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MicroArray Technology - Expression Profiling of MRNA and MicroRNA in Breast Cancer

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1. Introduction

Breast cancer is the most common form of cancer among women. In 2009, an estimated 194,280 new cases of breast cancer were diagnosed in the United States; breast cancer was estimated to account for 27% of all new cancer cases and 15% of cancer-related mortality in women (Jemal et al, 2009). Similarly, in Europe in 2008, the disease accounted for some 28% and 17% of new cancer cases and cancer-related mortality in women respectively (Ferlay et al, 2008). The increasing incidence of breast cancer worldwide will result in an increased social and economic burden; for this reason there is a pressing need from a health and economics perspective to develop and provide appropriate, patient specific treatment to reduce the morbidity and mortality of the disease. Understanding the aetiology, biology and pathology of breast cancer is hugely important in diagnosis, prognostication and selection of primary and adjuvant therapy. Breast tumour behaviour and outcome can vary considerably according to factors such as age of onset, clinical features, histological characteristics, stage of disease, degree of differentiation, genetic content and molecular aberrations. It is increasingly recognised that breast cancer is not a single disease but a continuum of several biologically distinct diseases that differ in their prognosis and response to therapy (Marchionni et al, 2008; Sorlie et al, 2001). The past twenty years has seen significant advances in breast cancer management. Targeted therapies such as hormonal therapy for estrogen receptor (ER) positive breast tumours and trastuzumab for inhibition of HER2/neu signalling have become an important component of adjuvant therapy and contributed to improved outcomes (Fisher et al, 2004; Goldhirsch et al, 2007; Smith et al, 2007). However, our understanding of the molecular basis underlying breast cancer heterogeneity remains incomplete. It is likely that there are significant differences between breast cancers that reach far beyond the presence or absence of ER or HER2/neu amplification. Patients with similar morphology and molecular phenotype based on ER, PR and HER2/neu receptor status can have different clinical courses and responses to therapy. There are small ER positive tumours that behave aggressively while some large high grade ER negative, HER2/neu receptor positive tumours have an indolent course. ER-positive tumours are typically associated with better clinical outcomes and a good response to

hormonal therapies such as tamoxifen (Osborne et al, 1998). However, a subset of these patients recur and up to 40% develop resistance to hormonal therapy (Clarke et al, 2003). Furthermore, clinical studies have shown that adding adjuvant chemotherapy to tamoxifen in the treatment of node negative, ER positive breast cancer improves disease outcome (Fisher et al, 2004). Indeed, treatment with tamoxifen alone is only associated with a 15% risk of distant recurrence, indicating that 85% of these patients would do well without, and could be spared the cytotoxic side-effects of adjuvant chemotherapy.

The heterogeneity of outcome and response to adjuvant therapy has driven the discovery of further molecular predictors. Particular attention has focused on those with prognostic significance which may help target cancer treatment to the group of patients who are likely to derive benefit from a particular therapy. There has been a huge interest in defining the *gene expression profiles* of breast tumours to further understand the aetiology and progression of the disease in order to identify novel prognostic and therapeutic markers. The sequencing of the human genome and the advent of high throughput molecular profiling has facilitated comprehensive analysis of transcriptional variation at the genomic level. This has resulted in an exponential increase in our understanding of breast cancer molecular biology. Gene expression profiling using microarray technology was first introduced in 1995 (Schena et al, 1995). This technology enables the measurement of expression of tens of thousands of mRNA sequences simultaneously and can be used to compare gene expression within a sample or across a number of samples. Microarray technology has been productively applied to breast cancer research, contributing enormously to our understanding of the molecular basis of breast cancer and helping to achieve the goal of individualised breast cancer treatment. However as the use of this technology becomes more widespread, our understanding of the inherent limitations and sources of error increases. The large amount of data produced from such high throughput systems has necessitated the use of complex computational tools for management and analysis of this data; leading to rapid developments in bioinformatics.

This chapter provides an overview of current gene expression profiling techniques, their application to breast cancer prognostics and the bioinformatic challenges that must be overcome to generate meaningful results that will be translatable to the clinical setting. A literature search was performed using the PubMed database to identify publications relevant to this review. Citations from these articles were also examined to yield further relevant publications.

2. Microarray technology – principles & technical considerations

2.1 High throughput genomic technology

There are a multitude of high throughput genomic approaches which have been developed to simultaneously measure variation in thousands of DNA sequences, mRNA transcripts, peptides or metabolites:

- DNA microarray measures gene expression
- Microarray comparative genomic hybridisation (CGH) measures genomic gains and losses or identifies differences in copy number for genes involved in pathological states (Oosterlander et al, 2004)
- Single nucleotide polymorphism (SNP) microarray technology (Huang et al, 2001) has been developed to test for genetic aberrations that may predispose an individual to disease development.

- CpG arrays (Yan et al, 2000) can be used to determine whether patterns of specific epigenetic alterations correlate with pathological parameters.
- Protein microarrays (Stoll et al, 2005) consisting of antibodies, proteins, protein fragments, peptides or carbohydrate elements, are used to detect patterns of protein expression in diseased states.
- ChIP-on-chip (Oberley et al, 2004) combines chromatin immunoprecipitation (ChIP) with glass slide microarrays (chip) to detect how regulatory proteins interact with the genome.

All of these approaches offer unique insights into the genetic and molecular basis of disease development and progression.

This chapter focuses primarily on gene expression profiling and cDNA microarrays, however many of the issues raised, particularly in relation to bioinformatics are also applicable to the other “-omic” technologies.

Gene expression which is a measurement of gene “activity” can be determined by the abundance of its messenger RNA (mRNA) transcripts or by the expression of the protein which it encodes. ER, PR and HER2/neu receptor status are determined in clinical practice using immunohistochemistry (IHC) to quantitate protein expression or fluorescence in situ hybridisation (FISH) to determine copy number. These techniques are semi-quantitative and are optimal when determining the expression of individual or a small number of genes.

Microarray technology is capable of simultaneously measuring the expression levels of thousands of genes in a biological sample at the mRNA level. The abundance of individual mRNA transcripts in a sample is a reflection of the expression levels of corresponding genes. When a complementary DNA (cDNA) mixture reverse transcribed from the mRNA is labelled and hybridised to a microarray, the strength of the signal produced at each address shows the relative expression levels of the corresponding gene.

cDNA microarrays are miniature platforms containing thousands of DNA sequences which act as gene specific probes, immobilised on a solid support (nylon, glass, silicon) in a parallel format. They are reliant on the complementarity of the DNA duplex i.e. reassembly of strands with base pairing A to T and C to G which occurs with high specificity. There are microarray platforms available containing bound libraries of oligonucleotides representing literally all known human genes e.g. Affymetrix GeneChip (Santa Clara, CA), Agilent array (Santa Clara, CA), Illumina bead array (San Diego, CA). When fluorescence-labelled cDNA is hybridised to these arrays, expression levels of each gene in the human genome can be quantified using laser scanning microscopes. These microscopes measure the intensity of the signal generated by each bound probe; abundant sequences generate strong signals and rare sequences generate weaker signals. Despite differences in microarray construction and hybridization methodologies according to manufacturing, microarray-based measurements of gene expression appear to be reproducible across a range of different platforms when the same starting material is used, as demonstrated by the MicroArray Quality Control project (Shi et al, 2006).

2.2 Experimental approach

There are experimental design and quality control issues that must be considered when undertaking a microarray experiment. The experiment should be designed appropriately to answer a specific question and samples must be acquired from either patients or cultured cells which are appropriate to the experimental setup. If the aim of a microarray experiment

is to identify differentially expressed genes between two groups of samples i.e. “experiment” and “control”, it is critical that the largest source of variation results from the phenotype under investigation (e.g. patient characteristic or treatment). The risk of confounding factors influencing the results can be minimised by ensuring that the groups of samples being compared are matched in every respect other than the phenotype under investigation. Alternatively, large sample numbers can be used to increase the likelihood that the experimental variable is the only consistent difference between the groups.

For a microarray experiment, fresh frozen tissue samples are required which have been snap-frozen in liquid nitrogen or collected in an RNARetain™ or RNA Later™ solution to preserve the quality of the RNA. Formalin-fixed and paraffin embedded tissue samples are generally unsuitable for microarray studies as the RNA in the sample suffers degradation during tissue processing (Cronin et al, 2004; Masuda et al, 1999, Paik et al, 2005).

Due to the omnipresence of ribonucleases and the inherent instability of RNA, it is essential to measure the integrity of RNA after extraction. Only samples of the highest integrity should be considered for reverse transcription to cDNA and hybridisation to the microarray platform (figure 1). Once obtained, intensity readings must be background adjusted and transformed; this data is then normalised and analysed and results are generally interpreted according to biological knowledge. The success of microarray experiments is highly dependent on replication. Technical replication refers to the repeated assaying of the same biological sample to facilitate quality assessment. Even more important is biological replication on larger sample sets. The accuracy of microarray expression measurements must be confirmed using a reliable independent technology, such as real-time quantitative PCR, and validated on a larger set of independent biological samples. It is independent validation studies that determine the strength or clinical relevance of a gene expression profile.

