A Review of the Use of DNA Microarrays for the Analysis of Pituitary Neoplasms

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INTRODUCTION
DNA microarray analysis is a recent technological development [1]. To date, few such studies have investigated pituitary neoplasms. The method could potentially advance the field as it permits thorough and simultaneous analysis of gene expression and could shed light on factors involved in pituitary tumorigenesis.

Pituitary adenomas are the most frequent of intracranial neoplasms. On balance, genetic studies have shown them to be monoclonal in nature [2]. The pathogenesis of these typically slow-growing tumors is unknown, but studies suggest evidence for a role for hormonal stimulation and/or molecular defects. While the monoclonality of most human adenomas indicates that genetic changes underlie the process of cell transformation, tumor progression appears to be sustained by various stimuli including, hormones and growth factors. Proteomic analyses have revealed several presumed mediators of pituitary tumorigenesis. Differential expression of oncogene proteins, including Gs-alpha proteins first described in somatotroph adenomas, ras mutations, first associated with pituitary carcinomas, protein kinase C (PKC), and members of the epidermal growth factor receptor family, have all been involved in pituitary tumorigenesis (Figure 3).

Also associated with neoplastic transformation in the pituitary is inactivation of several tumor suppressor genes, including multiple endocrine neoplasia type 1 (MEN-1), p53, cyclins and cyclin-dependent kinases (cdks), as well as cdk inhibitors inclusive of p15, p16, p27, and p57 [2].

Microarray analysis permits high throughput, high precision simultaneous analysis of thousands of genes. Few technologies offer such potential of providing new insights into pituitary cell biology and tumorigenesis. In this review we discuss the utility of microarray analysis as it relates to the clinical assessment, therapy and prognosis of pituitary neoplasms.

The Microarray Method
DNA microarray technology permits relative measurement and analysis of gene expression at the genomic level. The potential of microarray analysis in classifying disease phenotypes, predicting therapeutic response and clinical outcome, revealing molecular pathways, and forwarding our understanding of tumor pathogenesis is promising [3-5]. Expression profiling provides a simultaneous view of thousands of genes, permits a comprehensive analysis of ones differentially expressed, and demonstrates mutations. The method (Figures 1 and 2) exploits the ability of mRNA to hybridize to known DNA sequences. mRNA extracted from a tissue or cell line (normal or abnormal), is used to generate cDNA fragments termed probes. Thousands of target DNA sequences or short expressed sequence tags (EST) in the tissue under study are immobilized in a specific sequence on a solid phase support, either a glass microscope slide or a nylon membrane. PCR-amplified and fluorescence-labeled cDNA probe fragments are then allowed to bind to target DNA sequences. Thereafter, the quantity of bound probe is determined by laser scanning [6]. The resultant data can then be analyzed to quantify expression of any number, even thousands of genes. Normal and disease-associated cDNA sequences are labeled differentially to permit quantification of relative gene expression levels by laser scanning and bioinformatics methods. In summary, DNA microarray technology permits a comprehensive analysis of gene expression profiles.

Presently, cDNA doses of up to 20,000 mouse and 40,000 human genes are commercially available. Presynthesized oligonucleotide sequences can also be arrayed. A

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KEYWORDS:
DNA microarray analysis, laser scanning, fluorescence, molecular genetics, pituitary neoplasms

ACKNOWLEDGEMENTS
The authors thank the Jaridowsky Foundation and the Lloyd-Carr-Harris Foundation for their generous support, and Mrs. Denise Chase for her excellent secretarial support.

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Microscopy and Analysis 20(6):9-12 (UK), 2006

Figure 1: Flowchart schematic of the interacting components required for RNA expression analysis using DNA microarrays generated by robotic ampying of PCR products from cDNA clone sets. Adapted from Nature Genetics 21:25-32,1999 with permission of Macmillan Publishers Ltd.
PCR and proteomics analyses can be used to of thousands of transcripts simultaneously. RT- microarray analysis permits the examination purposes has traditionally been applied to sequences, thus complicating the comparison lem that can only be addressed by synthesizing short length oligonucleotide probes, a prob- genes. It is of note, however, that specificity detecting specific variants of homologous hybridization and are thus advantageous in coding mRNA transcript. Oligonucleotide and validation of results by R T-PCR. Table 1. stratification. The variable results of these fication as well as therapeutic and prognostic eesis and suggested its use for molecular classiﬁcation as well as therapeutic and prognostic stratiﬁcation. The variable results of these studies underline the need for standardization and validation of results by RT-PCR. Table 1, summarizes genes that were differentially expressed in microarray analysis, and were subsequently validated by RT-PCR.

