CMLS, Cell. Mol. Life Sci. 59 (2002) 1274–1279
1420-682X/02/081274-06
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CMLS Cellular and Molecular Life Sciences

Differential display analysis of gene expression in mammals: a p53 story

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Abstract. Differential display is used worldwide as a method to identify changes in gene expression and to discover novel genes that are involved in important biological pathways. The principle of differential display is the systematic amplification of the 3′ termini of messenger RNAs by using anchored oligo-dT primers in combination with upstream arbitrary primers. The separation of the polymerase chain reaction products by gel electrophoresis and their direct comparison allows the identification of differentially regulated genes. Recently, fluorescent differential display was established as the first nonradioactive differential display system with equivalent sensitivity to originally ^{33}P isotopic labeling method. Because of its simplicity, sensitivity, reproducibility and automation, which increase the throughput and accuracy, differential display has become one of the most widely used gene-screening methods in biomedical research involving mammals. This chapter provides a glimpse of the application of differential display in search of target genes of the p53 tumor suppressor gene.

Key words. Differential gene expression; fluorescent differential display; mRNA differential display; p53 target genes; tumor suppressor gene p53.

Introduction

The original ^{33}P-based differential display (DD) methodology was invented 10 years ago [1, 2]. This has been the most commonly used DD technology because of its sensitivity, simplicity, versatility and reproducibility. Since its description, numerous differentially expressed genes have been successfully identified in diverse biological fields, particularly in mammalian systems. Recently, a very similar sensitive DD technique was established by fluorescent labeling [3]. This was the first nonradioactive DD system with equivalent sensitivity to that of the ^{33}P isotopic labeling method [4, 5]. Fluorescent labeling is optimal for automation, which can greatly increase the throughput and accuracy of DD.

The general strategy for fluorescence differential display (FDD), which is very similar to the traditional radioactive DD, is outlined in figure 1. In brief, the first essential step before carrying out DD is the removal of all chromosomal DNA from the isolated total RNA or messenger RNA (mRNA) from two or more cell cultures and tissues. The principle of the DD method is to detect different gene expression patterns by performing simple reverse-transcription (RT) PCR reactions [6]. RT uses one of three individual one-base anchored oligo-dT primers. The following FDD-PCR reaction uses fluorescent-labeled anchored oligo-dT primers in combination with one of the various arbitrary primers. The resulting products are separated on a denaturing polyacrylamide gel [4]. By using a fluorescent laser scanner, the pattern of bands will be visible. Side-by-side comparisons of complementary DNA patterns between or among relevant RNA samples would reveal differences in gene expression. The cDNA fragments of interest can be retrieved from the gel, purified and reamplified. For further molecular studies, the obtained unlabeled reamplified PCR fragments can be cloned or directly sequenced. DNA sequence analysis of these cDNA fragments by a Blast Search of GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) may provide information whether a gene identified by DD is a known, homologous to known, or a novel gene. The general final step of the DD procedure is to confirm the differential expression of the obtained

