that keratinocytes did not influence the differential gene expression of FABP7 and GDF15 in NN and MM lesions. As for some tumour samples again no PCR product could be detected after 35 cycles, downregulation of FABP7 and GDF15 was even more profound.

To provide an indication of the overall fold change of FABP7 and GDF15 differential expression in NN and MM lesions, we calculated the average relative expression of NN and MM samples obtained by qPCR and compared these values. We found a 164-fold downregulation of FABP7 expression in MM compared to NN, whereas GDF15 showed a 53-fold upregulated expression. These fold changes are much higher than those found by custom array analyses (Table 3).

DISCUSSION

In this study, custom made oligonucleotide arrays were designed, based on previous Affymetrix high-density oligonucleotide array results, in which differential expression was analysed in human melanoma cell lines 1F6 and Mel57 (de Wit et al, 2002). The highly comparable results obtained by both array techniques, concerning differential gene expression in 1F6 and Mel57, indicate that our custom arrays are a reliable tool for analysis of differential gene expression.

Previous studies already showed that linear RNA amplification provides the opportunity to perform array analysis with minute amounts of starting material (Puskas et al, 2002; Gomes et al, 2003; Klur et al, 2004; Schneider et al, 2004). We experienced that the indirect labelling method, which is based on linear RNA
Table 2  Differential gene expression between NN and MM samples determined by dye-swap array experiments

| UniGene ID | Gene name                          | Gene symbol | Experiment no. | Up-regulation (# experiments) |
|------------|------------------------------------|-------------|----------------|-------------------------------|
| 2030       | Thrombomodulin                      | THBD        | NN 9**       | 8/9 1/9                       |
| 405913     | Likely ortholog of mouse gene rich cluster, C10 | GRCC10      | MM 1/9        |                               |
| 26770      | Fatty acid binding protein 7, brain | FABP7       | NN 9/9        | 8/9 0/9                       |
| 37627      | H2A histone family, member J       | H2AFJ       | MM 0/9        | 9/9 0/9                       |
| 75219      | Tyrosinase-related protein 1        | TYRP1       | MM 0/9        | 9/9 0/9                       |
| 77385      | Myosin, light polypeptide 6, alkali| MYL6        | MM 0/9        | 8/9 0/9                       |
| 79404      | DNA segment on chromosome 4, 234 expressed seq | D4S234E     | MM 8/9        | 8/9 1/9                       |
| 446471     | CD74 antigen                        | CD74        | MM 0/9        | 8/9 0/9                       |
| 107387     | Chromosome 7 open reading frame 20  | C7orf20     | MM 0/9        | 8/9 0/9                       |
| 415997     | Collagen, type VI, alpha 1 precursor| COL6A1      | MM 0/9        | 8/9 0/9                       |
| 184542     | Yippe protein                       | CGI-127     | MM 0/9        | 8/9 0/9                       |
| 238679     | Ras-related GTP binding D          | RRAGD       | MM 0/9        | 8/9 0/9                       |
| 380906     | Myeloid-associated differentiation marker | MYADM      | MM 0/9        | 8/9 0/9                       |
| 272367     | Hairless homolog (mouse)            | HR          | MM 0/9        | 9/9 0/9                       |
| 19333      | Hypothetical protein FLJ10349       | FLJ10349    | MM 0/9        | 9/9 0/9                       |
| 439639     | ADP-ribosylation factor 4           | ARF4        | MM 0/9        | 0/9 8/9                       |
| 418123     | Cathepsin L precursor (Major excreted protein) | CTSL      | MM 0/9        | 0/9 9/9                       |
| 78771      | Phosphoglycerate kinase 1           | PGK1        | MM 0/9        | 0/9 9/9                       |
| 83758      | CDC28 protein kinase regulatory subunit 2 | CKS2      | MM 0/9        | 0/9 9/9                       |
| 84113      | Cyclin-dependent kinase inhibitor 3 | CDKN3      | MM 0/9        | 0/9 8/9                       |
| 101895     | NCK adaptor protein 2               | NCK2        | MM 0/9        | 0/9 8/9                       |
| 149846     | Integrin, beta 5                    | ITGB5       | MM 0/9        | 0/9 8/9                       |
| 396283     | Ubiquitin carrier protein E2        |UBE2S        | MM 0/9        | 0/9 8/9                       |
| 298654     | Dual specificity protein phosphatase 6 (MAPK3) | DUSP6    | MM 0/9        | 0/9 8/9                       |
| 296638     | Growth differentiation factor 15 (NAG-1 / MIC-1) | GDF15   | MM 0/9        | 1/9 8/9                       |