Animal pituitary neoplasms Goidin et al. first applied microarray technol-ogy to rat pituitary [9]. The expression of 588 genes were screened by using RNA from pitu-taries of three month-old rats and tumor-bearing 28 month-old rats to generate cDNA. Microarray analysis revealed 79 differentially expressed genes; the results were conﬁrmed by RT-PCR in 14 of the 15 genes tested. The expression proﬁles of these genes were in keeping with our current understanding of pathophysiological processes. Numerous genes up-regulated in the adenoma-bearing pituitary of older rats have been linked to pitu- itary tumorigenesis (Table 2).

Microarray analysis is not only useful in demonstrating differential expression of genes of known interest, but can also hint at unsuspected disease associations and pathobiological mechanisms. For example, in older rats, Goidin et al., found up-regulation of pre-senilin 1 (PS1), a gene associated with Alzheimer’s disease. This gene is involved in lipid metabolism as well as the production of proinhibin BAP 32 which serves to attenuate DNA synthesis [9].

Recently, Wood et al. studied 1176 genes in thyroid stimulating hormone (TSH)-secrating pituitary adenomas in order to evaluate changes preceding thyroid hormone-induced tumor involution [10]. Microarray analysis revealed up-regulation of 7 genes, and down-regulation of 40. Northern blot analysis of RNA transcripts validated the results. Several differentially expressed genes were involved in cell growth and apoptosis. A search for cell cycle regulators revealed only a few relevant genes. For example, CDK2, which can increase levels of p27 and both cyclin A isoforms, was under-expressed. Thyroid hormones had sev-eral effects (Table 2). One such effect was down-regulation of genes involved in cell mobility by TSH, including cadherin 4, delta- catenin and the calpain light chain important to neuronal outgrowth. Cadherins and catenins, intermediates in Wnt signaling, are described as being part of a model of pituitary tumorigenesis [11] (see below). Other Wnt related down-regulated genes included Wnt receptor homologs. Further studies using microarray analysis in transgenic and knock-out animal models are needed to shed light on pituitary tumorigenesis.

Human pituitary neoplasms Evans et al. were the first to study human pitu- itary tumors by microarray analysis [1]. In total, 7075 genes in normal pituitary and in pituitary adenomas were studied using microarray analysis [1]. In the adenoma group, expression proﬁles of 128 genes were altered. Differences in gene expression patterns were noted in the four groups, including non-functioning, PRL, GH, ACTH adenomas, thus permitting classiﬁcation. In non-functioning adenomas, 22 genes were over-expressed and 8 were under-expressed. Differential expression of four genes were conﬁrmed by RT-PCR (Table 1). Pro-lactin (PRL), growth hormone (GH), and adrenocorticotropic hormone (ACTH)-secret- ing adenomas also demonstrated unique dif-ferential gene expression proﬁles. These ﬁnd- ings suggest the possibility of classifying pitu- itary adenomas on the basis of gene proﬁles. Furthermore, differential expression of unique genes in each tumor subtype may carry with it the potential for novel diagnostic-therapeutic strategies. Non-functioning adenomas showed up-regulation of the folate receptor (FR). This was validated in subsequent studies by the same group [12]. Further, a folate-hap- ten complex has been shown to generate humoral and cellular immune responses lead- ing to tumor destruction [5]. Hence, the FR might be of efﬁcacy in the chemo- and immuno-therapeutic treatment of non-func-tioning adenomas, a subtype of adenomas presently resistant to targeted drug treat- ment.