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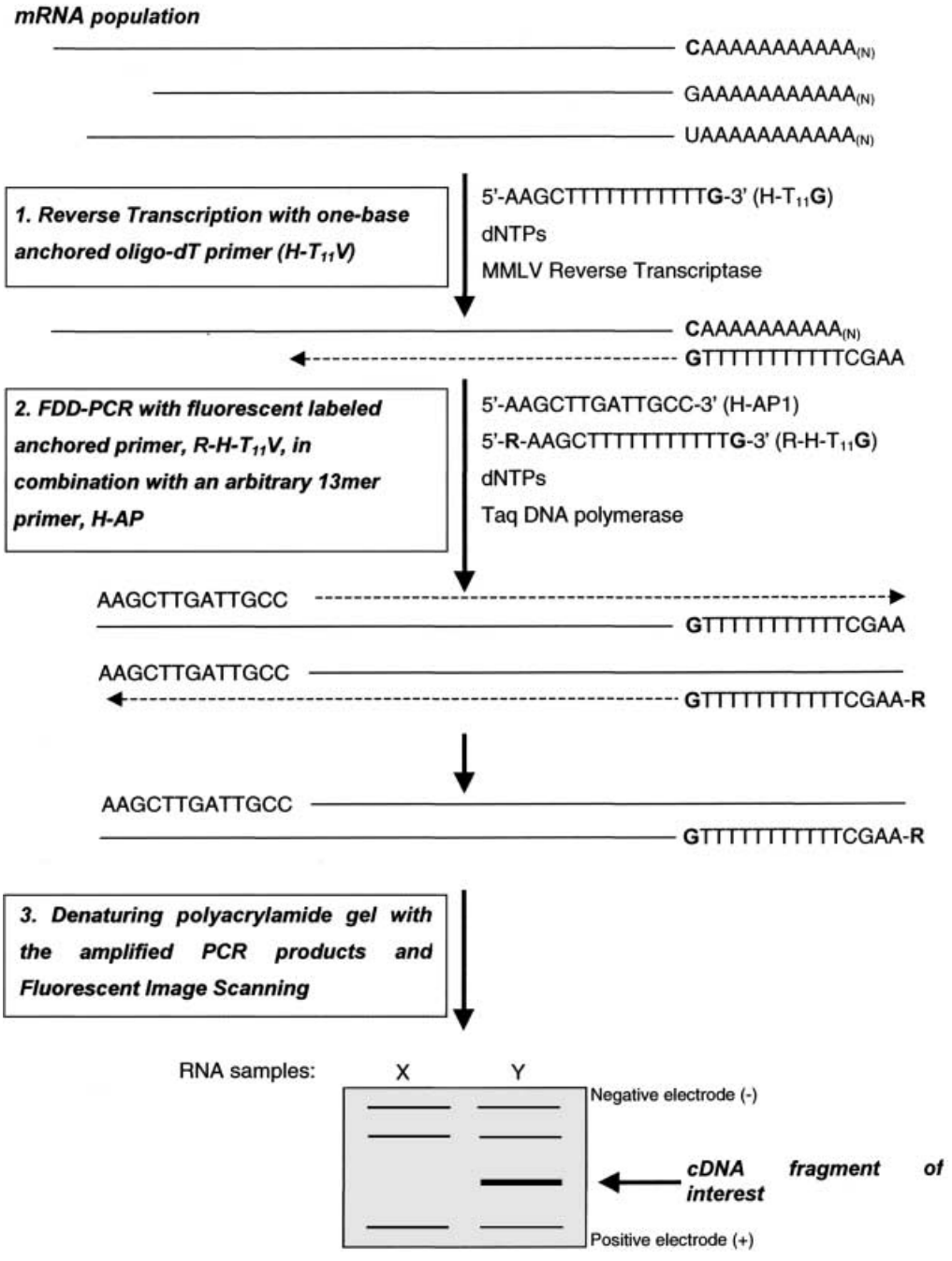

Figure 1. Representation of FDD method. After isolation of total RNA or mRNA from cell cultures or tissues, the following RT reaction used one of the three specific one-based anchored oligo-dT primers (H-T_{11}V; V may be dA, dC, dG). The FDD reaction is performed by using the corresponding fluorescent-labeled anchored oligo-dT primers (R-H-T_{11}V; R, rhodamine labeling) in different combinations with arbitrary 13mer primers. The amplified PCR products are separated on a denaturing polyacrylamide gel. The fluorescent-labeled cDNA fragments are visible by using a fluorescence scanner.

partial cDNAs (e.g. by Northern blotting). The advantage of this sensitive analysis provides not only confirmation by a method independent of DD but also information about the size of the gene. After confirmation, the cloned cDNA probe can be used to screen a cDNA library for a full-length clone, which is helpful for characterizing the function of the gene.

In this chapter we focus on the use of the FDD procedure to identify p53 target genes. p53 is a tumor suppressor whose expression is dramatically induced by a variety of cellular stresses such as heat shock, hypoxia, osmotic shock and DNA damage; this, in turn, leads to growth arrest and apoptosis (programmed cell death). Apoptosis is likely to be the most important function of p53 in sup-

pressing tumor formation. However, the mechanism by which p53 actually induces apoptosis remains to be determined. Recently, several p53 target genes were reported, which appear to contribute to p53-dependent apoptosis pathways [e.g. 7–14]. Remarkably, many of these p53 target genes were found by the DD method. However, the identification of additional, if not all, p53 target genes remains of great importance; these genes could provide the missing link between p53-mediated apoptosis and tumor suppression.