NN = nevus nevocellularis; MM = melanoma metastasis; *dye-swap experiment using individual NN and MM samples; **dye-swap experiment using pooled NN and MM samples; black boxes = upregulated expression was detected in dye-swap experiment; ND = no differential expression could be detected in dye-swap experiment. Differential gene expression was determined by visual analysis and by comparing the F-B values of Cy3 and Cy5 dyes.
Table 3  Fold changes of differentially expressed genes in a dye-swap experiment using pooled NN and MM samples

| UniGene ID | Gene name | Gene symbol | log2 of \( \frac{\text{median of ratios (Cy5/Cy3)}}{\text{NN-Cy5/MM-Cy3}} \) | log2 of \( \frac{\text{median of ratios (Cy5/Cy3)}}{\text{NN-Cy3/MM-Cy5}} \) |
|------------|-----------|-------------|---------------------------------|---------------------------------|
| 2030       | Thrombomodulin | THBD        | 0.72 ± 0.09                     | -1.35 ± 0.08                    |
| 405913      | Likely ortholog of mouse gene rich cluster, C10 | GRCC10 | 1.42 ± 0.10                     | -1.07 ± 0.34                    |
| 26770       | Fatty acid binding protein 7, brain | FABP7 | 2.55 ± 0.15                     | -1.89 ± 0.11                    |
| 36727       | H2A histone family, member J | H2AFJ | 3.36 ± 0.33                     | -2.73 ± 0.21                    |
| 75219       | Tyrosinase-related protein 1 | TYRP1 | 1.81 ± 0.27                     | -0.87 ± 0.42                    |
| 77385       | Yosin, light polypeptide 6, alkali | MYL6 | 0.68 ± 0.16                     | -1.69 ± 1.16                    |
| 79404       | DNA segment on chromosome 4, 234 expressed seq | D4S234E | 0.77 ± 0.15                     | -0.64 ± 0.19                    |
| 446471      | CD74 antigen | CD74 | 0.50 ± 0.07                     | -0.52 ± 0.16                    |
| 107387      | Chromosome 7 open reading frame 20 | C7orf20 | 0.45 ± 0.12                     | -0.65 ± 0.12                    |
| 415997      | Collagen, type VI, alpha 1 precursor | COL6A1 | 1.23 ± 0.14                     | -0.67 ± 0.10                    |
| 184542      | Yippee protein | CGI_127 | 0.78 ± 0.09                     | -1.63 ± 0.24                    |
| 238679      | Ras-related GTP binding D | RRAGD | 0.88 ± 0.12                     | -1.45 ± 0.13                    |
| 380906      | Myeloid-associated differentiation marker | MYADM | 1.80 ± 0.19                     | -1.47 ± 0.07                    |
| 272367      | Hairless homolog (mouse) | HR | 2.04 ± 0.14                     | -1.89 ± 0.17                    |
| 19333       | Hypothetical protein FLJ10349 | FLJ10349 | -0.81 ± 0.12                    | 0.67 ± 0.10                     |
| 435639      | ADP-ribosylation factor 4 | ARF4 | -1.62 ± 0.08                     | 1.05 ± 0.20                     |
| 418123      | Cathepsin L precursor (Major excreted protein) | CTSL | -0.63 ± 0.12                    | 0.90 ± 0.14                     |
| 78771       | Phosphoglycerate kinase 1 | PGK1 | -0.95 ± 0.05                     | 0.69 ± 0.16                     |
| 83758       | CDC28 protein kinase regulatory subunit 2 | CKS2 | -2.10 ± 0.04                     | 2.60 ± 0.25                     |
| 84113       | Cyclin-dependent kinase inhibitor 3 | CDKN3 | -1.83 ± 0.14                     | 3.29 ± 0.43                     |
| 101695      | NCK adaptor protein 2 | NCK2 | ND | 0.91 ± 0.23                     |
| 149846      | Integrin, beta 5 | ITGB5 | ND | 0.38 ± 0.08                     |
| 396393      | Ubiquitin carrier protein E2 | UBE2S | -1.37 ± 0.07                     | 2.66 ± 0.24                     |
| 298654      | Dual specificity protein phosphatase 6 (MAPK3) | DUSP6 | -1.10 ± 0.18                     | 0.40 ± 0.27                     |
| 296638      | Growth differentiation factor 15 (NAG-1 / MIC-1) | GDF15 | -1.78 ± 0.07                     | 2.15 ± 0.38                     |