Although the study of Evans et al. found PRL-secreting adenomas to exhibit over-expression of several other genes, these results were not conﬁrmed by RT-PCR. Nonetheless, the potential of microarray data in establishing diagnostic criteria is underscored, suggest- ing therapeutic approaches, and predicting prognosis. Further investigation of gene pro- ﬁles in pituitary neoplasms is dearly warranted.

Inconsistencies in the expression of genes associated with neoplastic transformation in various tissues emphasize the need for valida- tion of microarray analysis data by proteomics. For example, over-expression of cellular retinoic acid-binding protein 2 and actin-binding LIM protein in non-functioning pituitary adenomas, as well as under-expression of syn-decan 2 in PRL and ACTH adenomas was demonstrated, while the reverse of this expression pattern is observed in prostate and colon cancer, in addition to several other human cancers [1]. It is of note that gene expression levels are not necessarily reﬂected in their respective protein levels.

Moreno et al. investigated gene expression in nonfunctional pituitary adenomas, ﬁnding over-expression of 115 genes and down-regu- lation of 169 [11]. RT-PCR conﬁrmed the dif- ferential expression of four of these genes (Table 1) and proteomic analysis revealed an overall 43% agreement with microarray data. These results highlight the signiﬁcance of post-translational changes and translational efﬁciencies when interpreting microarray data. Thus, coupling microarray analyses with RT-PCR and proteomic studies is an exemplary model for validating microarray data.

The expression pattern of some genes was entirely consistent with our current under- standing of pituitary tumorigenesis [11]. For example, folate receptor 1 (FOLR1) was up-
regulated, whereas the genes for growth hormone (GH), prolactin (PRL) and their transcription factor (pit-1) were down-regulated. Furthermore, the general expression pattern of several functional gene groups has validated present models of tumorigenesis, tumor suppressor genes and apoptosis inducers being down-regulated, while anti-apoptotic genes were up-regulated, excluding BCL2 which was under-expressed. In addition, positive and negative cell cycle regulators were differentially expressed to favor proliferation. Further, genes involved in increased cell movement and tumor invasiveness were preferentially over-expressed in nonfunctional adenomas, tumors noted for their indolence. For example, N-cadherin 2 levels were increased. Patterns of N-cadherin expression are related to invasive behavior in pituitary adenomas with increased proliferation indices. Its localization to cell cytoplasm is associated with loss of cell adhesion [3]. In the case of non-functioning adenomas, expression levels alone may not reflect the actual function of N-cadherin in pituitary adenomas. Ephrins are implicated in neuronal migration, blood vessel formation and tumorigenesis [11]. It is of note that ephrin 3 was found to be over-expressed in nonfunctional pituitary adenomas.

Among the largest group of differentially expressed genes were transcriptional factors. Of these, inhibitors of DNA-binding were under-expressed, even though they are known to contribute to tumorigenesis by inhibiting cell differentiation, stimulating proliferation and facilitating angiogenesis [11]. Levels of several other transcription factors implicated in oncogenesis, such as FOS and JUNB, were also decreased. While these apparent discrepancies suggest caution in the interpretation of DNA microarray analysis results, they underscore both the differential expression and function of the various genes expressed in pituitary neoplasms.

As noted, the study of Moreno et al. found differential expression of four genes, the activity of which was confirmed by RT-PCR and proteomic analysis [11]. These included IDH1, PITX2, NOTCH3, and DLK1. The latter is a potential ligand of NOTCH3; their expression pattern was inverse with high levels of NOTCH3 (4.26 fold) and low levels of DLK1 (917.43 fold). Interactions between DLK proteins and Notch receptors are thought to play a role in lung cancer tumorigenesis via inhibition of differentiation. Studies have also shown inverse expression levels of DLK1 and NOTCH3 in neuroblastomas [11]. Elevated NOTCH3 levels in non-functioning pituitary adenomas suggest a possible method of treating such adenomas with inhibitors of Notch processing, such as the gamma secretase inhibitors implicated in the treatment of Notch-activated acute T-cell lymphoblastic leukemia. It is of note that ASCL1, a substance involved in the regulation of DLK1 and NOTCH3 levels, was also down-regulated (116.82 fold). It has been shown that DLK1 expression levels depend on ASCL1 gene expression and that increased NOTCH3 levels lead to down-regulation of ASCL1. Taken together, these findings suggest a pathway incorporating these elements, one in which elevated Notch levels result in reducing ASCL1 and DLK1. Moreno et al. proposed a model that incorporates the Wnt and Notch signaling pathways in parallel and is explained by differential expression of these genes in nonfunctional pituitary adenomas [11]. In this model, the Notch pathway is linked to a Wnt signaling cascade that results in proliferation and arrest of differentiation. Microarray analysis has revealed up-regulation of SFRP1, beta-catenin, PITX2 and cyclin D1 genes, all elements of the Wnt signaling pathway. This observation supports the model of parallel Notch and Wnt signaling cascades. Another up-regulated gene, TLE2, is also thought to link these pathways that it interacts with HE3-1 which in turn may be inhibited by DLK1. Thus, Notch maintains an "undifferentiated state" in nonfunctional pituitary adenomas via parallel Wnt and Notch pathways linked by TLE2 [11].