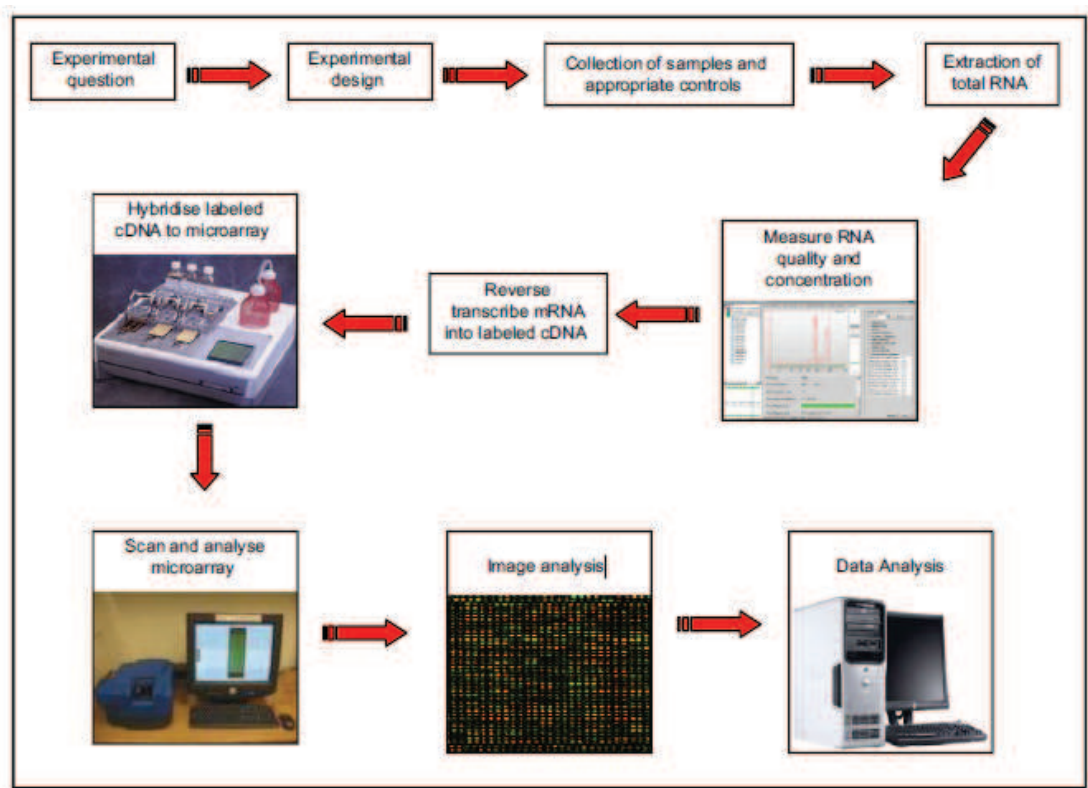


Fig. 1. The steps involved in a cDNA microarray experiment

3. Molecular profiling – unlocking the heterogeneity of breast cancer

Breast cancer researchers were quick to adopt high throughput microarray technology, which is unsurprising considering the opportunity it provides to analyse thousands of genes simultaneously.

3.1 Class discovery

Microarray studies can be used in three different manners;

- class comparison
- class prediction
- class discovery (Simon et al, 2003)

All of these approaches have been applied to the study of breast cancer.

Class discovery involves analyzing a given set of gene expression profiles with the goal of discovering subgroups that share common features. The early gene expression profiling studies of breast cancer (Perou et al, 2000; Sorlie et al, 2001) were class discovery studies. Researchers used an unsupervised method of analysis, in which tumours were clustered into subgroups by a 496-gene “intrinsic” gene set that reflects differences in gene expression between tumours without using selection criteria. The tumour subtype groupings consist of luminal like subtypes which are predominantly ER and PR positive, basal-like subtypes which are predominantly triple negative for ER, PR and HER2/neu, HER2/neu-like subtypes which have increased expression of the HER2/neu amplicon and a normal-like subtype (Perou et al, 2000). Subsequent studies from the same authors, on a larger cohort of patients with follow-up data showed that the luminal subgroup could be further subdivided into at least two groups, and that these molecular subtypes were actually associated with distinct clinical outcomes (Sorlie et al 2001). These molecular subtypes of breast cancer have been confirmed and added to in subsequent microarray datasets (Hu et al, 2006; Sorlie et al, 2003; Sotiriou et al, 2003). Given the importance of the ER in breast cancer biology, it is not surprising that the most striking molecular differences were identified between the ER-positive (luminal) and ER-negative subtypes. These differences have been repeatedly identified and validated with different technologies and across different platforms (Fan et al, 2006; Farmer et al, 2005; Sorlie et al, 2006). The luminal subgroup has been subdivided into two subgroups of prognostic significance:

- *luminal A tumours* which have high expression of ER -activated genes, and low expression of proliferation related genes
- *luminal B tumours* which have higher expression of proliferation related genes and a poorer prognosis than luminal A tumours (Geyer et al, 2009; Paik et al, 2000; Parker et al, 2009; Sorlie et al, 2001, 2003).

The ER negative tumours are even more heterogeneous and comprise the:

- *basal-like subgroup* which lack ER and HER2/neu expression and feature more frequent overexpression of basal cytokeratins, epidermal growth factor receptor and c-Kit (Nielsen et al, 2004)
- *HER2/neu subgroup* which overexpress HER2/neu and genes associated with the HER2/neu pathway and/or the HER2/neu amplicon on chromosome 17.

The HER2/neu and basal-like subtypes have in common an aggressive clinical behaviour but appear to be more responsive to neoadjuvant chemotherapy than the luminal subtypes (Carey et al, 2007; Rouzier et al, 2005). Also clustering with the ER negative tumours are the normal-like breast cancers; these are as yet poorly characterised and have been shown to

cluster with fibroadenoma and normal breast tissue samples (Peppercorn et al, 2008). It is important at this point to acknowledge the limitations of this molecular taxonomy; intrasubtype heterogeneity has been noted despite the broad similarities defined by these large subtypes (Parker et al, 2009). In particular the basal-like subgroup can be divided into multiple additional subgroups (Kreike et al, 2007; Nielsen et al, 2004). Additionally, although the luminal tumours have been separated into subgroups of prognostic significance, meta-analysis of published expression data has suggested that these luminal tumours actually form a continuum and their separation based on expression of proliferation genes may be subjective (Shak et al, 2006; Wirapati et al, 2008). Furthermore, the clinical significance of the normal-like subtype is yet to be determined; it has been proposed that this subgroup may in fact represent an artefact of sample contamination with a high content of normal breast tissue (Parker et al, 2009; Peppercorn et al, 2008). Due to these limitations and the subjective nature of how the molecular subtypes were identified, the translation of this taxonomy to the clinical setting as a definitive classification has been difficult (Pustzai et al, 2006). The development of a prognostic test based on the intrinsic subtypes has not been feasible to date. However, the seminal work by Sorlie and Perou (Perou et al, 2000; Sorlie et al, 2001) recognized for the first time the scale of biological heterogeneity within breast cancer and led to a paradigm shift in the way breast cancer is perceived.

3.2 Class comparison

A number of investigators undertaking microarray expression profiling studies in breast cancer have since adopted class comparison studies. These studies employ supervised analysis approaches to determine gene expression differences between samples which already have a predefined classification. The “null hypothesis” is that a given gene on the array is not differentially expressed between the two conditions or classes under study. The alternative hypothesis is that the expression level of that gene is different between the two conditions. An example of this approach is the microarray experiments that have been undertaken to define differences between invasive ductal and invasive lobular carcinomas (Korkola, 2003; Weigelt, 2009; Zhao, 2004), between hereditary and sporadic breast cancer (Berns, 2001; Hedenfalk, 2001) and between different disease stages of breast cancer (Pedraza, 2010).

3.3 Class prediction

Perhaps the most clinically relevant use of this technology, however, are the microarray class prediction studies which have been designed to answer specific questions regarding gene expression in relation to clinical outcome and response to treatment. The latter approach attempts to identify predictive markers, as opposed to the prognostic markers which were identified in the “intrinsic gene-set”. There is frequently some degree of confusion regarding the terms of “prognostic” and “predictive biomarkers”. This is partially due to the fact that many prognostic markers also predict response to adjuvant therapy. This is particularly true in breast cancer where, for example, the ER is prognostic, and predictive of response to hormonal therapy, but also predictive of a poorer response to chemotherapy (Carey 2007; Kim, 2009; Rouzier 2005).