NN = nevus nevocellularis; MM = melanoma metastasis; *median of ratios values (GenePix Pro 4.0) were transformed by taking the log2 and averaged from octaplicate gene-specific spots (mean ± s.d); ND = no detectable differential expression by analysis of log2 of median of ratios.
amplification, is indeed a valuable and reliable technique, although we determined a reduction in number of analysable spots. This might be explained by lack of optimal amplification efficiency, as the quality of total RNA can negatively influence the procedure. However, we found that signal intensities of the spots could be analysed were generally hardly diminished. Besides linear RNA amplification by in vitro transcription (IVT), alternative methods have recently been described to reduce the amount of staring material for array analysis. For instance, amplification of full-length double-stranded cDNA by PCR has been shown to be useful in various studies (Saghizadeh et al., 2003; Becker et al., 2004; Rihl et al., 2004), and also application of single-stranded linear amplification protocol (SLAP) has been reported, which combines linear amplification and PCR (Stirewalt et al., 2004). However, it has to be noted that for identification of differential gene expression by array analysis, it is highly preferable that each sample is equally treated prior to hybridisation, as every amplification method has the possibility to introduce transcript-dependent biases, which even increase as the starting amount of RNA decreases (Stirewalt et al., 2004).

Using fresh NN and MM lesions for custom array analyses, we determined whether the differential gene expression that was previously found in melanoma cell lines 1F6 and Mel57 could also be detected in the in vivo situation. Differential gene expression was analysed in two ways: by comparing F-B values of the Cy dyes and by calculating the log2 of the median of ratios for each dye swap experiment. For the latter method, which provided an indication of the fold change of the differential gene expression, the ‘median of ratios’ was chosen over the commonly used ‘ratio of ratios’ values (Brody et al., 2002) determined that the ‘median of ratios’ provided a more consistent measurement. However, as the size of the spots still influences the accuracy of the measurement of the ‘median of ratios’ values (Brody et al., 2002), in our study we attached higher importance to the F-B software analysis. Moreover, comparison of F-B values of the Cy dyes showed a better correlation with visual analysis of the arrays. In this way, 25 genes were identified showing a highly reproducible constant differential expression pattern in NN vs MM lesions. The dye-swap experiment using two NN samples for hybridisation showed that the differential expression of most of these genes was not patient specific.

For nine of the 25 genes, namely THBD, FABP7, H2AFJ, RRAGD, MYADM, HR, CKS2, NCK2 and GDF15, our semiquantitative RT–PCR results were in accordance with the differential expression that was found by custom array analyses. However, the possibility remains that for some of the 13 genes that were now only tested in a pilot experiment using semiquantitative RT–PCR, verification of differential expression can still be achieved using an expanded series of melanocytic tumour lesions. For instance, DUSP6 was very recently also found to be differentially expressed in normal melanocytes compared to melanoma cells by Hoek et al. (2004). This indicates it might be an interesting gene for further studies. Additionally, also genes that showed a differential expression in five out of nine, six out of nine and seven out of nine custom array experiments might still be potential players in melanocytic tumorigenesis.

Real-time qPCR, performed for FABP7 and GDF15, strengthened the data of our semiquantitative RT–PCR analyses, as both techniques provided comparable results. Moreover, it confirmed differential gene expression found by custom array analysis, although fold changes that were found by performing qPCR were much higher than those obtained using arrays (log2 of the median of ratios, Table 3). The tendency to underestimate fold change ratios by array analysis is also reported in other studies (Rajeevan et al., 2001; Yuen et al., 2002).

Our differential gene expression data showed minimal overlap with previously described microarray experiments in which also gene expression patterns were studied in cutaneous melanoma progression (Brem et al., 2001; Baldi et al., 2003b; Carr et al., 2003; Dooley et al., 2003; Rumpler et al., 2003; Becker et al., 2004; Nambiar et al., 2004). This could be explained by the fact that design of our custom made oligonucleotide arrays was based on previously found differential gene expression in melanoma cell lines. This way, we already made a considerable selection of genes to be examined for differential gene expression, probably missing some genes that might also be involved in melanocytic tumour progression. Moreover, high variability can be introduced between related array studies, as arrays designed with different types of oligonucleotides/cDNAs (e.g. 50-/60-/70-mer oligo’s, full-length cDNA) can be used and distinct methods can be selected for labelling of target probes. Therefore, numerous comparable microarray studies are described with different outcomes (Nambiar et al., 2004). This emphasises the necessity of verification of the array data using more conventional techniques, like (semi-) quantitative RT–PCR, to prove that differential gene expression is really present.

