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Expression profiling via novel multiplex assay allows rapid assessment of gene regulation in defined signalling pathways

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ABSTRACT

The current interest in expression of groups of functionally related genes creates a demand for novel experimental tools. We describe a multiplex ligation-dependent amplification procedure (RT-MLPA), which accurately quantifies up to 45 transcripts of interest in a one-tube assay. The output, a set of fluorescent DNA fragments, is analysed via capillary sequencer and spreadsheet software. The procedure is highly sensitive and reproducible over a 100-fold range of input RNA, with excellent compatibility with RT-PCR and microarrays. We targeted two comprehensive sets of human genes: 35 apoptosis regulators and 30 genes involved in inflammation. Both probe sets accurately assessed specific changes in gene expression in two relevant model systems. Stimulation of lymphocytes with various Toll-like receptor (TLR) ligands induced distinct inflammatory profiles. Furthermore, osteosarcoma cells treated with cytostatic drugs showed as primary response strong up-regulation of the apoptotic p53-inducible PUMA transcript. Suppression by RNAi validated that indeed Puma expression was responsible for apoptosis induction. Thus, RT-MLPA enables relevant changes in transcription patterns to be quickly pinpointed and guide further experiments. This can be an advantage compared to hypothesis-free whole genome screens where large numbers of differentially expressed genes can obscure functional interpretation.

INTRODUCTION

At present, there is intense interest to monitor and interpret transcriptional changes in cellular biology. Changes in gene expression are at the core of development and differentiation, and play a decisive role in many pathological processes. Consequently, there is a great requirement to reliably monitor the ‘transcriptome’ as a whole, but also in a more restricted fashion by focusing on a set of genes involved in defined biological processes. The latter becomes especially attractive as knowledge on the human genome and the full complement of genes involved in defined pathways is on the verge of completion.

Nucleic acid-based analysis techniques to be introduced in both research and routine diagnostics should be simple to perform, sensitive and robust, and should preferably give quantitative results. Importantly, many applications require detection and quantification of less than 50 sequences, which makes a multiplex format attainable. The recently described Multiplex Ligation-dependent Probe Amplification (MLPA) method (1) fulfills these criteria and has already proved useful for analysis of human DNA sequences (2–4). MLPA permits the simultaneous PCR amplification of up to 45 different sequences with the use of a single primer pair. MLPA probes consist of two oligonucleotides that anneal to adjacent sites on a target sequence and are then ligated by a heat stable ligase. Each ligated probe gives rise to an amplification product of unique length. The procedure requires only a thermocycler and standard sequencing equipment and software for identification and relative quantification of the amplicons.

The principle of MLPA offers various advantages compared to current expression profiling techniques such as northern blotting, ‘real time’ PCR and microarrays. Here we describe application of a novel format of the MLPA technique, modified to quantify gene transcripts over a wide expression range. The new procedure was tested on mRNAs that regulate two defined biological processes. The first set of target genes plays a central role in the response of cells to inflammatory conditions. The second probe set is designed to comprehensively monitor genes involved in direct regulation of apoptosis. Both these target gene sets are intensely studied in fundamental and applied research. Basic conditions are defined for high throughput relative quantification of these mRNAs in total RNA samples derived from human cells. The two probe sets were able to reliably measure expression profiles and to detect strong induction of transcription in two relevant model systems. Specifically, the ligation of Toll-like...
receptors (TLRs) on peripheral blood lymphocytes by various TLR ligands could be demonstrated to result in distinct cytokine gene induction profiles. Secondly, profiling of osteosarcoma cells after cytostatic drug treatment pinpointed the p53-responsive PUMA transcript as a primary target among all apoptosis regulators monitored.

MATERIALS AND METHODS

Reagents and cells

Cell lines. Jurkat T cells and U2OS osteosarcoma cells were cultured in IMDM plus 10% fetal calf serum (FCS) supplemented with penicillin and streptomycin.