One of the first microarray studies designed to identify a gene-set predictive of prognosis in breast cancer was that undertaken by van't Veer and colleagues (van't Veer et al, 2002). They

developed a 70-gene set capable of predicting the development of metastatic disease in a group of 98 patients made up of 34 who had developed metastasis within 5-years of follow-up, 40 patients who remained disease-free at 5-years, 18 patients with a BRCA-1 mutation, and 2 patients with a BRCA-2 mutation. The 70-gene signature was subsequently validated in a set of 295 breast cancers, including the group used to train the model, and shown to be more accurate than standard histopathological parameters at predicting outcome in these breast cancer patients (van de Vijver et al, 2002). The signature includes many genes involved in proliferation, and genes associated with invasion, metastasis, stromal integrity and angiogenesis are also represented. This 70-gene prognostic signature classifies patients based on correlation with a “good-prognosis” gene expression profile; a coefficient of greater than 0.4 is classified as good prognosis. The signature was initially criticised for the inclusion of some patients in both the discovery and validation stages (van de Vijver et al, 2002). However, it has been subsequently validated in multiple cohorts of node-positive and node-negative patients and has been shown to outperform traditional clinical and histological parameters at predicting prognosis (Buyse et al, 2006; Mook et al, 2009).

3.3.1 Mammaprint assay

The 70-gene signature was approved by the FDA to become the MammaPrint Assay (Agendia BV, Amsterdam, The Netherlands); the first fully commercialized microarray based multigene assay for breast cancer. This prognostic tool is now available and can be offered to women under the age of 61 years with lymph node negative breast cancer. The MammaPrint test results are dichotomous, indicating either a high or low risk of disease recurrence, and the test performs best at the extremes of the spectrum of disease outcome i.e. identifying patients with a very good or a very poor prognosis.

The MammaPrint signature is a purely prognostic tool, and its role as a predictive marker for response to therapy was not examined at the time it was developed. Its’ clinical utility is currently being assessed, however, in a prospective clinical trial called microarray in node negative and 1 to 3 positive lymph node disease may avoid chemotherapy (MINDACT) trial (Cardoso et al, 2008). The trial aims to recruit 6000 patients, all of whom will be assessed by standard clinicopathologic prognostic factors and by the MammaPrint assay. In cases where there is concordance between the standard prognostic factors and the molecular assay, patients will be treated accordingly with adjuvant chemotherapy with or without endocrine therapy for poor prognosis patients. If both assays predict a good prognosis, no adjuvant chemotherapy is given, and adjuvant hormonal therapy is given alone where indicated. In cases where there is discordance between the standard clinicopathological prognostic factors and the MammaPrint assays’ prediction of prognosis the patients are randomised to receive adjuvant systemic therapy based on either the clinicopathological or the MammaPrint prognostic prediction results. The expected outcome is that there will be a reduction of 10-15% in the number of patients requiring adjuvant chemotherapy based on the MammaPrint assay prediction. It is envisaged that this trial will answer the questions of what patients can be spared chemotherapy and still have a good prognosis, thus accelerating progress towards the goal of more tailored therapy for breast cancer patients.

3.3.2 Oncotype Dx assay

While MammaPrint was developed as a prognostic assay, the other most widely established commercialized multigene assay Oncotype Dx was developed in a more context specific

manner as a prognostic *and* predictive test to determine the benefit of chemotherapy in women with node-negative, ER-positive breast cancer treated with tamoxifen (Paik et al, 2004). The authors used published microarray datasets, including those that identified the intrinsic breast cancer subtypes and the 70-gene prognostic signature identified by the Netherlands group to develop real time quantitative polymerase chain reaction (RQ-PCR) tests for 250 genes. Research undertaken by the National Surgical Adjuvant Breast and Bowel Project (NSABP) B14 protocol using three independent clinical series, resulted in the development of an optimised 21-gene predictive assay (Paik et al, 2004). The assay has been commercialised as Oncotype® DX by Genomic Health Inc¹ and consists of a panel of 16 discriminator genes and 5 endogenous control genes which are detected by RQ-PCR using formalin-fixed paraffin embedded (FFPE) sections from standard histopathology blocks. The ability to use FFPE tissue facilitates clinical translation and has allowed retrospective analysis of archived tissue in large cohorts with appropriate follow up data. The assay has been used to generate Recurrence Scores (RS) by differentially weighting the constituent genes which are involved in:

- proliferation (MKI67, STK15, BIRC5/Survivin, CCNB1, MYBL2)
- estrogen response (*ER*, *PGR*, *SCUBE2*)
- HER2/neu amplicon (HER2/neu/ERBB2, GRB7),
- invasion (MMP11, CTSL2)
- apoptosis (*BCL2*, *BAG1*)
- drug metabolism (*GSTM1*)
- macrophage response (*CD68*).

The assay was evaluated in 651 ER positive lymph node negative breast cancer patients who were treated with either tamoxifen or tamoxifen and chemotherapy as part of the NSABP B20 protocol (Paik et al, 2006). It was found that patients with high recurrence scores had a large benefit from chemotherapy, with a 27.6% mean decreased in 10 year distant recurrence rates, while those with a low recurrence score derived virtually no benefit from chemotherapy. The RS generated by the expression of the 21 genes is a continuous variable ranging from 1-100, but has been divided into three groups for clinical decision making; low (<18), intermediate (18-31) and high (>31). It has been shown in a number of independent datasets that ER positive breast cancer patients with a low RS have a low risk of recurrence and derive little benefit from chemotherapy. Conversely, ER positive patients with high RS have a high risk of recurrence but do benefit from chemotherapy (Goldstein, 2006; Habel, 2006; Mina, 2007; Paik, 2006). The ability of the 21-gene signature to so accurately predict prognosis has led to the inclusion of the Oncotype Dx assay in American Society of Clinical Oncology (ASCO) guidelines on the use of tumour markers in breast cancer as a predictor of recurrence in ER-positive, node-negative patients. However, despite the accurate performance of the assay for high and low risk patients, there remains uncertainty regarding the management of patients with intermediate RS (18-31). This issue is being addressed in a prospective randomized trial assigning individual options for treatment (TAILORx) sponsored by the National Cancer Institute (Lo et al, 2007). This multicentre trial aims to recruit 10,000 patients with ER -positive, lymph node negative breast cancer who are assigned to one of three groups based on their RS; low<11, intermediate 11-25 and high >25. Notably, the RS criteria have been changed for the TAILORx trial, with the intermediate

¹<http://www.genomichealth.com/OncotypeDX>

range being changed from RS 18-30 to RS 11-25 to avoid exluding patients who may derive a small benefit from chemotherapy (Sparano et al, 2006). Patients in the intermediate RS group are randomly assigned to receive either adjuvant chemotherapy and hormonal therapy, or hormonal therapy alone. The primary aim of the trial is to determine if ER positive patients with an intermediate RS benefit from adjuvant chemotherapy or not. The MammaPrint and Oncotype Dx gene signatures both predict breast cancer behaviour, however there are fundamental differences between them (outlined in table 1). This chapter has focused on these signatures as they were the first to be developed, have been extensively validated, and are commercially available. However it is important to note that there are other multi-gene based assays that have been developed and commercialized but are not discussed in detail as they are not yet as widely utilized (Loi et al, 2007; Ma et al, 2008; Ross et al, 2008; Wang et al, 2005).

Assay	MammaPrint	Oncotype Dx
Manufacturer	Agendia BV	Genomic Health, Inc.
Development of Signature	From candidate set of 25,000 genes in 98 patients	From candidate set of 250 genes in 447 patients
Gene signature	70 genes	21 genes
Patient cohort	Stage I & II breast cancer Lymph node negative <55yrs	Stage I & II breast cancer Lymph node negative ER positive Receiving Tamoxifen
Platform	cDNA Microarray	RQ-PCR
Sample requirements	Fresh frozen tissue or collected in RNA preservative	FFPE tissue
Outcome	5-year distant relapse free survival	10-year distant relapse free survival
Test Results	Dichotomous correlation coefficient >4.0 = good prognosis <4.0 = poor prognosis	Continuous recurrence score <18 = low risk 18-31= intermediate risk >31 = high risk
Predictive	No; purely prognostic	Yes
Prospective Trial	MINDACT	TAILORx
FDA approved	Yes	No
ASCO Guidelines	No	Yes

Table 1. Comparison of commercially available prognostic assays MammaPrint and Oncotype Dx

4. Microarray data integration

4.1 Setting standards for microarray experiments

It must be acknowledged that despite the multitude of breast cancer prognostic signatures available, the overlap between the gene lists is minimal (Ahmed, 2005; Brenton, 2005; Fan et

al, 2006; Michiels et al, 2005). This lack of concordance has called into question the applicability of microarray analysis across the entire breast cancer population. In order to facilitate external validation of signatures and meta-analysis in an attempt to devise more robust signatures, it is important that published microarray data be publicly accessible to the scientific community. In 2001 the Microarray Gene Expression Data Society proposed experimental annotation standards known as minimum information about a microarray experiment (MIAME), stating that raw data supporting published studies should be made publicly available in one of a number of online repositories (table 2), these standards are now upheld by leading scientific journals and facilitating in depth interrogation of multiple datasets simultaneously.