Until now, minimal information is available in literature of most of the genes that we found differentially expressed by our custom array analyses as well as by RT–PCR. However, GDF15 and THBD were previously described to be involved in tumorigenesis. GDF15, which is also called macrophage inhibitory cytokine-1 (MIC-1), NSAID-activated protein (NAG-1) or prostate differentiation factor (PLAB), is a divergent member of the tumour growth factor β (TGF-β) superfamily (Bootcov et al., 1997; Hromas et al., 1997; Baek et al., 2001). The major function of GDF15 is still uncertain, but there are indications that it plays a role in growth inhibition and induction of apoptosis in several tumour cell lines (Li et al., 2000; Albertoni et al., 2002; Yang et al., 2003). Controversially however, several studies have reported an upregulated (secreted) expression in (advanced and more aggressive) tumours compared to noncancerous tissues or less aggressive tumours (Welsh et al., 2001; Brown et al., 2003; Karan et al., 2003; Nakamura et al., 2003). This is in accordance with our data, as we also found an upregulated expression during melanoma progression. THBD is a thrombin receptor, which is mostly found on the surface of vascular endothelial cells and epidermal keratinocytes. However, its presence is also reported on tumour cells of several types of cancer, such as, hepatocellular carcinoma, ovarian cancer, breast...
Figure 3  Analysis of protein expression of THBD, GDF15 and FABP7 in human melanoma cell lines 1F6 and Mel57. Magnification (× 400) of AgarCyto’s (A–H) and cultured 1F6 and Mel57 cells (I, J). (A, B) Negative control of 1F6 and Mel57, respectively, leaving out specific antisera. (C, D, I, J) IHC using THBD specific antibodies; in AgarCyto’s as well as cultured cells most 1F6 cells showed a (membranous) THBD specific staining (C, I), whereas Mel57 cells were completely negative (D, J). (E, F) IHC using GDF15 specific antibodies; a more intense cytoplasmic GDF15 staining could be detected in cell line Mel57 (F), compared to 1F6 cells (E). (G, H) IHC using FABP7 specific antibodies; 1F6 cells showed a higher immunoreactivity for FABP7 (cytoplasmic) (G) than Mel57 cells (H). (K) Western blot analysis of GDF15; a distinct preferential protein expression could be detected in Mel57 compared to 1F6. The 35 kDa band represents the pro-form of GDF15 (pro-peptide + mature protein), whereas the 25 kDa band shows the cleaved GDF15 pro-peptide.
cancer and squamous cell carcinoma (Suehiro et al., 1995; Kim et al., 1997; Tabata et al., 1997; Wilhelm et al., 1998). In these tumours, the expression level of THBD is inversely correlated with malignancy of cancer. As for melanoma, it was previously reported that THBD isolated from human urine could suppress experimental lung metastasis of murine melanoma cells (B16F10 cells) in vivo (Hosaka et al., 2000). Also for human melanoma cell lines, a negative correlation was described between THBD expression and cell proliferation in vitro and in vivo (Zhang et al., 1998). This is in accordance with our findings, as we determined a downregulated THBD expression in MM lesions by array analysis and RT–PCR. Moreover, our IHC

**Figure 4** Verification of differential gene expression in human melanocytic tumour progression lesions by semiquantitative RT–PCR. (A) For 12 differentially expressed genes, relative expression to GAPDH was determined by semiquantitative RT–PCR as shown for FABP7. (B) For MYL6 and C7orf20 differential expression could hardly be detected. THBD, FABP7, H2AFJ, RRAGD, MYADM and HR showed a downregulated expression during melanoma progression, with a preferable decrease in MM lesions. The latter was especially true for H2AFJ. Expression of CKS2, NCK2 and GDF15 showed an upregulation associated with increased malignancy of melanocytic tumour lesions. For THBD, FABP7, H2AFJ, RRAGD, MYADM, HR, CKS2, NCK2 and GDF15 differential expression found by semiquantitative RT–PCR was in accordance with our custom array data, whereas CTSL showed an inverse differential expression.
Data showed membranous expression in human melanoma cell line 1F6, whereas the more malignant cell line Mel57 remained negative. Although, previous studies report THBD expression in keratinocytes, our RT–PCR data of NS and laser dissected melanocytic NN cells indicated that epidermal cells were not responsible for the differential expression that we determined in NN and MM lesions. Obviously, for both GDF15 and THBD, additional studies are necessary to elucidate their role in cancer biology and determine its potential clinical significance.