Healthy volunteers and patients. Blood was collected from the antecubital vein of healthy volunteers or from B cell chronic lymphocytic leukemia (B-CLL) patients using heparin (LEO Pharmaceutical, Weesp, The Netherlands; final concentration, 10 U/ml) or EDTA as anticoagulant. CD8+ T cells from peripheral blood and naive tonsil B cells were obtained as described (5,6). Human blood samples were obtained upon informed consent.

Whole blood stimulation. Stimulation of whole blood with ligands for TLR2, 3, 4, 7 or 9 was performed essentially as described previously (7). Heparinised whole blood (0.5 ml) was diluted with an equal volume of RPMI-1640 (GibcoBRL, Invitrogen, Breda, The Netherlands) or RPMI-1640 containing various stimuli and incubated in polypropylene tubes for 2 h at 37°C. Stimuli used were the TLR2 ligand LTA (10 µg/ml final, kindly provided by Dr T. Hartung, University of Konstanz, Germany), TLR3 ligand poly(I-C) (50 µg/ml final, Sigma, Zwijndrecht, The Netherlands), TLR4 ligand LPS (1 µg/ml final, E. coli LPS 0111:B4, Sigma), TLR7 ligand imiquimod (1 µM final, Sequoia Research Products Ltd, Oxford, UK), TLR9 ligand CpG-2006 oligodeoxynucleotides or control non-CpG-2006flip (10 µM final, Eurogentec, Seraing, Belgium). After incubation, cells were collected and processed for RNA isolation.

RNA isolation. Total nucleic acids were isolated from whole blood according to a solid-phase extraction method as described previously (8). Briefly, 100 µl whole blood was mixed with 900 µl lysis buffer [50 mM Tris–HCl (pH 6.4), 20 mM EDTA, 1.3% (wt/vol) Triton X-100, 5.25 M guanidine thiocyanate]. Next, 50 µl of activated silica suspension (1 µg/ml) was added to the lysis mixture. After washing and drying the silica, nucleic acid was eluted with 50 µl elution buffer and stored at −70°C. Alternatively, for T and B cell samples and cell lines, Trizol reagent (Gibco) was used to isolate total RNA according to the manufacturer’s instructions. Commercial RNA samples from prostate cells were obtained from Clontech (Erembodegem, Belgium).

MLPA probes and oligonucleotides

Each MLPA probe consists of one short synthetic oligonucleotide (Biologio, Malden, The Netherlands) and one phage M13 derived long probe oligonucleotide. Preparation of the M13 derived MLPA probe oligonucleotides has been described (1). All probes used in this study were designed to detect cDNA, and are described in detail in the Supplementary Material (Table 1). In order to avoid detection of contaminating DNA fragments, all target sequences have an exon boundary close to the probe ligation site. Splice variants present in the mRNA and EST databases were taken into consideration and where possible splice junctions within the coding sequence selected. For some genes, probes were included to detect multiple splice variants where this was seen to be functionally necessary. For each probe target sequence, a specific Reverse Transcription primer was designed that is complementary to the RNA sequence immediately downstream of the probe target sequence. Tm of all RT primers was between 55 and 60°C. Sequence information available from the public databases was used.