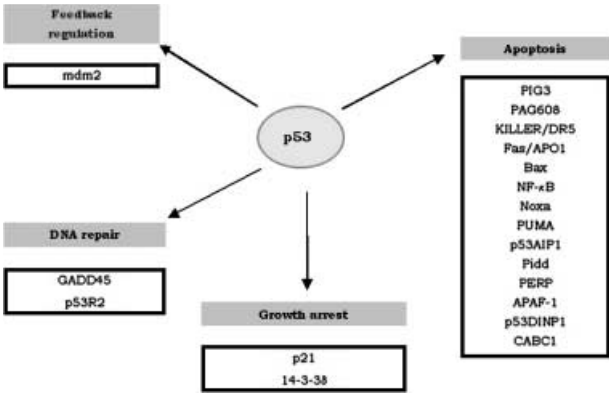

Figure 2. Multiple roles of the tumor suppressor gene p53.

The p53 tumor suppressor

Tumor suppressor genes are needed to keep cell growth under control [15]. In the late 1970s, the first tumor suppressor gene, p53, was described. Wild-type (wt) p53 protects cells from malignant transformation and is mutated or deleted more often than any other known gene in human cancer (uncontrolled cell proliferation) [16]. The fact that the loss of p53 function or disruption of the p53 pathway plays a critical role in the development of most tumor types implicates this gene in a major role in suppressing tumor formation. The human p53 gene encodes a 393-amino acid tetrameric polypeptide. p53 can be divided into three distinct functional domains based on numerous structural analyses: (i) an acidic N-terminal transactivation domain, (ii) a central DNA binding domain and (iii) a basic C-terminal oligomerization domain [17]. p53 is a transcription factor that can mediate many downstream pathways by the activation or repression of target genes (fig. 2) [18]. Levels of the wt p53 protein are very low in normal, nonstressed cells; however, p53 is activated and stabilized by a variety of intracellular and extracellular stresses, including DNA-damaging agents (such as ultraviolet light, γ-irradiation, chemotherapeutic drugs) [17]. In response to these events, which lead to numerous subsequent posttranslational modifications of the wt p53 protein, p53 induces cell cycle arrest or apoptosis. In contrast to p53-mediated cell cycle arrest, the mechanism of p53-induced apoptosis appears to be the most important component of p53-related tumor suppression. Moreover, the therapeutic interest in p53 as the molecular target of anticancer agents lies in its apoptotic pathways [19]. Therefore, to verify the p53-mediated apoptosis pathway(s), particular interest has been focused on p53 target genes that are involved in this process. To date, a number of different p53-induced genes have been identified and implicated in p53-mediated cell death, including Pidd [7], Noxa [8], p53AIP1 [9], p53DINP1 [10], PERP [11], APAF-1 [12], PUMA [13, 14], Bax [20, 21], Fas/Apo-1 [22], Killer/DR5 [23], PIGs [24], PAG608 [25] and CABC1 [26]. These genes were found by various screening methods (table 1); however, many p53-induced target genes were identified by the DD method [7–10, 25, 27–29]. While most of these genes have been

Table 1. List of p53-induced genes implicated mainly in apoptosis.

Gene(s)	Definition/Function	References	used screening method
PIG3	NADPH-quinone oxidoreductase homologue, oxidative stress, apoptosis[a]	[24]	SAGE
PAG608	Novel gene, encodes a nuclear zinc finger protein, apoptosis	[25]	DD
TP53TG1	Novel gene, DNA-damage	[27]	DD
TP53TG3	Novel gene, cell cycle checkpoint	[28]	DD
p53R2	Ribonucleotide reductase, DNA repair	[29]	DD
PERP	Novel member of the PMP-22/gas family, pro-apoptotic transmembrane protein	[11]	SH
Noxa	Novel gene, pro-apoptotic BH3 protein	[8]	DD
Pidd	Novel gene, death-domain protein	[7]	DD
p53AIP1	Novel gene, apoptosis, induced after phosphorylation of p53 at Serine-46	[9]	DD
APAF-1	Pro-caspase 9 activation, apoptosis	[12]	Chip
p53DINP1	Novel gene, apoptosis, role in p53 phosphorylation at Serine-46	[10]	DD
PUMA	Novel gene, Pro-apoptotic BH3-protein	[13, 14]	SAGE, Chip
CABC1	Chaperone-ABC1 homologue, mitochondrial protein, apoptosis[a]	[26]	Chip

mRNA Differential Display (DD), DNA-Microarray (Chip), Serial Analysis of Gene Expression (SAGE), subtractive hybridization (SH)
[a] Suggested function in p53-mediated apoptosis.