Public Database for Microarray Data	URL	Organization	Description
Array Express	http://www.ebi.ac.uk/arrayexpress/	European Bioinformatics Institute (EBI)	Public data deposition and queries
GEO Gene Expression Omnibus	http://www.ncbi.nlm.nih.gov/geo/	National Centre for Biotechnology Information (NCBI)	Public data deposition and queries
CIBEX Center for Information Biology Gene Expression Database	http://cibex.nig.ac.jp/index.jsp	National Institute of Genetics	Public data deposition and queries
ONCOMINE Cancer Profiling Database	http://www.oncomine.org/main/index.jsp	University of Michigan	Public queries
PUMAdb Princeton University MicroArray database	http://puma.princeton.edu/	Princeton University	Public queries
SMD Stanford Microarray Database	http://genome-www5.stanford.edu/	Stanford Univeristy	Public queries
UNC Chapel Hill Microarray database	https://genome.unc.edu/	University of North Carolina at Chapel Hill	Public queries

Table 2. List of Databases with Publicly Available Microarray Data

4.2 Gene ontology

The volume of data generated by high throughput techniques such as microarray poses the challenge of how to integrate the genetic information obtained from large scale experiments with information about specific biological processes, and how genetic profiles relate to functional pathways. The development of the Gene Ontology (GO) as a resource for

experimentalists and bioinformaticians has contributed significantly to overcoming this challenge (Ashburner et al, 2000). The GO Consortium was established with the aim of producing a structured, precisely defined, common, controlled vocabulary for describing the roles of genes and gene products in any organism. Initially a collaboration between three organism databases: Flybase (The Flybase Consortium, 1999), Mouse Genome Informatics (Blake et al, 2000) and the Saccharomyces Genome Database (Ball et al, 2000), the GO Consortium has grown to include several of the world's major repositories for plant, animal and microbial genomes.

The Gene Ontology provides a structure that organizes genes into biologically related groups according to three criteria. Genes and gene products are classified according to:

- Molecular Function: biochemical activity of gene products at the molecular level
- Biological Process: biological function of a gene product
- Cellular Component: location in the cell or extracellular environment where molecular events occur

Every gene is described by a finite, uniform vocabulary. Each GO entry is defined by a numeric ID in the format GO#####. These GO identifiers are fixed to the textual definition of the term, which remains constant. A GO annotation is the specific association between a GO identifier and a gene or protein and has a distinct evidence source that supports the association. A gene product can take part in one or more biological process and perform one or more molecular functions. Thus, a well characterized gene product can be annotated to multiple GO terms in the three GO categories outlined above. GO terms are related to each-other such that each term is placed in the context of all of the other terms in a node-directed acyclic graph (DAC). The relationships used by the GO are: "is_a", "part_of", "regulates", "positively_regulates", "negatively_regulates" and "disjoint_from". Each term in the DAC may have one or more parent terms and possibly one or more child nodes, and the DAC gives a graphical representation of how GO terms relate to each other in a hierarchical manner.

The development of Gene Ontology has facilitated analysis of microarray gene sets in the context of the molecular functions and pathways in which they are involved (Blake & Harris, 2002). GO-term analysis can be used to determine whether genetic "hits" show enrichment for a particular group of biological processes, functions or cellular compartments. One approach uses statistical analysis to determine whether a particular GO is over or under-represented in the list of differentially expressed genes from a microarray experiment. The statistical tests used for such analysis include hypergeometric, binomial or Chi-square tests (Khatrı et al, 2005).

An alternative approach known as "gene-set testing" has been described which involves beginning with a known set of genes and testing whether this set as a whole is differentially expressed in a microarray experiment (Lamb et al, 2003; Mootha et al, 2003). The results of such analyses inform hypotheses regarding the biological significance of microarray analyses.

Several tools have been developed to facilitate analysis of microarray data using GO, and a list of these can be found at: <http://www.geneontology.org/GO.tools.microarray.shtml>

Analysing microarray datasets in combination with biological knowledge provided by GO makes microarray data more accessible to the molecular biologist and can be a valuable strategy for the selection of biomarkers and the determination of drug treatment effect in breast cancer (Arciero et al, 2003; Cunliffe et al, 2003).

4.3 Microarray meta-analysis – combining datasets

Meta-analyses have confirmed that different prognostic signatures identify similar biological subgroups of breast cancer patients (Fan et al, 2006) and have also shown that the designation of tumours to a “good prognosis”/“low risk” group or a “poor prognosis”/“high risk” group is largely dependent on the expression patterns of proliferative genes. In fact, some of these signatures have been shown to have improved performance when only the proliferative genes are used (Wirapati, 2008). Metanalyses of the signatures have also proposed that the prognostic ability of the signatures is optimal in the ER positive and HER2-negative subset of breast tumours (Desmedt, 2008; Wirapati, 2008), the prognosis of this group of tumours being governed by proliferative activity.

Despite obvious clinical application, none of these prognostic assays are perfect, and they all carry a false classification rate. The precise clinical value for these gene expression profiles remains to be established by the MINDACT and TAILORx trials. In the interim the performance of these assays is likely to be optimised by combining them with data from traditional clinicopathological features, an approach which has been shown to increase prognostic power (Sun et al, 2007).

Microarray technology has undoubtedly enhanced our understanding of the molecular mechanisms underlying breast carcinogenesis; profiling studies have provided a myriad of candidate genes that may be implicated in the cancer process and are potentially useful as prognostic and predictive biomarkers or as therapeutic targets. However, as yet there is little knowledge regarding the precise regulation of these genes and receptors, and further molecular categories are likely to exist in addition to and within the molecular subtypes already delineated. Accumulating data reveal the incredible and somewhat foreboding complexity and variety of breast cancers and while mRNA expression profiling studies are ongoing, a new player in breast cancer biology has come to the fore in recent years; a recently discovered RNA species termed MiRNA (miRNA) which many scientists believe may represent a crucial link in the cancer biology picture.

5. MicroRNA - a recently discovered layer of molecular complexity

It has been proposed that the discovery of miRNAs as regulators of gene expression represents a paradigm changing event in biology and medicine. This discovery was made in 1993 by researchers at the Ambros laboratory in Dartmouth Medical School, USA at which time it was thought to be a biological entity specific to the nematode *C. Elegans* (Lee et al, 1993). In the years following this discovery, hundreds of miRNAs were identified in animals and plants. However it is only in the past 5 years that the field of miRNA research has really exploded with the realisation that miRNAs are critical to the development of multicellular organisms and the basic functions of cells (Bartel, 2004). MiRNAs are fundamental to genetic regulation, and their aberrant expression and function have been linked to numerous diseases and disorders (Bartel, 2004; Esquela-Kerscher & Slack, 2006). Importantly, miRNA have been critically implicated in the pathogenesis of most human cancers, thus uncovering an entirely new repertoire of molecular factors upstream of gene expression.

5.1 MicroRNA - novel cancer biomarkers

The first discovery of a link between miRNAs and malignancy was the identification of a translocation-induced deletion at chromosome 13q14.3 in B-cell Chronic Lymphocytic

Leukaemia (Calin et al, 2002). Loss of *miR-15a* and *miR-16-1* from this locus results in increased expression of the anti-apoptotic gene *BCL2*. Intensifying research in this field, using a range of techniques including miRNA cloning, quantitative PCR, microarrays and bead-based flow cytometric miRNA expression profiling has resulted in the identification and confirmation of abnormal miRNA expression in a number of human malignancies including breast cancer (Heneghan et al, 2010; Lowery et al, 2007). MiRNA expression has been observed to be upregulated or downregulated in tumours compared with normal tissue, supporting their dual role in carcinogenesis as either oncogenic miRNAs or tumour suppressors respectively (Lu et al, 2005). The ability to profile miRNA expression in human tumours has led to remarkable insight and knowledge regarding the developmental lineage and differentiation states of tumours. It has been shown that distinct patterns of miRNA expression are observed within a single developmental lineage, which reflect mechanisms of transformation, and support the idea that miRNA expression patterns encode the developmental history of human cancers. In contrast to mRNA profiles it is possible also to successfully classify poorly differentiated tumours using miRNA expression profiles (Volinia et al, 2006). In this manner, miRNA expression could potentially be used to accurately diagnose poorly differentiated tissue samples of uncertain histological origin, e.g. metastasis with an unknown primary tumour, thus facilitating treatment planning.