MLPA reaction

MLPA reactions were performed in 200 µl tubes in a thermocycler with a heated lid. Total RNA samples (2.5 µl of a 50 ng/µl solution) were mixed with Reverse Transcriptase (RT) buffer (final concentration 50 mM Tris–HCl pH 8.5; 75 mM KCl; 3 mM MgCl2, 10 mM DTT), dNTPs (1.25 nmol) and 500 fmol of each probe specific RT primer in a final volume of 5 µl. After heating to 80°C for 1 min and incubation at 45°C for 5 min, 30 U (0.5 µl) MMLV Reverse Transcriptase (Promega, Leiden, The Netherlands) was added and samples were incubated for 15 min at 37°C. After heating for 1 min at 98°C, 1.5 µl salt solution (1500 mM KCl, 300 mM Tris–HCl pH 8.5, 1 mM EDTA) mixed with 1.5 µl probe mix (4 fmol of each synthetic oligonucleotide and M13-derived oligonucleotide in TE) was added. Samples were heated for 1 min at 95°C and then incubated for 16 h at 60°C. Ligation of annealed oligonucleotides was performed at 54°C by diluting the samples to 40 µl with dilution buffer (2.6 mM MgCl2, 5 mM Tris–HCl pH 8.5; 0.013% non-ionic detergents) containing 0.2 mM NAD and 1 U Ligase-65 and incubation for 15 min. Ligase-65 is a Bacillus derived NAD-dependent DNA-Ligase, similar to Tth-Ligase and Taq-Ligase but with more favourable temperature-optimus and heat denaturation characteristics. Ligase-65 is a product of MRC-Holland, Amsterdam, The Netherlands. The ligase enzyme was inactivated by heating at 98°C for 5 min and ligation products were amplified by PCR. For most experiments, 10 µl of the ligation reaction was added to 30 µl PCR buffer. While at 60°C, 10 µl of a buffered solution containing the PCR primers (10 pmol), dNTPs (2.5 nmol) and 2.5 U Taq polymerase were added. PCR was for 33 cycles (30 s at 95°C; 30 s at 60°C and 1 min at 72°C). Samples amplified with one unlabelled and one FAM labelled primer were analysed on an Applied Biosystems 3100 capillary sequencer (Applied Biosystems, Warrington, UK). Samples amplified with one unlabelled and one D4-labelled primer (Research Genetics) were analysed using a Beckman CEQ20000 eight capillary electrophoresis system (Beckman Coulter, Mijdrecht, The Netherlands). The sequences of the labelled primer is 5'-GGGGTTCCCTTAAAGGGTTGGA-3' and that of the unlabelled primer is 5'-GGGGTTCCCTTAAAGGGTTGGA-3'. For situations where off-scale signals needed to be reduced to a level within the dynamic range of the capillary sequencer being used, competitor oligos were applied. A competitor oligo for a particular target consists of only the hybridising part of the synthetic short half-probe without the PCR primer binding.
site, and thus cannot be amplified in PCR. Where required, a range of competitor to probe ratios were tested and the amount of competitor that yielded signals within the dynamic range of the sequencer was then included in the mix. The targets compensated with competitors and the ratios used are supplied in Table 1 of the Supplementary Material.

**Data analysis**

After the PCR stage, aliquots of samples were mixed with GeneScan-500 ROX size standards and run on an ABI 3100 capillary sequencer in GeneScan mode. Data were analysed with GeneScan and Genotyper software packages (ABI), successively. Category tables containing the area for each assigned peak (scored in arbitrary units) were compiled in Genotyper and exported for further analysis with Microsoft Excel spreadsheet software. Alternatively, after the PCR stage, samples were mixed with D1-labelled CEQ DNA 600 size standards and analysed on a Beckman CEQ2000 in formamide. Data were analysed with the CEQ2000 fragment analysis software and directly exported and analysed with Microsoft Excel. Depending on the experiment, two slightly different ways of calculating data and representation of results were applied. In circumstances using unpurified cells such as whole blood stimulation, where the cell populations as well as expression pattern vary hugely, results from all target genes were calculated relative to the signal of β2-microglobulin. In the case of purified cell populations, there were minimal variations in expression of housekeeping genes during an experiment, and the sum of all peak data was set at 100% to correct for fluctuations in total signal between samples, and individual peaks were calculated relative to the 100% value. Both methods yield the same results qualitatively, but the first method corrects for changes in levels of housekeeping genes. For the experiment with TLR ligands which used whole blood stimulation and applied both probe sets, all data were first calculated relative to the β2M signal, and subsequently expressed as values relative to the signal in the untreated medium sample. Signals below the detection limit in medium were assigned a value corresponding to the threshold value for noise cut-off in GeneScan.

