The use of mRNA differential display for discovery of novel therapeutic targets in cardiovascular disease

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Abstract

Recent advances in molecular biology techniques have provided powerful tools for discovery of novel genes relevant to both biological and pathological processes. mRNA differential display is an emerging technique for novel gene discovery and it has been successfully applied to many physiological and pathological conditions including normal development, cell differentiation, cancer, cardiovascular disease, inflammation and CNS disorders. In the present work, we briefly illustrate the critical procedure and highlight most recent technical improvements and modifications of this technology. Based upon the successful applications of this technique in cardiovascular research, it may provide a valuable and powerful investigational tool for the identification of novel therapeutic targets in cardiovascular diseases. © 1997 Elsevier Science B.V.

Keywords: Cardiovascular disease; Differential display; Gene expression; Therapeutic target

1. Introduction

Differential gene expression is essential for normal development and pathological processes. Therefore, discovery of differentially expressed genes is critical for the understanding of the molecular mechanisms involved in normal and pathological states, as well as providing new insights for discovery of novel molecular targets for pharmacological manipulation and gene therapy. A number of techniques including subtractive library screening, differential hybridization, representational difference analysis (RDA), mRNA differential display, serial analysis of gene expression (SAGE) and electronic subtraction have been developed for novel gene discovery to date. Subtractive library screening and differential hybridization are conventional methods [1] for the identification of molecules that differ in abundance between two pools of molecules. RDA [2,3] and mRNA differential display [4] are polymerase chain reaction (PCR)-based techniques and are sufficiently sensitive for the detection of differentially expressed genes. SAGE is a technique that allows a rapid and detailed analysis of thousands of small expressed sequence tags (ESTs) [5,6]. Electronic subtraction is the method comparing ESTs from experimental and control cDNA libraries [7–9], that also facilitates the identification of the abundant messages since the ESTs from a particular library only represent a small fraction of the total number of differentially expressed genes. Table 1 summarizes some basic features of these techniques for their utilization in novel gene discovery. In the last decade or so, these various techniques have been widely used to identify differentially expressed genes in many biological systems [10]. In particular, the mRNA differential display methodology has been adopted by a large number of laboratories as an important tool being applied for both in vitro and in vivo systems [11]. Detailed protocols for mRNA differential display have been described previously [12,13]. In the present article, we briefly outline the critical procedure of the mRNA differential display technique and its technical improvements and modifications, and summarize its suc-
<table>
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<tr>
<th>Key technique</th>
<th>Advantages</th>
<th>Disadvantages</th>
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<tr>
<td>Subtractive library</td>
<td>Hybridization-based subtraction to identify differentially expressed genes</td>
<td>Highly reproducible</td>
</tr>
<tr>
<td>Electronic subtraction</td>
<td>Computer-based ESTs subtraction to identify differentially expressed genes</td>
<td>Rapidity if ESTs available; know the sequence identity</td>
</tr>
<tr>
<td>SAGE</td>
<td>Comprehensive analysis for multiple short nucleotide sequence tags (9–10 bases)</td>
<td>Provide a rapid, detailed analysis of thousands of transcripts</td>
</tr>
<tr>
<td>RDA</td>
<td>Subtraction coupled to PCR amplification to identify differentially expressed genes</td>
<td>Very sensitive</td>
</tr>
<tr>
<td>Differential display expressed genes</td>
<td>Modified RT-PCR for the identification of differentially expressed genes</td>
<td>Sensitive; allows multiple, side-by-side comparison of both up- and down-regulated genes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>High incidence of false positives; many ESTs are 3’-UTR</td>
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The method of mRNA differential display consists of two basic steps (Fig. 2): (1) reverse transcription using a set of 3'-anchored primers, such as T$_{12}$MN [4] where M = G, A, C and N = G, A, T, or C, and (2) PCR amplification of cDNA fragments using arbitrary (upstream) primers and anchored (downstream) primers. For the RT reaction, total cellular RNA (usually DNase-treated to eliminate the possibility of genomic DNA contamination) is reverse-transcribed to yield the first strand cDNA primed with T$_{12}$MN oligonucleotides. This RT reaction enables all the mRNA species having a poly(A) tail to be reverse-transcribed. Typically, this reverse transcription reaction is divided into 4 subgroups each using a different T$_{12}$MN primer with G, A, T or C at the last base of the 3'-end. Because a large number of mRNA species are present in a cell, the division of subgroups for the RT allows a portion of the mRNA species to be displayed, which will increase the resolution of cDNA species after amplification [4].