MicroRNAs exhibit unique, inherent characteristics which make them particularly attractive for biomarker development. They are known to be dysregulated in cancer, with pathognomonic or tissue specific expression profiles and even a modest number of miRNAs is sufficient to classify human tumours, which is in contrast to the relatively large mRNA signatures generated by microarray studies (Lu et al, 2005). Importantly, miRNA are remarkably stable molecules. They undergo very little degradation even after processing such as formalin fixation and remain largely intact in FFPE clinical tissues, lending themselves well to the study of large archival cohorts with appropriate follow-up data (Li et al, 2007; Xi et al, 2007). The exceptional stability of miRNAs in visceral tissue has stimulated investigation into their possible preservation in the circulation and other bodily fluids (urine, saliva etc.). The hypothesis is that circulating miRNAs, if detectable and quantifiable would be the ideal biomarker accessible by minimally invasive approaches such as simple phlebotomy (Cortez et al, 2009; Gilad et al, 2008; Mitchell et al, 2008).

5.2 MicroRNA microarray

The unique size and structure of miRNAs has necessitated the modification of existing laboratory techniques, to facilitate their analysis. Due to the requirement for high quality large RNA molecules, primarily for gene expression profiling, many laboratories adopted column-based approaches to selectively isolate large RNA molecules, discarding small RNA fractions which were believed to contain degradation products. Modifications to capture miRNA have been made to existing protocols to facilitate analysis of the miRNA fraction. Microarray technology has also been modified to facilitate miRNA expression profiling. Labelling and probe design were initially problematic due to the small size of miRNA molecules. Reduced specificity was also an issue due to the potential of pre-miRNA and pri-miRNAs to produce signals in addition to active mature miRNA. Castoldi *et al* described a novel miRNA microarray platform using locked nucleic acid (LNA)-modified capture probes (Castoldi et al, 2006). LNA modification improved probe thermostability and increased specificity, enabling miRNAs with single nucleotide differences to be

discriminated - an important consideration as sequence-related family members may be involved in different physiological functions (Abbott et al, 2005). An alternative high throughput miRNA profiling technique is the bead-based flow cytometric approach developed by Lu *et al.*; individual polystyrene beads coupled to miRNA complementary probes are marked with fluorescent tags (Lu et al, 2005). After hybridization with size-fractionated RNAs and streptavidin-phycoerythrin staining, the beads are analysed using a flow-cytometer to measure bead colour and phycoerythrin, denoting miRNA identity and abundance respectively. This method offered high specificity for closely related miRNAs because hybridization occurs in solution. The high-throughput capability of array-based platforms make them an attractive option for miRNA studies compared to lower throughput techniques such as northern blotting and cloning; which remain essential for the validation of microarray data.

5.2.1 MicroRNA microarray - application to breast cancer

Microarray analysis of miRNA expression in breast cancer is in its' infancy relative to expression profiling of mRNA. However, there is increasing evidence to support the potential for miRNAs as class predictors in breast cancer. The seminal report of aberrant miRNA expression in breast cancer by Iorio et al. in 2005 identified 29 miRNAs that were differentially expressed in breast cancer tissue compared to normal, a subset of which could correctly discriminate between tumour and normal with 100% accuracy (Iorio et al, 2005). Among the leading miRNAs differentially expressed; *miR-10b*, *miR-125b* and *miR-145* were downregulated whilst *miR-21* and *miR-155* were consistently over-expressed in breast tumours. In addition, miRNA expression correlated with biopathological features such as ER and PR expression (*miR-30*) and tumour stage (*miR-213* and *miR-203*). Mattie et al. subsequently identified unique sets of miRNAs associated with breast tumors defined by their HER2/neu or ER/PR status (Mattie et al, 2006). We have described 3 miRNA signatures predictive of ER, PR and Her2/neu receptor status, respectively, which were identified by applying artificial neural network analysis to miRNA microarray expression data (Lowery et al, 2009). Blenkiron et al used an integrated approach of both miRNA and mRNA microarray expression profiling to classify tumours according to "intrinsic subtype". This approach identified a number of miRNAs that are differentially expressed according to intrinsic breast cancer subtype and associated with clinicopathological factors including ER status and tumour grade. Importantly, there was overlap between the differentially expressed miRNAs identified in these studies.

There has been interest in assessing the prognostic value of miRNAs, and expression studies in this regard have focused on detecting differences in miRNA expression between primary breast tumours and metastatic lymph nodes. This approach has identified numerous miRNA that are dysregulated in primary breast tumours compared to metastatic lymph nodes (Baffa et al 2009; Huang et al, 2008). MiRNA have also been identified that are differentially expressed in patients who had a "poor prognosis" or a short time to development of distant metastasis (Foekens et al, 2008); *miR-516-3p*, *miR-128a*, *miR-210*, and *miR-7* were linked to aggressiveness of lymph node-negative, ER-positive human breast cancer.

The potential predictive value of miRNA is also under investigation. Preclinical studies have reported associations between miRNA expression and sensitivity to adjuvant breast cancer therapy including chemotherapy, hormonal therapy and HER2/neu targeted therapy (Ma

et al, 2010; Tessel et al, 2010; Wang et al, 2010), prompting analysis of tumour response in clinical samples. Rodriguez-Gonzalez et al attempted to identify miRNAs related to response to tamoxifen therapy by exploiting the Foekens dataset (Foekens, 2008) which comprised miRNA expression levels of 249 miRNAs in 38 ER positive breast cancer patients. Fifteen of these patients were hormone naive and experienced relapse, which was treated with tamoxifen. Ten patients responded and five did not, progressing within 6 months. Five miRNAs (miR-4221, miR-30a-3p, miR-187, miR-30c and miR-182) were the most differentially expressed between patients who benefitted from tamoxifen and those who failed therapy. The predictive value for these miRNAs was further assessed in 246 ER positive primary tumours of hormone naive breast cancer patients who received tamoxifen as monotherapy for metastatic disease. MiR-30a-3p, miR-30c and miR-182 were significantly associated with response to tamoxifen, but only miR-30c remained an independent predictor on multivariate analysis (Rodriguez-Gonzalez, 2010).

Microarray-based expression profiling has also been used to identify circulating miRNAs which are differentially expressed in breast cancer patients and matched healthy controls. Zhao et al profiled 1145 miRNAs in the plasma of 20 breast cancer patients and 20 controls, identifying 26 miRNAs with at least two-fold differential expression which reasonably separated the 20 cases from the 20 controls (Zhao et al, 2010). This is the first example of genome-wide miRNA expression profiling in the circulation of breast cancer patients and indicates potential for development of a signature of circulating miRNAs that may function as a diagnostic biomarker of breast cancer.

At present diagnostic, prognostic and predictive miRNA signatures and markers remain hypothesis generating. They require validation in larger, independent clinical cohorts prior to any consideration for clinical application. Furthermore as additional short non-coding RNAs are continuously identified through biomarker discovery programmes, the available profiling technologies must adapt their platforms to incorporate newer potentially relevant targets. MicroRNAs possess the additional attraction of potential for development as therapeutic targets due to their ability to regulate gene expression. It is likely that future microarray studies will adopt an integrated approach of miRNA and mRNA expression analysis in an attempt to decipher regulatory pathways in addition to expression patterns.

6. Limitations of microarray technology & bioinformatic challenges

In addition to the great promises and opportunities held by microarray technologies, several issues need to be borne in mind and appropriately addressed in order to perform reliable and non-questionable experiments. As a result, several steps need to be addressed in order to identify and validate reliable biomarkers in the scope of potential future clinical application. This is one of the reasons why, despite the promises of using powerful high-throughput technologies as such as microarray, only very few useful biomarkers have been identified so far and/or have been translated to useful clinical assay or companion diagnostics (Mammaprint®, Oncotype DX®). There still remains a lack of clinically relevant biomarkers (Rifai et al, 2006). Amongst the limitations and pitfalls around the technology and the use of microarrays, some of the most important are the reported lack of reproducibility, as well as the massive amount of data generated, often extremely noisy and with an increasing complexity. As for example, in the recent Affymetrix GeneChip 1.0 ST microarray platform (designed to target all known and predicted exons in human, mouse and rat genomes), where there is approximately 1.2 million exon clusters corresponding to

over 1.4 million probesets (Lancashire et al, 2009). As a result, it appears clearly that extracting any relevant key component from such datasets requires robust mathematical and/or statistical models running on efficient hardware to perform the appropriate analyses.

With this in mind, it is clear that the identification of new biomarkers still requires a concerted, multidisciplinary effort. It requires the expertise of the biologist or pathologist, to extract the samples, the scientist to perform the analysis on the platform and then the bioinformatician/biostatistician to analyse and interpret the output. The data-mining required to cope with these types of data needs careful consideration and specific computational tools, and as such remains a major challenge in bioinformatics.