**Microarray, RT–PCR**

Custom made cDNA microarrays (Khuny, E., Reitsma, P.M. and Spek, C.A., manuscript in preparation) were probed with fluorescently labelled RNA mixtures derived from peripheral blood lymphocytes, according to standard procedures. Results were calculated using AIDA Array Metrix software (Raytest, Tilburg, The Netherlands). RT–PCR was performed using standard protocols, oligonucleotides used were, for GAPDH: sense GTGAAGGTCCAGTAGTCAACGC and antisense TGAAGGTCGGAGTCACCGTCAACGC. For A1/Bfl-1, sense ATGACAGACTGGAATTTGAG and antisense TCAACAGTAGTCTGTCAG. Scans of 1% agarose gels were analysed on a Lumi-Imager with Lumi-Analyst (Roche Diagnostics, Almere, The Netherlands).

**Transfection and RNAi**

U2OS cells were cultured in six-well plates and transfected by standard Ca2+-phosphate procedure using pcDNA3.1-eGFP as marker for transfected cells. Transfection efficiencies were between 30 and 50%. The pSuper plasmid was obtained from R. Agami. Puma sequences targeted were AGACAGGAATCCACGGCTT for Pumi1 and CGAGATGAGGCCCCAATTAG for Pumi3; these were incorporated in oligos encoding short hairpins and cloned in the pSuper vector as described (9). Cells were transfected with 1 μg of plasmid encoding eGFP plus 9 μg of pSuper, or a mixture of 4.5 μg pSuper-Pumi1 and 4.5 μg pSuper-Pumi3. Sixteen hours after transfection, medium was refreshed and fludarabine (300 μM) was added where required. Cells were incubated for another 24 h and analysed using a Leica DM IRBE microscope. Representative fields were digitally stored via Leica IM50 software.

**RESULTS**

**Principle and validation of RT-MLPA technique**

The recently described MLPA technique (1) is a high throughput, PCR based, method to determine the relative copy number of up to 45 DNA sequences in small samples of human DNA. It is based on the annealing of a mixture of hemi-probes on their cognate DNA sequences. One of the hemi-probes contains stuffer DNA of variable length (19–370 bp), and is generated after cloning in a series of specially created M13 vectors. Annealing of the two adjacent probes is followed by ligation at 54°C by a heat-stable ligase. All single-stranded DNA fragments thus obtained contain identical flags at their 5’- and 3’-ends. After amplification with a single fluorescent primer pair, samples containing mixed DNA fragments of 130–480 bp can be accurately quantified on a capillary sequencer. The principle of the technique is represented in Figure S1 in the Supplementary Material.

In order to enable sensitive detection of RNA transcripts, the original MLPA technique was modified by introducing an RT-step with gene-specific primers prior to the probe annealing stage. Since the NAD-requiring ligase used in MLPA is not capable of ligating DNA probes that are annealed to an RNA strand, the synthesis of cDNA was required. Hemi-probes were designed to span exon boundaries, precluding the detection of potential contaminating genomic DNA. Target genes for the inflammation and apoptosis probe sets are listed in Tables 1 and 2 (for exact sequence, location and length of the probes used in both probe sets, see Supplementary Material, Table 1). Further details are described elsewhere (1) and in Materials and Methods.

In order to reliably monitor changes in transcription of the target genes, two requirements must be met by a technique containing a PCR stage. First, the signal strength and ratios should not be influenced by the number of PCR cycles. Second, even large changes in expression of a certain gene should not influence the signals of the other probes. These requirements were met, as illustrated by the examples in Figure 1. Varying the number of PCR cycles from 28 to 40 did not significantly influence the signal ratios, both for high and low responses, while the total signal strength increased by a factor of 10. After 28 cycles, very low signals were not yet detectable, but these yielded a constant response from 30 cycles on. Importantly, from 34 to 40 cycles, the total signal hardly increased due to exhaustion of reagents (mostly primers), but during these additional cycles the signal ratios remained constant (Fig. 1A and B). Next, by titrating in an
unlabelled competitor of a high-response gene (BNIP3/NIP3), the relative signal was decreased from 12% to <0.5% of total. The competitor used was identical to the hybridizing part of the short probe oligonucleotide but lacked the PCR primer sequence. This large fluctuation in a single particular gene hardly influenced the other responses, as illustrated in Figure 1C (and by additional data described below). Thus, these results demonstrate that RT-MLPA is well suited to track changes in expression of the target genes. In the MLPA probe mixes used, we deliberately reduced the signal of some probes (listed in Table 1 of the Supplementary Material), such as the probe detecting the abundant beta-microglobulin transcript, up to 6-fold by the use of a mixture of short probe oligonucleotides and competitor oligonucleotide.