Amplification of all the cDNAs is carried out using an upstream arbitrary primer and a downstream anchored primer (identical to the one used for the RT) in the presence of a radioactive nucleotide ($^{32}$P-α-dATP or $^{35}$S-α-dATP). The upstream primer has been optimized to 10 bases in length composed of approximately 50% of G or C base contents [4]. In addition, a relatively low annealing temperature (42°C) is applied in the PCR so that the number of the amplified mRNA species is maximized. Using these conditions of amplification, it has been esti-
Table 2
Technical improvements/ modifications of mRNA differential display

<table>
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<tr>
<th>Key features/ issues in the initial protocol</th>
<th>Technical improvement/ modifications and advantages</th>
<th>References</th>
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<tbody>
<tr>
<td>Primer components</td>
<td>Two base-anchored 3' primers, e.g., T12 M, were used to reduce the number of primers for reactions</td>
<td>[14]</td>
</tr>
<tr>
<td>Primer length</td>
<td>Short up- and down-stream primers</td>
<td>[15–17]</td>
</tr>
<tr>
<td>Radioisotope</td>
<td>(13S)α-dATP used for PCR labeling could generate hazardous decomposition products</td>
<td>[18–21]</td>
</tr>
<tr>
<td>False positive</td>
<td>High incidence of false positive</td>
<td>[12,15,16,22]</td>
</tr>
<tr>
<td>Biased amplification</td>
<td>Biased amplification towards high copy number mRNA</td>
<td>[23,24]</td>
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To reduce the false positive, it is recommended to use DNase-treated RNA, apply multiple samples, and use longer primers for two-step PCR at higher annealing temperatures.

Use selected longer primers for PCR and apply quantitative RT-PCR for confirming studies.
made on the primers: a a single base anchored primer rat aortic artery stimulated with LPS. mRNA differential display was diagram of differential display products for RNA isolated from cultured play technique 4, a number of modifications have been noted for their differential expression in response to LPS resolved by electrophoresis Fig. 3B. Several bands were LPS-stimulated and unstimulated rat carotid artery using a thesis was carried out using cellular RNAs isolated from different display analy-sis was carried out using cellular RNAs isolated from cultured rat aortic artery stimulated with LPS. mRNA differential display was carried out using an upstream primer (5′-GACCGCTTGT-3′) in combination with downstream primers (T12MA, M = G, A or C) in the presence of [3P]ATP, and the PCR products were resolved in an 8 M urea/6% polyacrylamide DNA sequencing gel in the following order: lane 1, unstimulated and lane 2, stimulated carotid artery from spontaneously hypertensive rats, lane 3, unstimulated and lane 4, stimulated carotid artery from Wistar-Kyoto rats. The band(s) indicated with an arrowhead shows a marked induction in response to LPS stimulation.

Schematic illustration of the differential display PCR products after gel electrophoresis Fig. 3. Gel electrophoresis of differential display PCR products. A: 5 arbitrary primer 5'-GACCGCTTGT-3' and a 3' MA 12 s

Since the initial description of mRNA differential display technique [4], a number of modifications have been made on the primers: (a) a single base anchored primer was described [14] that required less primers to generate similar amplification results; (b) a primer with an extended restriction enzyme site was used to facilitate subcloning and direct sequencing of the PCR products [27]; (c) longer primers (18–20 mers) for RT and PCR, in combination with two step PCR amplification (the first several cycles done at low annealing temperatures and the subsequent cycles at higher annealing temperatures that improved the fidelity of the amplification and reduced the incidence of false positive [15,16,22]). Additional modification has been made to overcome a strong bias towards high copy number RNAs [23], using selected primers that preferentially isolate RNAs of moderate to low abundance [24].