Found to be up-regulated in the series of Moreno et al., IDH1 gene expression has also been connected to colon cancer. It may exert anti-apoptotic effects as well [11]. PITX2 overexpression is associated with poor prognosis.

Table 1. Microarray analysis and RT-PCR to validate differential gene expression in human pituitary neoplasms. (REG’N = regulation)

<table>
<thead>
<tr>
<th>STUDY</th>
<th>GENE</th>
<th>REG’N</th>
<th>IMPLICATIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C-mer proto-oncogene tyrosine kinase (C-MAP-1)</td>
<td>Up</td>
<td>cell growth and differentiation, anti-apoptotic and proliferative functions, tumorigenesis, a common pathway with neoplastic transformation in several tissues</td>
</tr>
<tr>
<td>Moreno et al [11]</td>
<td>Racaglutide dehydrogenase 1 (RAC1)</td>
<td>Up</td>
<td>IDH1 gene expression has also been connected to colon cancer; it may exert anti-apoptotic effects as well</td>
</tr>
<tr>
<td></td>
<td>paired-like homeodomain transcription factor 2 (PITX2)</td>
<td>Up</td>
<td>Wnt signaling pathway</td>
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<td></td>
<td>NOTCH3</td>
<td>Up</td>
<td>gamma secretase inhibitors of Notch processing implicated in the treatment of Notch-activated acute T-cell lymphoblastic leukemia</td>
</tr>
<tr>
<td></td>
<td>delta-like 1 homolog 1 (DLK1)</td>
<td>Down</td>
<td>interacts with Notch receptors in lung cancer tumorigenesis by inhibition of differentiation; inverse expression levels of DLK1 and NOTCH3 in neuroblastomas</td>
</tr>
<tr>
<td></td>
<td>BCL2-associated athanogene 1 (BAG1)</td>
<td>Up</td>
<td>a positive regulator of BCL-2 anti-apoptotic protein and several other proteins involved in tumorigenesis; BAG1 over-expression in many human malignancies makes it a potential candidate for therapeutic intervention</td>
</tr>
<tr>
<td>Reubel [15]</td>
<td>G aleen-3 (LGALS-3)</td>
<td>Up</td>
<td>cell growth and differentiation; tumor cell adhesion; angiogenesis; tumor progression and metastasis</td>
</tr>
<tr>
<td></td>
<td>Human aracho-ate-solute homologue-1 (HAS)</td>
<td>Up</td>
<td>a basic hepx-loop-hepx (BHLH) transcription factor; critical to neuroendocrine differentiation during development; over expressed in several neuroendocrine tumors including pulmonary carcinoid tumors and carcinomas, associated with poor prognosis</td>
</tr>
<tr>
<td></td>
<td>Inhibitor of DNA binding 2 (ID2)</td>
<td>Up</td>
<td>role in carcinogenesis by antagonizing B and LHL transcription factors and inhibiting differentiation; up-regulated by bone morphogenetic protein (BMP), an important factor in pituitary cell growth, and may be potential target in the treatment of pituitary carcinomas; knock-out leads to growth inhibition &amp; induction of transdifferentiation</td>
</tr>
<tr>
<td></td>
<td>Transducin-like enhancer of split-4 (TLE-4)</td>
<td>Up</td>
<td>up-regulated in pituitary adenomas as compared to ACTH containing pituitary carcinoma; mediates transcription; pituitary development in the mouse; associates with RUNT domain proteins expressed in pituitary tumors; transcriptional inactivation of chromatin</td>
</tr>
</tbody>
</table>
expression has similarly been documented by microarray, its up-regulation being confirmed at the mRNA level as well as by proteomics. The demonstration of PITX2 further strengthens the above-noted model of Wnt and Notch pathways, since PITX2 is a\(a\) induced by Wnt/beta-catenin signaling and b\(b\) is associated with cell proliferation during pituitary development via the activation and expression of cyclin D2 and D1, respectively. Both increased cyclin D1 levels and over-expression of SPRI, a positive regulator of Wnt under some conditions, also fit the proposed model as suggested by the finding of increased beta-catenin levels in pituitary adenomas[11].