Reproducibility and compatibility with other techniques

Reliability of results was tested in two ways: by comparing dilutions of identical input samples and by independent duplicate samples. The results in Figure 2 are displayed as the response of the various target genes relative to total signal in the sample (apoptosis probe set), or calculated with reference to the value for the β2M signal (inflammation probe set). Correlation of the data sets of diluted with undiluted samples was excellent (P > 0.99 for 1:10 and 1:100-fold dilution), both for low (<0.5% of total signal) and highly expressed genes. Reproducibility of independent duplicate samples was also satisfactory; interassay correlation between three representative data sets of independent samples was 0.96 and intra-assay variation between four independent samples was 0.97.

The response of various genes in RT-MLPA was compared to the results obtained with cDNA microarrays and standard RT–PCR. Representative examples in Figure 3 illustrate that both high and low variations in response are faithfully detected. Blood samples obtained at timed intervals after administration of LPS to healthy volunteers (10) showed a >10-fold peak response of IL-1β and IkxBα at 2 h (Fig. 3A). Similarly, after antigen receptor triggering of Ramos Burkitt lymphoma cells, expression levels of the protective Bcl-2 family member A1 peaked at 4 h with a relative 2.2- to 2.6-fold increase (Fig. 3B), as detected by both RT–PCR and RT-MLPA. For comparison, non-changing expression levels of housekeeping genes are also shown. In summary,

<table>
<thead>
<tr>
<th>Table 1. Genes and categories for the inflammation probe set</th>
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<tbody>
<tr>
<td>Cytokines</td>
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<tr>
<td>IL-1α</td>
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<td>IL-1β</td>
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<td>IL-1Ra</td>
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<tr>
<td>IFNγ</td>
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<td>TNF-α</td>
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</table>

Genes targeted by MLPA assay in the inflammation probe set. Detailed information regarding probe sequence and location, gene name and amplification product length can be found in the Supplementary Material. Probes for TNFα, IL-10 and p65 did not yield a signal in samples obtained from peripheral blood, and are therefore not represented in the figures. The various housekeeping genes included in the probe mix are indicated in the right column.

<table>
<thead>
<tr>
<th>Table 2. Genes and categories for the apoptosis probe set</th>
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<tr>
<td>Bcl-2 family</td>
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<tr>
<td>-------------</td>
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<tr>
<td>A1/Bfl-1</td>
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<tr>
<td>Bcl-W</td>
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<tr>
<td>Bcl-XI</td>
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<tr>
<td>Bcl-2</td>
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<tr>
<td>Mcl1-long</td>
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Genes targeted by MLPA assay in the apoptosis probe set. Detailed information regarding probe sequence and location, gene name and amplification product length can be found in the Supplementary Material. Pro-apoptotic Bax is targeted by probes discriminating two splice variants (see www.ncbi.nlm.nih.gov/LocusLink). The transcript encoding Apaf-XL was proposed to have anti-apoptotic function (35) and is for that reason tentatively presented as such. Various probes were designed and tested for IAP2, but for unknown reasons, these never gave a significant signal. Signals for Bcl-W, Bcl-GS, Livin (also called ML-IAP) were generally very low and/or rare, in accord with their restricted expression. Housekeeping genes included in the apoptosis probe set are β2M, FTL and PARN.
RT-MLPA represents a simple and robust technique to monitor (changes in) gene expression.