2.2. Band recovery and confirmation of the differentially expressed genes

Following mRNA differential display, the bands of interest may be recovered by applying the following steps: the DNA band is excised from the dried sequencing gel, isolated by extraction procedures, and reamplified using the same sets of primers as in the original PCR [4]. The recovered DNA band can serve as a probe to confirm mRNA expression by means of Northern blot analysis, and/or be subcloned into a vector for further analysis.

Confirmation of gene expression is one of the critical steps following mRNA differential display, in as much as a large number of false positive bands may be present on differential display. A variety of methods have been utilized in different laboratories to reduce false positives, of which the most commonly used method is Northern blot analysis. In addition, dot blot, quantitative RT/PCR, ribonuclease protection assays and other methods have also been used.

2.3. Sequence analysis of the differentially expressed genes

Sequencing of the differentially expressed cDNA is a logical next step. This step relies on DNA sequencing analysis of the recovered DNA band. Because multiple DNA species may be present in this recovered band and the differential display primers are too small to be used successfully for direct sequencing using standard protocols, the differential displayed DNA fragments are typically subcloned into a vector prior to sequencing analysis [4,28]. Recent modifications using elongated primers for direct differential display [15,16] or the reamplification following the original differential display method [27] allow direct sequencing analysis of the PCR products if only a single DNA species is present in a particular band. Based upon the sequence information, the identity of the differentially expressed genes can be determined by searching a computer database, such as GenBank. If it represents a novel gene, a cDNA library can be screened using this DNA probe in order to obtain the full length cDNA clone.
<table>
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<tr>
<th>Genes</th>
<th>RNA sources</th>
<th>Potential functions</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multiple genes</td>
<td>Bovine retinal pericyte cultures, glucose stimulated</td>
<td>The multiple glucose regulated genes may contribute to the effects of hyperglycemia on vascular cells</td>
<td>[31]</td>
</tr>
<tr>
<td>Elongation factor 2 and other genes</td>
<td>Bovine smooth muscle cells, glucose stimulated</td>
<td>These genes may related to glucose metabolism</td>
<td>[32]</td>
</tr>
<tr>
<td>Multiple genes (novel genes and cell-surface receptor)</td>
<td>Transplanted hearts</td>
<td>These genes may be associated with chronic cardiac rejection</td>
<td>[33]</td>
</tr>
<tr>
<td>Allograft inflammatory factor-1</td>
<td>Transplanted hearts</td>
<td>Upregulation of this gene may play a role in macrophage activation and function</td>
<td>[34]</td>
</tr>
<tr>
<td>IP-10</td>
<td>Cultured carotid artery stimulated with LPS</td>
<td>Induces smooth muscle cell proliferation and migration</td>
<td>[26]</td>
</tr>
<tr>
<td>Carnitine palmitoyltransferase I (CPT I)</td>
<td>Carnitine-deficient heart</td>
<td>Increased expression of CPT I gene was associated with carnitine deficiency</td>
<td>[35]</td>
</tr>
<tr>
<td>AdipoQ</td>
<td>Adipocytes and their differentiation</td>
<td>AdipoQ may function as a novel signaling molecule for adipose tissue</td>
<td>[36]</td>
</tr>
<tr>
<td>Nucleophosmin</td>
<td>Estrogen-stimulated vascular smooth muscle cells</td>
<td>May be associated with estrogen-regulated function</td>
<td>[37]</td>
</tr>
<tr>
<td>Immunoglobulin J chain and other genes</td>
<td>Aortic transplant</td>
<td>May be related to graft vasculopathy</td>
<td>[38]</td>
</tr>
<tr>
<td>Titin and other cytoskeletal proteins</td>
<td>Left ventricles (LV)</td>
<td>The differential regulation of the major cytoskeletal proteins may be related to LV pressure overload hypertrophy and failure</td>
<td>[39]</td>
</tr>
<tr>
<td>BART-1</td>
<td>Carotid artery after balloon angioplasty</td>
<td>The upregulation of BART-1 may be related to vascular trauma</td>
<td>[40]</td>
</tr>
<tr>
<td>Cyclooxygenase-2, manganese superoxide dismutase, and endothelial cell nitric oxide synthase</td>
<td>Human vascular endothelial cells in response to shear stress</td>
<td>These genes with potential atheroprotective activity may be associated with shear stress and atherosclerosis</td>
<td>[41]</td>
</tr>
<tr>
<td>Jagged ligand for the Notch receptor</td>
<td>Human endothelial cells exposed to fibrin</td>
<td>Jagged-Notch signaling may involve endothelial migration in vitro and angiogenesis in vivo</td>
<td>[42]</td>
</tr>
<tr>
<td>GRP78/Bip and novel genes</td>
<td>Vascular endothelial cells treated with homocysteine</td>
<td>Homocysteine can upregulate stress protein and other novel genes, which may contribute to atherogenesis</td>
<td>[43]</td>
</tr>
<tr>
<td>Aldose reductase</td>
<td>Vascular smooth muscle cells stimulated with hydrogen peroxide</td>
<td>Oxidative stress response</td>
<td>[44]</td>
</tr>
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</table>
It should also be noted that the cDNA fragments isolated by this method are in general small, and frequently located in the 3'-untranslated region (UTR). Therefore, in order to determine the identity of the differentially expressed gene, one may often require to isolate the full length cDNA clone.