shown to be required for p53-mediated apoptosis, no single target gene has been identified as pivotal in the apoptotic pathway. These results also suggest a role for both the death receptor and mitochondrial pathways in triggering p53-dependent apoptosis. There is convincing evidence that p53 is essential for the induction of apoptosis; however, there is still considerable uncertainty about how p53 expression is able to trigger apoptosis. Therefore, it is necessary to search for additional p53 target genes, which might be integrated in the p53-mediated programmed cell death pathway.

Identification of p53-induced and p53-repressed genes using FDD

Our aim in this present study is to identify p53-induced as well as p53-repressed target genes by FDD technology. In an attempt to screen p53 target genes, we used a tetracycline-regulated ('tet-off' system) p53-inducible human colon cancer cell line, DLD-1 [30]. This cell line contains an inactive endogenous wt p53 gene. The wt p53 protein in this system is induced rapidly after withdrawal of tetracycline (tet). The induction of wt p53 in this cell system stimulates the expression of the two well-known p53 target genes [31]: $p21^{WAF/CIP}$ and mdm2, which were confirmed by Western blot analysis (fig. 3 A). Mdm2 (murine double minute 2) is a protooncogene and a negative feedback regulator of the tumor suppressor gene p53. Mdm2 binds to the N-terminus of p53, represses p53-dependent transactivation of target genes and is also able to promote the rapid degradation of p53 through the ubiquitin-proteosome pathway. The transcriptional activity of p53 leads also to increased expression of $p21^{WAF/CIP}$ (cyclin-dependent kinase inhibitor, a universal cell cycle inhibitor). $p21^{WAF/CIP}$ is a direct p53 target gene, and deletion of this gene significantly reduces the cell cycle arrest response to p53.