Applications

The RT-MLPA probe sets presented here were designed to target two defined biological responses; inflammation and apoptosis. We assumed that specific expression profiles for the targeted genes in separate individuals or distinct cell types could be obtained. In addition, we postulated that the current knowledge with respect to the number of genes involved is sufficient to adequately monitor the most relevant changes in transcription patterns that may govern these processes. A crucial rationale behind the RT-MLPA technique is that it should provide, by relatively simple means, a clear and relevant basis for further experiments relating to the responses under investigation. These assumptions were tested by various approaches.

Profiles of patients and various cell types

In order to reliably compare (groups of) individuals or patients for response to a stimulus or drug in a desired setting, it is important that the expression profiles show equivalent baseline levels of target genes. This was validated for the inflammation probe set in RNA obtained from peripheral blood leucocytes of 16 healthy volunteers before undergoing experimental low grade endotoxemia. The variation in expression levels observed was generally <20% of the total signal for a particular gene, either for low- and highly expressed genes (Fig. S2 in the Supplementary Material).

Conversely, valuable information concerning signalling pathways operating in distinct cell types can be obtained by profiling, especially if the expression patterns are typical for that cell type. To test whether such signature profiles can be obtained by MLPA, we compared naive tonsil B cells and naive CD8 T cells from several donors using the apoptosis probe set. As representatives of malignant B and T cells that may have disturbed responses to apoptotic signals, we included ex vivo samples of chronic lymphocytic leukemia (B-CLL) and Jurkat T cell lines maintained in three separate laboratories. The results are summarised in Figure S3 in the Supplementary Material, and clearly show that indeed a particular cell type displays a specific profile. For example,
already within the group of pro-apoptotic BH3-only members of the Bcl-2 family, striking differences were observed, yielding interesting clues about the apoptotic mechanisms that can be active. For more details, see legend to Figure S3 in the Supplementary Material.

**Induction of inflammation or apoptosis**

In order to utilise RT-MLPA to accurately determine alterations in gene expression during an inflammatory response, we analysed mRNA expression in unfractonated PBLs stimulated ex vivo with ligands of several innate immunity receptors of the TLR family, known to induce differential gene expression. Incubation of whole blood for 2 h with LTA, pIC, LPS, imiquimod or CpG, which are ligands for TLR2, 3, 4, 7 and 9, respectively (11), triggered the expression of a restricted set of inflammatory and apoptosis regulatory genes. Induction of expression ranging from 10- to 200-fold was observed for IL-1β, IL-6 and TF (Fig. 4A), as well as MIP-1α and MCP-1 (data not shown) after stimulation with ligands for TLR2, 3, 4 and 9, but not TLR7 and 9. The distinct gene expression profiles may well be explained by the differential TLR expression on subsets of PBL and diversity in TLR signalling pathways. The TLRs for LTA and LPS are present on monocytes and PMNs, whereas the TLRs targeted by imiquimod and CpG are present on circulating B cells and plasmacytoid dendritic cells (12-14). Furthermore, TLR2, 7 and 9 signalling induces NFκB and p38 MAPK activation, whereas TLR4 signalling induces also IFN-β-inducible genes (11,15). An unexpected observation was that pIC induced a gene profile that closely mimicked the profiles induced by LTA and LPS (including the induction of monocyte-specific Tissue Factor). Although it was previously shown that peripheral blood mononuclear cells are pIC responsive (16), the expression of TLR3 in PBL is still controversial (12,13,17,18). Moreover, the signalling pathway downstream of TLR3 is different from that of TLR2 and 4 (19). Our data, however, imply that pIC acts on unfractonated PBL in a similar manner to LTA or LPS.

Besides the induction of inflammatory genes, altered expression of a number of apoptosis regulatory genes was found in PBL after treatment with ligands for TLR2, 3 or 4 (Fig. 4B). The most notable effect was found for the granzyme B inhibitor PI-9, which was strongly up-regulated by LTA, pIC and LPS, confirming and extending recent findings that PI-9 is induced in liver cells by LPS under control of NFκB and AP-1 (20). Other genes induced by the same stimuli were Bid and IAP1, but in contrast transcripts for Puma and Apaf were suppressed approximately 5-fold (not shown). Taken together, these data indicate that the RT-MLPA method efficiently detects changes in expression of major effectors during inflammatory processes, and is well suited to monitor differential patterns of gene induction and repression.