2.4. Functional analysis

The identification of the biological functions for the differentially expressed genes is one of the most challenging tasks. The gene may encode a secreted, cytoplasmic, or nuclear protein. To predict its function, one should first isolate the full length cDNA and predict the peptide sequence, and then explore the functional aspects based upon the encoding peptide. For example, the gene identified in Fig. 3B by differential display was further pursued for full length cloning and found to be a rat homologue of interferon inducible protein 10 (IP-10), a C–X–C chemokine [26]. Based upon structural and functional similarities of IP-10 to other cytokines and chemokines such as IL-1β and IL-8 (which have been demonstrated to involve SMC proliferation and migration) [29,30], we hypothesized that IP-10 may have a role in vascular smooth muscle cell biology. IP-10 was then demonstrated to be a potent mitogen and chemotactic factor for vascular smooth muscle cells, two features of smooth muscle cell phenotypes that are believed to contribute to the pathogenesis of atherosclerosis and neoointima formation [26].

3. Applications of mRNA differential display in cardiovascular research

Despite the relative advantages and disadvantages of mRNA differential display, it has been demonstrated as a powerful tool for novel gene discovery and investigation of the mechanisms involved in many biological and pathological functions. A number of laboratories have successfully applied this technique in cardiovascular research, including in vitro and in vivo studies which are summarized in Table 3.

4. Summary

mRNA differential display is one of the most flexible and comprehensive methods available for the detection of differentially expressed genes in cells and tissues. Since its initial description, this technique has been established in many laboratories and successfully applied for the identification of genes in vitro and vivo systems.

It also should be pointed out that similar to differential display, other methods, such as subtractive library screening, differential hybridization, electronic subtraction, SAGE and RDA, have also been successfully used for novel gene discovery. The application of these techniques will no doubt facilitate the discovery of novel therapeutic targets and help to understand the molecular mechanisms of disease. On the other hand, however, it should be pointed out that this is the first of many steps required in discovery of a novel therapeutic target especially as the function of the novel gene is likely to be unknown. Therefore, further actions should be taken to characterize the functions of the differentially expressed gene, including isolation of the full length cDNA, expression of the gene product for functional studies and target validation for the importance of this gene in disease processes.

References