6.1 Problems with the analysis of microarray data

6.1.1 Dimensionality and false discovery

The statistical analysis of mRNA or miRNA array data poses a number of challenges. This type of data is of extremely high dimensionality *i.e.* has a large number of variables. Each of these variables represents the relative expression of a mRNA or miRNA in a sample. Each of these components contain noise, are non-linear may not follow a normal distribution through a population and may be strongly correlated with other probes in the profile. These characteristics mean that the data may violate many of the assumptions of conventional statistical techniques, particularly with parametric tests.

The dimensionality of the data poses a significant problem, and remains as one of the most critical when analysing microarray data. When one analyses this type of data, one has to consider what is referred to as *the curse of dimensionality*, firstly described by Bellman in 1961 as the “*exponential growth of the search space as a function of dimensionality*” (Bellman, 1961; Bishop, 1995). This occurs in highly dimensional systems where the number of dimensions masks the true importance of an individual single dimension (variable). It is particularly true in a microarray experiment when the number of probes representing the number of miRNA/mRNA studied far exceeds the number of available samples. So there is the potential for a probe that is in reality of high importance to be missed when considered with a large number of other probes. This problem is overcome by breaking down the analysis into single or small groups of variables and repeating the analysis rather than considering the whole profile in one single analysis. Other methods consists of using pre-processing methods and feature extraction algorithms in order to only analyse a subset of the data supposed to hold the most relevant features (Bishop, 1995), as determined by the pre-processing steps.

High dimensionality also creates problems due to false discovery. The false discovery rate (FDR) introduced by Benjamini and Hochberg (Benjamini and Hochberg, 1995) is a measure of the number of features incorrectly identified as “differential” and various approaches have been suggested to accurately control the FDR. In this case if one has a high number of dimensions and analyses each singly (as above) a proportion can appear to be of high importance due to random chance considering the distribution, even when they are not. To overcome this one has to examine a rank order of importance and when testing for significance one has to correct the threshold for significance by dividing it by the number of dimensions. So for example when analysing the significance of single probes from a profile with 4,000 probes in it the threshold becomes $P < 0.05$ divided by 4,000 *i.e.* $P < 0.0000125$.

6.1.2 Quality and noise

Noise also poses a problem in the analysis of mRNA or miRNA data. The inherent technical and biological variability necessarily induces noise within the data, eventually leading to biased results. The noise may lead to misinterpretation of sample groups that may actually have no biological relevance. As a consequence extreme care needs to be taken to address the problem of noise.

Noise may be random where it is applied to all parts of the miRNA equally or systematic where particular probes inherently have more noise than others because of the nature of the component miRNA or genomic code that they represent.

It is now widely acknowledged that the reported high level of noise found in microarray data is the most critical pull-back of microarray-based studies, as it is pointed by the MAQC Consortium (Shi et al, 2006; Klebanov and Yakovlev, 2007).

6.1.3 Complexity and non-normality

Because of the complex nature of the profile a particular mRNA or miRNA may be non-normally distributed through a population. Such non-normality will immediately invalidate any statistical test that uses parametric statistics i.e. depends on the assumption of a normal distribution. Invalidated tests would include ANOVA and t-test. To overcome this, the data would have to be transformed mathematically to follow a normal distribution or an alternative non parametric test would have to be employed. Examples of non-parametric tests include Kruskal-Wallis and Mann Whitney U which are ANOVA and unpaired T-Test alternatives respectively. Generally non-parametric tests lack power compared to their parametric alternatives and this may prove to be a problem in high dimensional space due to the reasons described previously.

6.1.4 Reproducibility

Reproducibility has a marked effect on the accuracy of any analysis conducted. Furthermore reproducibility has a profound effect on the impact of other issues such as dimensionality and false detection. Robust scientific procedures requires that the results have to be reproducible in order to reduce the within sample variability, the variability between sample runs and the variability across multiple reading instruments. Aspects of variability can be addressed using technical and experimental replicates. The averaging of samples profiles can be used to increase the confidence in the profiles for comparison (Lancashire et al., 2009). Technical replicates provide information on the variability associated with instrumental variability whilst experimental (or biological) replicates give a measure of the natural sample to sample variation. Problems in data analysis occur when the technical variability is high. In this situation the problem in part can be resolved by increasing the number of replicates. If however the technical variation is higher than the biological variation then the sample cannot be analysed.

6.1.5 Auto-correlation or co-correlation

Auto correlation exists when two components within a system are strongly linearly correlated with one another. In any complicated system there are likely to be a number of components that are auto correlated. This is especially true in array profiling of biological samples. Firstly due to biological processes one protein in a set of samples is likely to interact or correlate with another through a population.

Auto correlation becomes a problem when using linear based regression approaches. This is because one of the assumptions of regression using multiple components is that the components are not auto correlated. If intensity for multiple miRNA probes are to be added into a regression to develop a classifier these components should not be auto correlated. Auto correlation can be tested for using the Durbin Watson test.

6.1.6 Generality

The whole purpose of biomarker (or set of biomarkers) identification, using high-throughput technologies or any other, is to provide the clinicians with an accurate model in order to assess a particular aspect. However, a model is only as good as its ability to generalize to unseen real world data. A model only able to explain the population on which it was developed would be purely useless for any application.

As a result, if one is to develop classifiers from mRNA or miRNA array data the features identified should be generalised. That is they will predict for new cases in the general population of cases. When analysing high dimensional data there is an increased risk of over fitting, particularly when the analysis methods imply supervised training on a subset of the population. So for example, when a large number of mRNA or miRNA are analysed there is the potential for false detection to arise. If a random element identified through false detection is included as a component of a classifier (model) then the generality of that classifier will be reduced; i.e. it is not a feature that relates to the broader population but is a feature specific to the primary set of data used to develop the classifier. Standards of validation required to determine generality have been defined by Michiels et al, 2007.

Generality of classifiers can be increased by the application of bootstrapping or cross validation approaches.

Some algorithms and approaches, that usually involve supervised training, suffer from over-fitting (sometimes called memorisation). This is a process where a classifier is developed for a primary dataset but models the noise within the data as well as the relevant features. This means that the classifier will not accurately classify for new cases i.e. it does not represent a general solution to the problem which is applicable to all cases. This is analogous, for example, to one developing a classifier that predicts well the risk of metastasis for breast cancer patients from Nottingham but will not predict well for a set of cases from Denmark. Over fitted classifiers seldom represent the biology of the system being investigated and the features identified are often falsely detected.

One of the most common solutions to avoid over-fitting is to apply a Cross Validation technique in combination with the supervised training. Random sample cross validation is a process of mixing data. Firstly the data are divided into two or three parts (figure 2); the first part is used to develop the classifier and the second or second and third parts are used to test the classifier. These parts are sometimes termed training, test and validation data sets respectively. In certain classifiers such as Artificial Neural Network based classifiers the second blind set is used for optimisation and to prevent over fitting. In random sample cross validation the random selection and training process is repeated a number of times to create a number of models each looking at the global dataset in a number of different ways (figure 2). Often the mean performance of these models is considered.

Leave one out cross validation is an approach also used to validate findings. In this case one sample is left out of the analysis. Once training is complete the sample left out is tested. This process is repeated a number of times to determine the ability of a classifier to predict

unseen cases. This approach of random sample cross validation drives the classifier solution to a generalised one by stopping the classifier from training too much on a seen dataset and stopping the training earlier based on a blind dataset.

7. Methods used to analyse microarray data and their limitations

With the advent of cutting edge new technologies such as microarrays, the analysis tools for the data produced need to be appropriately applied. Although expression arrays have brought high hopes and expectations, they have brought tremendous challenges with them. They have been proven to suffer from different limitations as previously discussed. However, innovative computational analysis solutions have been developed and have been proven efficient and successful at identifying markers of interest regarding particular questions. This section presents some of the most common methods employed to overcome the limitations discuss above, and to analyse expression array data.

7.1 Application of ordination techniques

If we are to utilise the mRNA or miRNA profile we have to identify robust features despite its high dimensionality that are statistically valid for the general population not just for a subset. Ordination techniques are used to map the variation in data. They are not directly predictive and cannot classify directly unless combined with another classification technique.