To monitor gene induction during apoptosis, we utilised U2OS osteosarcoma cells treated with various cytotoxic agents. These cells are a well-known model system and have an intact p53 response (21,22). After treatment with the DNA-modifying prodrug fludarabine, the topoisomerase inhibitor etoposide or RNA-synthesis inhibitor actinomycin D, expression of apoptotic regulators was determined via RT-MLPA. The results in Figure 5 show a distinct and prominent
induction of the transcript encoding the BH3-only protein Puma. This apoptosis inducer was characterised recently based on its early upregulation upon p53 activation (21,23), and was shown to trigger cytochrome c release from mitochondria. Additional genes reported to be p53-reactive such as Bax and Noxa (24) were generally induced to a much lower extent, in good agreement with a very recent report on the p53 response in colorectal cancer cells (25). Certain genes were clearly down-regulated in response to all three agents, such as the IAP family members Survivin and Apollon (data not shown). The obvious and testable conclusion from this experiment is that after cytostatic drug treatment in these cells, upregulation via p53 of PUMA is the prime inducer of apoptosis. This was verified by an RNAi approach to specifically block PUMA expression. U2OS were transfected with empty pSuper vector (9) or two constructs targeting the Puma transcript, and subsequently treated with fludarabine for 24 h (Fig. 6). The panels show, respectively, healthy adherent cells transfected with GFP plasmid only (left), non-adherent dead cells in the presence of fludarabine (middle), and again predominantly healthy cells by combining Puma RNAi with drug treatment. Clearly, suppression of Puma expression resulted in increased survival of cells in the presence of the drug. This approach thus quickly yields insight into the most relevant biological responses of malignant cells to cytostatic drugs.

DISCUSSION

We describe and validate a novel technique to perform easy and reliable expression profiling of concise sets of genes. Relevant example sets presented here concern two crucial biological processes: inflammation and apoptosis. RT-MLPA has important advantages over current techniques such as northern blotting, real-time PCR, RNase protection assays and even large scale microarray approaches. Numerous samples can be quickly processed in a standard PCR 96-well format, which can be easily adapted for automation. Analysis is straightforward and yields quantitative information on a medium-sized gene number. These are obvious improvements compared to northern blotting, real-time PCR and RNase protection assays, which are either quite laborious, less sensitive and/or more limited in scope. In addition, these techniques may require cumbersome radioactive techniques for maximum sensitivity. Another method based on ligation-dependent PCR (26) is the recently described RASL technique (27), which also affords highly sensitive multiplex expression analysis using total RNA as input. The reported RASL procedure focuses on alternatively spliced transcripts and used oligos linked to randomly distributed glassbeads on fiber-optic arrays. Bead identity is subsequently assigned by serial hybridisation prior to the actual experiment. After the annealing step, the RASL technique includes poly(A)+ selection in order to remove the excess of non-hybridised probes, and requires special equipment for the experiment and analysis. In comparison, for RT-MLPA only specialised synthesis of the long hemi-probes in M13 vectors is applied (1). Once probe mixes are available, it requires fewer experimental steps and allows routine use with large numbers of samples, using equipment present in most laboratories.

Because a predefined set of genes is targeted by MLPA instead of the whole genome, a trade-off is made between scope of the analysis and ease of interpretation. Although microarray techniques potentially sample the whole transcriptome, in practice functional interpretation is limited when hundreds of genes are differentially expressed, which is often the case (28,29). In many instances, lists of gene products identified by microarray analysis cover every aspect of cellular biochemistry and function. In addition, the time consuming design and fabrication of large scale solid phase microarrays may preclude incorporation of novel genes, while with RT-MLPA new probes can be quickly included in an existing probe mix should this need arise. To illustrate this point, of the apoptosis regulators represented in the current probe mix, a significant number (9-11, i.e. 28-34%) is lacking on two microarrays we have investigated (Netherlands Cancer Institute www.microraars.nki.nl, and lymphochip www.llmpp.nih.gov). Most prominent among these was PUMA, which we could pinpoint with RT-MLPA as the prime apoptosis inducer in a model of cytostatic drug treatment. In addition, both microarrays lacked representation of Bcl-rambo, Bcl-GS, Apollon, Livin, Map-1 and Smac-Diablo, and various others were absent from a single array.