In summary, this study provides a reliable, solid indication that CKS2, NCK2, GDF15 THBD, FABP7, RRAGD, MYADM, HR and H2AFJ are candidate players in melanocytic tumour progression, as similar differential expression patterns are demonstrated for these genes by custom array analysis, semiquantitative RT–PCR and even real-time qPCR. The genes that showed a preferable expression in benign melanocytic tumour stages and loss of expression during tumour progression might be functional markers to facilitate early diagnosis of melanocytic lesions before onset of the metastatic phase. Genes with an elevated expression in advanced, more malignant melanocytic lesions are probably more suitable for development of novel therapeutics for melanoma patients. Moreover, regarding prognostic settings, the downregulated genes can be potential indicators for a relatively good prognosis, whereas the upregulated genes might be of opposite prognostic value. Nevertheless, to ensure that the genes that we found to be differentially expressed in this study are indeed useful for diagnostic, prognostic and/or therapeutical applications, further investigation is necessary.

**ACKNOWLEDGEMENTS**

We thank the Microarray Facility Nijmegen for their help in conducting our custom oligonucleotide array experiments. Furthermore, we thank the Department of Hematology of Radboud University Nijmegen Medical Centre for their practical support in performing real-time PCR. This study was partly supported by the Dutch Cancer Society (NUKC 98-1782).
Albertoni M, Shaw PH, Nozaki M, Godard S, Tenan M, Hamou MF, Fairlie DW, Breit SN, Paralkar VM, de Tribolet N, Van Meir EG, Hegi ME (2002) Anoxia induces macrophage inhibitory cytokine-1 (MIC-1) in glioblastoma cells independently of p53 and HIF-1. Oncogene 21: 4212 – 4219
Bautz A, Kim KS, Nixon JB, Wilson LC, Elling TE (2001) Cyclooxygenase inhibitors regulate the expression of a TGF-beta superfamily member that has proapoptotic and antimicrobial activities. Mol Pharmacol 59: 901 – 908
Baldi A, Battista T, De Luca A, Santini D, Rosselli L, Baldi F, Natali PG, Lombardi D, Piccardo M, Felsani A, Paggi MG (2003a) Identification of genes down-regulated during melanoma progression: a cDNA array study. Exp Dermatol 12: 212 – 218
Baldi A, Santini D, De Luca A, Paggi MG (2003b) cDNA array technology in melanoma: an overview. J Cell Physiol 196: 219 – 223
Becker B, Roesch A, Hafner C, Stolz W, Dugas M, Landthaler M, Vogt T (2004) Discrimination of melanocytic tumors by cDNA array hybridization of tissues prepared by laser pressure catapulting. J Invest Dermatol 122: 361 – 368
Bootcov MR, Bauskin AR, Valenzuela SM, Moore AG, Bansal M, He XY, Zhang HP, Donnellan M, Mahler S, Pryor K, Walsh BJ, Nicholson RC, Fairlie WD, Po SB, Robbins JM, Breit SN (1997) MIC-1, a novel macrophage inhibitory cytokine, is a divergent member of the TGF-beta superfamily. Proc Natl Acad Sci USA 94: 11514 – 11519
Breit SN, Jarsch M, Vagner S, Weidle UH (2001) Identification of metastasis-associated genes by transcriptional profiling of a metastasizing versus a non-metastasizing human melanoma cell line. Anticancer Res 21: 1731 – 1740
Brody JP, Williams BA, Wold BJ, Quake SR (2002) Significance and statistical errors in the analysis of DNA microarray data. Proc Natl Acad Sci USA 99: 12975 – 12978
Brown DA, Ward RL, Buckhaults P, Liu T, Romans KE, Hawkins NJ, Bauskin AR, Knizler KW, Vogelstein B, Breit SN (2003) MIC-1 serum and experimental metastasis of murine melanoma cells independently of p53 and HIF-1. J Cell Physiol 196: 219 – 223