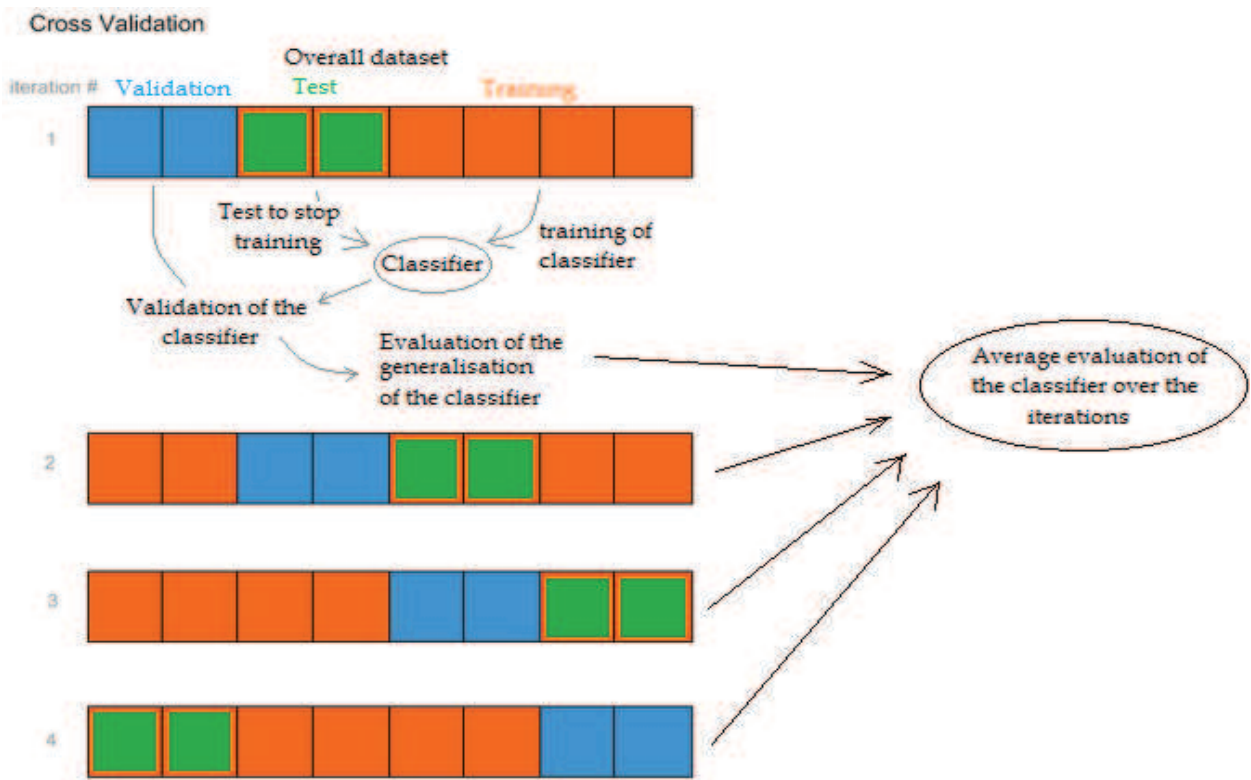


Fig. 2. Illustration of Cross Validation technique, here with three subsets: the training subset used to train the classifier, the test subset used to stop the training when it has reached an optimal performance on this subset, and a validation subset to evaluate the performance (generalization ability) of the trained classifier.

7.1.1 Principal components analysis

PCA is usually a method of choice for dimensionality reduction. It is a multivariate exploratory technique used to simplify complex data space (Raychaudhuri et al, 2000) by translating the data space into a new space defined by the principal components. It works by identifying the main (principal) components that explain best the shape (variance) of a data set. Each principal component is a vector (line) through the data set that explains a proportion of the variance, it is the expression of a linear combination of the data. In PCA the first component that is added is the one that explains the most variance the second component added is then orthogonal to the first. Subsequent orthogonal components are added until all of the variation is explained. The addition of vectors through a multidimensional data set is difficult to visualise in print, we have tried to illustrate it with 3 dimensions in figure 3. In mRNA/miRNA profile data where thousands of dimensions exist, PCA is a useful technique as it reduces the dimensionality to a manageable number of principal components. If the majority of the variance is explained in 2 or 3 principal components these can be used to visualise the structure of the population using 2 or 3 dimensional plots. A limited parameterisation can also be conducted to determine the contribution of each parameter (miRNA) to each of the principal components. This however suffers from the curse of dimensionality in high dimensional systems. Thus the main limitation of using PCA for gene expression data is the inability to verify the association of a principal component vector with the known experimental variables (Marengo et al, 2004). This often makes it difficult to accurately identify the importance of the mRNA or miRNA in the system, and make it a valuable tool only for data reduction.

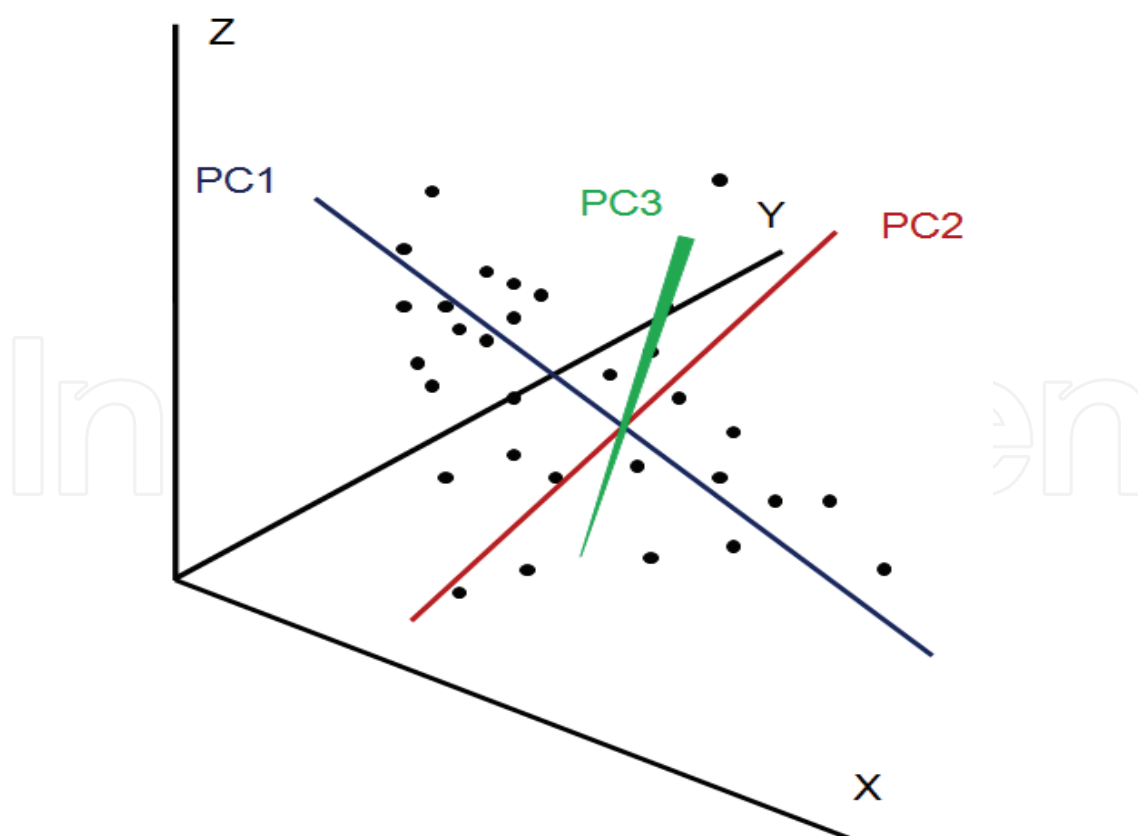


Fig. 3. Example of a 3 dimension PCA with the 3 orthogonal PCs.

7.1.2 Hierarchical clustering

Although several clustering techniques exist, the most used in the context of microarray data analysis is hierarchical clustering. Hierarchical clustering is used to identify the structure of a given population of cases or a given set of markers such as proteins. Every case is considered to have a given position in multidimensional space. Hierarchical clustering determines the similarity of cases in this space based on the distance between points. There are various linkage methods used for calculating distance, such as single linkage, complete linkage and average linkage. Single linkage computes the distance as the distance between the two nearest points in the clusters being compared. Complete linkage computes the distance between the two farthest points, whilst average linkage averages all distances across all the points in the clusters being compared. One commonly used distance measure is Euclidian distance which is the direct angular distance between two points. In fact it considers the distance in multidimensional space between each point and every other point. In this way a hierarchy of distances is determined. This hierarchy is plotted in the form of a dendrogram (figure 4). From this dendrogram we can identify clusters of cases or markers that are similar at a given distance.

The one major problem concerning clustering is that it suffers from the curse of dimensionality when analysing complex datasets. In a high dimensional space, it is likely that for any given pair of points within a cluster there will exist dimensions on which these points are far apart from one another. Therefore distance functions using all input features equally may not be truly effective (Domeniconi et al, 2004). Furthermore, clustering methods will often fail to identify coherent clusters due to the presence of many irrelevant and redundant features (Greene et al, 2005). Additionally, the important number of different distance measure may add an additional bias: it has been reported that the choice of a distance measure can greatly affect the results and produce different outcomes after the analysis (Quackenbush, 2001). Dimensionality is also of importance when one is examining the structure of a population through ordination techniques. This is particularly the case when utilising hierarchical cluster analysis. This approach is of limited suitability for high dimensional data as in a high dimensional space the distance between individual cases reaches convergence making all cases appear the same (Domeniconi et al, 2004). This makes it difficult to identify the real structure in the data or clusters of similar cases.