Morris et al. used oligonucleotide arrays of 14,500 genes to study their expression in pituitary adenomas. Analysis revealed differential expression of 3906 genes and 351 expressed sequence tags (EST) [14]. Their results were in close agreement with those of the prior study of Evans et al. Nonetheless, total agreement with expression patterns of candidate genes involved in pituitary tumorigenesis was not achieved. PITX2 was over-expressed in ACTH-secreting adenomas in the series, expression of GADD45-S-gamma and BMP-4, two genes involved in pituitary tumorigenesis, was not demonstrated.

Ruebel et al. used oligonucleotide arrays containing over 15,000 characterized genes in an effort to examine their expression in an ACTH-secreting pituitary carcinoma as well as in four pituitary adenomas of various type [15]. For the purpose of validation, tissue microarray analysis was applied to a larger series of adenomas and carcinomas for validation. Fully 4298 genes were differentially expressed in adenomas as compared to 2057 over-expressed and 2241 under-expressed genes in carcinomas. The observations demonstrated differential expression of several genes known to be associated with pituitary tumorigenesis and progression, including PTG-1, TP53, MEG-3, DAPK-1, TLE, and hASH-1. Ruebel et al. selected four genes (LGALS-3, ASH-1, ID2, and TLE-4) in order to validate their differential expression in both adenomas and carcinomas arising from the ACTH-producing carcinoma using RT-PCR. Validation of the differential expression of some of these genes provided insight into their potential role in pituitary tumorigenesis. For example, over-expression of LGALS-3 and ASH-1 in PRL- and ACTH-secreting adenomas as compared to GH, FSHLH and null cell adenomas was observed. This finding may relate to carcinogenesis in that up to 70% of pituitary adenomas arise from PRL- and ACTH-secreting adenomas [15]. DNA microarray analysis also revealed over-expression of galectins (gal) 3 and 8 in numerous tumors, thus confirming the results of other recent RT-PCR studies of pituitary adenomas [15]. Gal-3 is a beta-galactoside binding protein associated with various biological pathways, such as cell proliferation and adhesion, apoptosis, tumor progression and metastasis [15]. Its over-expression was demonstrated in specific pituitary adenomas suggesting a potential role for gal-3 as a therapeutic target. Indeed, gal-3 inhibitors have been used in clinical trials [15].

### CHALLENGES AND THE FUTURE

As in the case of any new technology, microarray analysis provides not only insights into biological processes in health and disease but challenges as well. Disadvantages encountered are both technical and non-technical in nature. Technical issues include contamination of RNA samples with heterogeneous cell lines or tissues, as well as lack of validation techniques applicable across various platforms. Although high-precision microarray methodology can reduce variability in fluorescence intensity to less than 10%, the role of biological factors is of paramount importance. The inherent biologic characteristics of various tissues, cell lines, transgenic and knock-out animal models, as well as the effects of environment upon gene expression, proliferative activity, and clonal variation in samples all contribute to differential gene expression [8]. Validation of microarray analysis by RT-PCR and proteomics must be undertaken, as gene expression is one only aspect of structure and function.

### REFERENCES


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