Carr KM, Bittner M, Trent JM (2003) Gene-expression profiling in human cutaneous melanoma. Oncogene 22: 3076 – 3080
de Vries TJ, Verheijen JH, de Bart AC, Weidle UH, Ruiter DJ, van Muijen GN (1996) Decreased expression of both the low-density lipoprotein receptor-related protein-alpha(2)-macroglobulin receptor and its receptor-associated protein in late stages of cutaneous melanocytic tumor progression. Cancer Res 56: 1432 – 1439
de Wit NJ, Burtscher HJ, Weidle UH, Ruiter DJ, van Muijen GN (1996) Decreased expression of both the low-density lipoprotein receptor-related protein-alpha(2)-macroglobulin receptor and its receptor-associated protein in late stages of cutaneous melanocytic tumor progression. Cancer Res 56: 1432 – 1439
Dooley TP, Curto EV, Davis RL, Grammatico P, Robinson ES, Wilborn TW (2003) DNA microarrays and likelihood ratio bioinformatic methods: discovery of human melanocyte biomarkers. Pigment Cell Res 16: 245 – 253
Gomes LI, Silva RL, Stolf BS, Cristo EB, Hirata R, Soares FA, Reis LF, Neves EJ, Carvalho AF (2003) Comparative analysis of amplified and nonamplified RNA for hybridization in cDNA microarray. Anal Biochem 321: 244 – 251
Hoek K, Rimm DL, Williams KR, Zhao H, Aryan S, Lin A, Kluger HM, Berger AJ, Cheng E, Trombetta ES, Wu T, Niinobe M, Yoshikawa K, Hannigan GE, Halaban R (2004) Expression profiling reveals novel pathways in the transformation of melanocytes to melanomas. Cancer Res 64: 5270 – 5282
Hosaka Y, Higuchi T, Tsumagari M, Ishii H (2000) Inhibition of invasion and experimental metastasis of murine melanoma cells by human soluble thrombomodulin. Cancer Lett 161: 231 – 240
Hromadka R, Hufford M, Sutton J, Xu D, Li Y, Lu L (1997) PLAB, a novel placental bone morphogenetic protein. Biochim Biophys Acta 1354: 40 – 44
Karan D, Chen SJ, Johansson SL, Singh AP, Paralkar VM, Lin MF, Batra SK (2003) Dysregulated expression of MIC-1/PDF in human prostate tumor cells. Biochem Biophys Res Commun 308: 598 – 604
Kerstens HM, Robbers P, De Peddegh P, Melchers WJ, Boonstra H, de Wilde PC, Macville MV, Hanselaar AG (2000) AgarCyto: a novel cell-processing method for multiple molecular diagnostic analyses of the uterine cervix. J Histochem Cytochem 48: 709 – 718
Kim SJ, Shiba E, Ishii H, Inoue T, Taguchi T, Tanji Y, Kimo Y, Izukura M, Takai S (1997) Thrombomodulin is a new biological and prognostic marker for breast cancer: an immunohistochemical study. Anticancer Res 17: 2319 – 2323
Klur S, Toy K, Williams MP, Certa U (2004) Evaluation of procedures for amplification of small-size samples for hybridization on microarrays. Genomics 83: 508 – 517
Li PX, Wong J, Ayed A, Ngo D, Brade AM, Arrowsmith C, Austin RC, Klamut HJ (2000) Placental transforming growth factor-beta is a downstream mediator of the growth arrest and apoptotic response of tumor cells to DNA damage and p53 overexpression. J Biol Chem 275: 20127 – 20135
Livesik KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(Delta Delta C(T)) method. Methods 25: 402 – 408
Nakamura T, Scirilas A, Stephan C, Yousef GM, Kristiansen G, Jung K, Diamandis EP (2003) Quantitative analysis of macrophage inhibitory cytokine (MIC-1) gene expression in human prostatic tissues. Br J Cancer 88: 1101 – 1104
Namibrari S, Mirmohammadssadegh A, Bar A, Bardenheuer W, Roeder G, Henge UR (2004) Applications of array technology: melanoma research and diagnosis. Expert Rev Mol Diag 4: 549 – 557
Puslak LS, Zvara A, Hackler JR, L M, Tischk V, van Hummelen P (2002) Production of bulk amounts of universal RNA for DNA microarrays. Biotechniques 33: 898 – 900, 902 – 904