Figure 5. Induction of apoptosis in U2OS cells by cytostatic regimen. Osteosarcoma cells (U2OS) were stimulated with actinomycin D, etoposide or fludarabine for 24 h. RT-MLPA was performed with the apoptosis probe set, and results are expressed relative to untreated cells (set as 1). Only subsets of data of BH3-only proteins containing the most relevant changes in expression are depicted. The signals of the remaining probes displayed less than 2-fold changes upon the various stimuli. Results and standard deviations of three separate experiments are shown.

Figure 6. Suppression of Puma expression prevents drug-induced apoptosis. U2OS cells were transiently transfected with vectors encoding eGFP and empty pSuper RNAi vectors, or together with two RNAi constructs targeting the Puma transcript. The left panel depicts untreated eGFP-expressing cells, middle panels show cells treated with fludarabine, and the right panel shows the combination of fludarabine and Puma RNAi. Compared to the right and left panels, the middle panel show typical signs of apoptosis; cells are rounded off and detached from the well (and therefore sometimes out of focus). Data are shown from one experiment of two.
Curiously, omissions were not limited to novel or obscure genes, suggesting that completeness within a specified area is difficult to achieve with unbiased large scale set-ups.

Using RT-MLPA, nanogram quantities of total input RNA can yield reliable quantitative information (Fig. 2). This makes possible direct analysis of minute samples obtained from, for example, cell sorting or needle biopsies, without the need for prior amplification. Although amplification strategies improve RNA yield to levels that allow labelling and detection by microarray, this still requires substantially more (≥50-fold) starting material than RT-MLPA. Furthermore, although amplification may preserve general overall representation, a certain distortion is inevitable and especially low abundance transcripts may be lost (30). Also, solid phase hybridization techniques with cDNA microarrays often cannot distinguish highly homologous genes, while with MLPA such genes can be targeted by specific probes designed to discriminate such sequences. Thus, for various current purposes, the sensitivity and the focus on an entire set of genes provided by MLPA may in fact be superior to microarray techniques.

The unbiased representation of genomes by microarrays is, however, highly useful in settings where starting from (pathological) tissue samples a ‘signature-set’ can be derived that is characteristic for a specific disease or that allows finer classification and diagnosis (28,31–34). A promising application for MLPA is to apply this knowledge of signatures to design (combinations of) disease-specific probe sets. This would enable quick and large scale use of the information gathered from an unbiased sampling of expression profiles, thereby combining the specific advantages of both techniques.

The RT-MLPA procedure proved sensitive, consistent and highly concordant with RT–PCR and microarray results over a wide range of input RNA concentrations. The highest induction observed in the experiments described here was over 100-fold. Obviously, such large increases will only be observed when basal levels are relatively low. Near-maximal responses in fluorescent read-out in both MLPA and micro-arrays are limited in dynamic range. In addition, highly represented mRNAs (yielding strong signals) are unlikely to be subject to strong induction and/or regulation. Nevertheless, by including non-amplifiable competitor oligos in an MLPA probe-mix, high signals can be attenuated to a desired level, as exemplified here in Figure 1C. Ongoing studies will guide final adaptations of the existing initial probe mixes presented here regarding competitor-oligos and inclusion of novel target genes.

In conclusion, RT-MLPA provides a valuable contribution to expression profiling. Application of RT-MLPA to monitor responses of malignant cells to cytostatic drugs successfully pinpointed Puma as a prime inducer of apoptosis. The technique is well suited for high-throughput automated applications, such as delineation of gene expression in large case-control studies of anti-inflammatory drugs. A second promising application is the assembly of potentially discriminating profiles in responders versus non-responders to chemotherapy in cancer treatment.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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