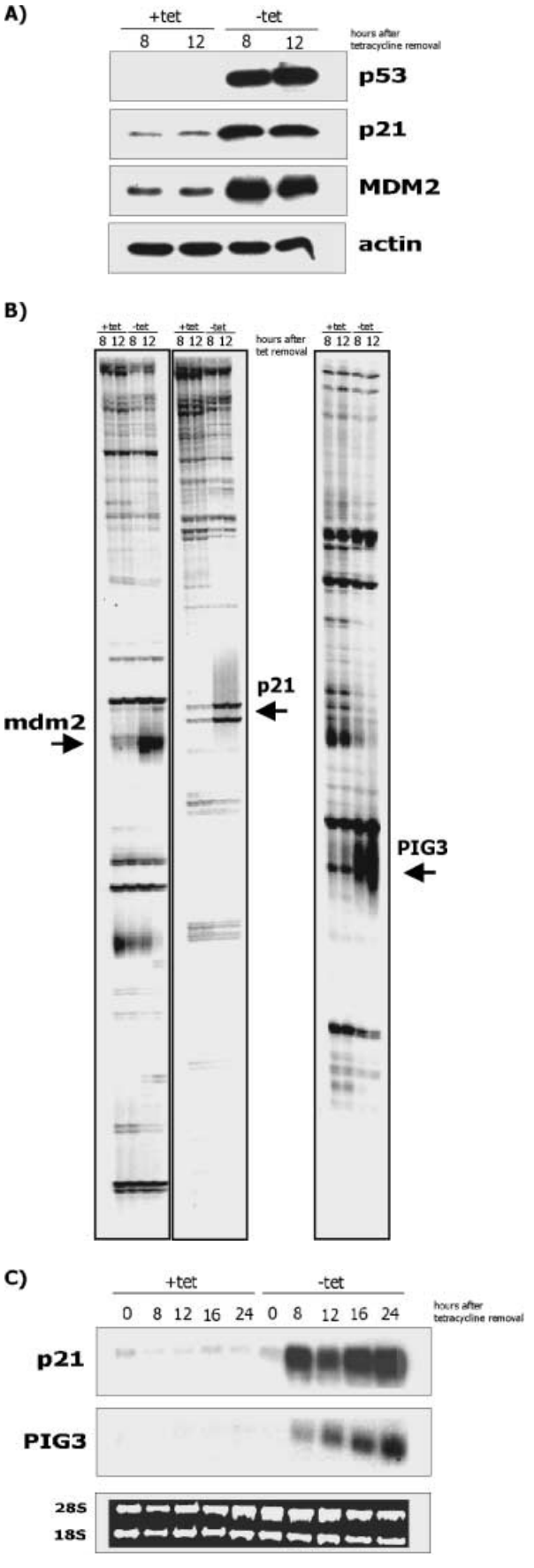

Figure 3. Induction of p53 target genes. (*A*) Tetracycline (tet) was removed at different time points (8 h –tet and 12 h –tet) in DLD-1 'tet-off' cells, and the resulting protein levels for p53, p21 and MDM-2 were analyzed by Western blotting. The polyclonal antibody pAb 1801 against p53 was used. The antibodies against MDM2 (SMP-14) and p21 (C-19) were purchased from Santa Cruz Biotechnology (CA, USA). As control for equivalent protein loading, anti-actin antibody was used (A2066, Sigma-Aldrich, MO, USA). (*B*) Mdm2, p21 and PIG3 gene expression were identified by comprehensive FDD screening. The known p53 downstream target genes p21 and PIG3 were displayed using specific 13mer primers in combination with an A-anchored and C-anchored primer, respectively. Arbitrary primer H-AP10 with A-anchored oligo-dT primer amplified Mdm2, the p53 general regulator. (*C*) The FDD results for the p53-induced target genes p21 and PIG3 were confirmed by performing Northern blot analysis. As control for equivalent RNA loading (each lane 10 µg total RNA), the 18S and 28S rRNA bands are shown.

The cell system, which we used in this study appears to be also appropriate to investigate the process of p53-mediated apoptosis. The first apoptotic features, long before any visible changes appear in the morphological characteristics of apoptosis, could be obtained approximately 8 h after removal of tet. Furthermore, the expression of known apoptotic genes such as PIGs (p53-inducible genes), and the pro-apoptotic Bax protein could be confirmed in this cell system [30]. To perform the FDD, first total RNA was isolated from the cells at different time points either with tet (+tet, no p53 induction), or after withdrawal of tet (–tet, p53 induction). As a positive control for our FDD system, a specific arbitrary 13mer primer recognizing p21 was used in combination with an A-anchored oligo-dT primer to confirm the induction of this gene by p53 (fig. 3B). Mdm-2 also displayed by A-anchored oligo-dT with arbitrary primer H-AP10 (fig. 3B). To check that p53 triggers apoptosis after removal of tet, we also examined the p53-inducible gene 3 (PIG3) expression by FDD, using a specific arbitrary primer in combination with a C-anchored oligo-dT primer (fig. 3B). PIG3 is one of the recently described panel of 14 PIGs that are activated after oxidative stress and is also suggested to play an important role in apoptotic signaling pathways [24]. However, the precise biological functions of the PIGs, especially PIG3, are still unknown, and their role in apoptotic signaling awaits further characterization. The expression of both known p53 downstream target genes, $p21^{WAF/CIP}$ and PIG3, were also confirmed by Northern blot analysis (fig. 3C).

We have identified additional p53 novel target genes [unpublished data], which are either induced or repressed by p53 using comprehensive FDD screening. Interestingly, over 50% of these genes represent novel and uncharacterized genes [unpublished data]. This is in contrast to DNA microarray (chip) technology, which can only recognize known gene sequences. Other advantages of DD over DNA microarrays are the requirement of much less RNA, the ability to compare more than two different RNA samples simultaneously and the ability to detect rare mRNAs.

In summary, our results and also the outcomes from other colleagues provide evidence that DD is an elegant methodology to identify changes in gene expression and to discover novel genes that are involved in important biological pathways. Furthermore, FDD also allows analysis of digital gene expression profiling and precise quantification of gene expression differences.

Acknowledgment. This work was supported in part by NIH grant no. CA76969 to P.L., and by the Deutsche Akademie der Naturforscher-Leopoldina (Halle, Sachsen-Anhalt, Germany). We thank Drs J. Pietenpol for the polyclonal p53 antibody (pAb 1801) and B. Vogelstein for the tet-p53-regulated DLD-1 cell line, and R. J. Jackson II for valuable comments and critical reading of the manuscript.

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