Rajeevan K, Ranamukhaarachchi DG, Vernon SD, Unger ER (2001) Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies. Methods 25: 443 – 451
Rihl M, Baeten D, Seta N, Gu J, De Keyser F, Veys EM, Kuipers JG, Zeidler H, Yu DT (2004) Technical validation of cDNA based microarray as screening technique to identify candidate genes in synovial tissue biopsy specimens from patients with spondyloarthropathy. Ann Rheum Dis 63: 498 – 507
Rumpler G, Becker B, Hafner C, McClelland M, Stolz W, Landthaler M, Schmitt R, Bosscherhoff A, Vogt T (2003) Identification of differentially expressed genes in models of melanoma progression by cDNA array analysis: SPARC, MIF and a novel cathepsin protease characterize aggressive phenotypes. Exp Dermatol 12: 761 – 771
Saghizadeh M, Brown DJ, Tajbakhsh J, Chen Z, Kenney MC, Farber DB, Nelson SF (2003) Evaluation of techniques using amplified nucleic acid probes for gene expression profiling. Biomol Eng 20: 97 – 106
Schneider J, Buness A, Huber W, Volz J, Kriosch P, Hafner M, Poustka A, Sultmann H (2004) Systematic analysis of T7 RNA polymerase based in vitro linear RNA amplification for use in microarray experiments. BMC Genomics 5: 29
Streitfeld DL, Pogoosova-Agadjanian EL, Khalid N, Hare DR, Ladne PA, Sala-Torra O, Zhao LP, Radich JP (2004) Single-stranded linear amplification protocol results in reproducible and reliable microarray data from nanogram amounts of starting RNA. Genomics 83: 321 – 331
Suzuki T, Shimada M, Matsutama T, Takeotomi A, Yamamoto K, Sugimachi K (1995) Thrombomodulin inhibits intrahepatic spread in human hepatocellular carcinoma. Hepatology 21: 1285 – 1290
Tabata M, Sugihara K, Yonezawa S, Yamashita S, Maruyama I (1997) An immunohistochemical study of thrombomodulin in oral squamous cell carcinoma and its association with invasive and metastatic potential. J Oral Pathol Med 26: 258 – 264
Welsh JB, Sapinoso LM, Su AI, Korn SG, Wang-Rodriguez J, Moskaluk CA, Frierson Jr HF, Hampton GM (2001) Analysis of gene expression identifies candidate markers and pharmacological targets in prostate cancer. Cancer 65: 1394 – 1397
Westphal JR, van’t-Hullenaar RA, van-der-Laan JA, Cornelissen IM, Schalkwijk LJ, van-Muijen GN, Wesseling P, de-Wilde PC, Ruiter DJ, de-Waal RM (1997) Vascular density in melanoma xenografts correlates with vascular permeability factor expression but not with metastatic potential. Br J Cancer 76: 561 – 570
Wilhelm S, Schmitt M, Parkinson J, Kuhn W, Graeff H, Wilhelm OG (1998) Thrombomodulin, a receptor for the serine protease thrombin, is decreased in primary tumors and metastases but increased in ascitic fluids from patients with advanced ovarian cancer FIGO IIIc. Int J Oncol 13: 645 – 651
Winer J, Jung CK, Shackel I, Williams PM (1999) Development and validation of real-time quantitative reverse transcriptase-polymerase chain reaction for monitoring gene expression in cardiac myocytes in vitro. Anal Biochem 270: 41 – 49

REFERENCES
Yang H, Filipovic Z, Brown D, Breit SN, Vassilev LT (2003) Macrophage inhibitory cytokine-1: a novel biomarker for p53 pathway activation. *Mol Cancer Ther* 2: 1023–1029

Yuen T, Wurmbach E, Pfeffer RL, Ebersole BJ, Sealfon SC (2002) Accuracy and calibration of commercial oligonucleotide and custom cDNA microarrays. *Nucleic Acids Res* 30: e48

Zhang Y, Weiler-Guettler H, Chen J, Wilhelm O, Deng Y, Qiu F, Nakagawa K, Klevesath M, Wilhelm S, Bohrer H, Nakagawa M, Graeff H, Martin E, Stern DM, Rosenberg RD, Ziegler R, Nawroth PP (1998) Thrombomodulin modulates growth of tumor cells independent of its anticoagulant activity. *J Clin Invest* 101: 1301–1309