7.2 Application of modelling techniques

This second part of the section focusing on analysis tools considers more evolved techniques with what is known as *machine learning*. There are however a number of other techniques that can be employed in a predictive or classification capacity. Others include hidden Markov and Bayesian methods. These are widely described in the literature.

7.2.1 Decision tree based methodologies

Decision tree methodologies include, boosted decision trees, classification and regression trees, random forest methodologies. This approach is based on splitting a population into groups based on a hierarchy of rules (figure 5). Thus a given case is split into a given class based on a series of rules. This approach has been modified in a number of ways. Generally, a decision is made based on a feature that separates classes (one branch of the cluster dendrogram from another) within the population. This decision is based on a logical or numerical rule. Although their use in the analysis of miRNA data has been limited, decision

trees have been used in the analysis of miRNA data derived to classify cancer patients (Xu, et al, 2009).

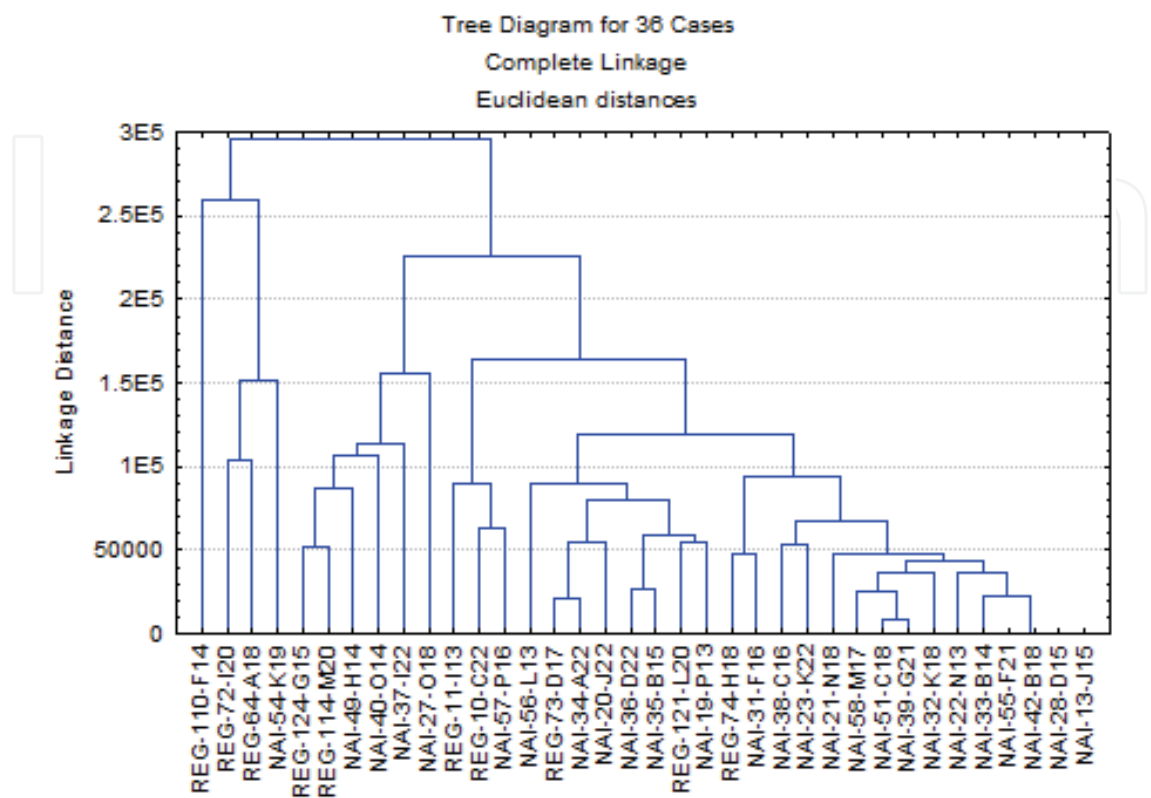


Fig. 4. Example of a hierarchical clustering analysis result aiming to find clusters of similar cases.

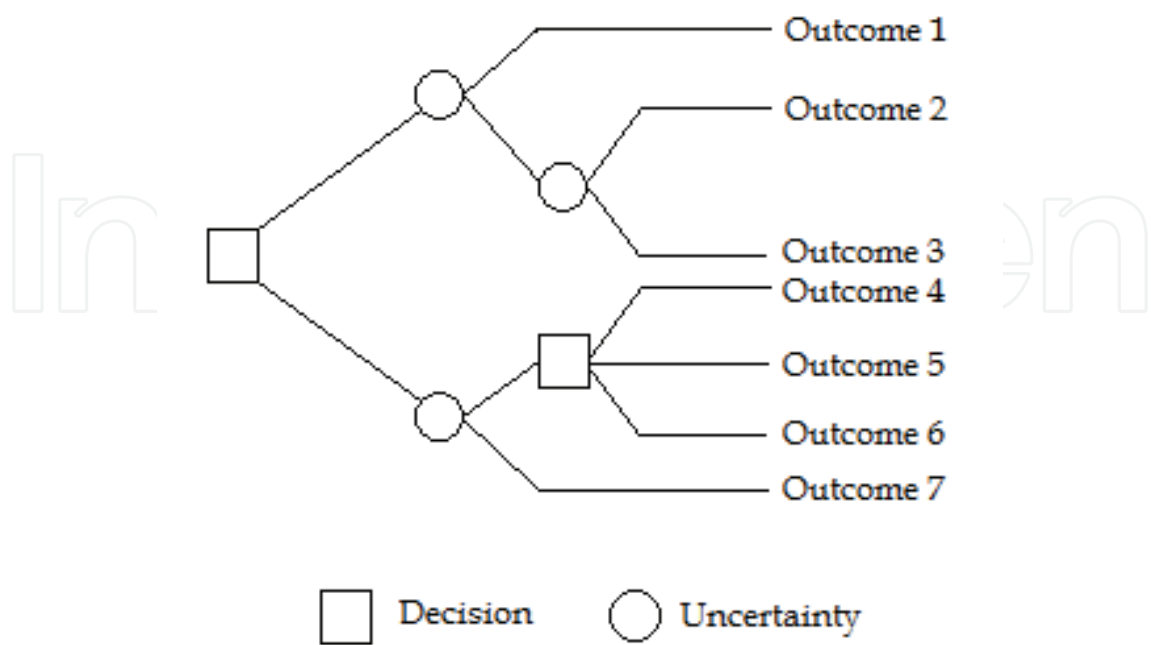


Fig. 5. Schematic example of the basic principle of Decision Trees

Boosted decision trees take the primary decision tree algorithm and boost it. Boosting is a process where classifiers are derived to allow prediction of those not correctly predicted by earlier steps. This means that a supervised classification is run where the actual class is known. A decision tree is created that classifies correctly as many cases as possible. Those cases that are incorrectly classified are given more weighting. A new tree is then created with these boosted weights. This process is similar to the iterative learning that is conducted with the Artificial Neural Network back propagation algorithm.

Random forest approaches take the basic decision tree algorithm and couple it with random sample cross validation. In this way a forest of trees is created. Integration of a number of decision trees identifies a combined decision tree which, as it is developed on blind cases, represents what approaches a generalised solution for the problem being modelled (Breiman et al, 2001). This approach has been shown to be very good at making generalised classifications. The approach essentially derives each tree from a random vector with equivalent distribution from within the data set, essentially an extensive form of cross validation. Yousef et al, (2010) have used random forest as one method for the identification of gene targets for miRNAs. Segura et al (2010) have used random forests as a part of an analysis to define post recurrence survival in melanoma patients.

7.2.2 Artificial Neural Networks

Artificial Neural Networks are a non linear predictive system that may be used as a classifier. A popular form of ANN is the multi-layer perceptron (MLP) and is used to solve many types of problems such as pattern recognition and classification, function approximation, and prediction. The approach is a form of artificial intelligence in that it “learns” a solution to a problem from a preliminary set of samples. This is achieved by comparing predicted versus actual values for a seen data set (the training data set described earlier) and using the error of the predicted values from the ANN to iteratively develop a solution that is better able to classify. In MLP ANNs, learning is achieved by updating the weights that exist between the processing elements that constitute the network topology (figure 6). The algorithm fits multiple activation functions to the data to define a given class in an iterative fashion, essentially an extension of logistic regression. Once trained, ANNs can be used to predict the class of an unknown sample of interest. Additionally, the variables of the trained ANN model may be extracted to assess their importance in the system of interest. ANNs can be coupled with Random sample cross validation or any other cross validation method (LOO or MCCV) in order to ensure that the model developed is not over fitted. One of the advantages of ANNs is that the process generates a mathematical model that can be interrogated and explored in order to elucidate further biological details and validate the model developed on a wide range of cases. A review of their use in a clinical setting presented in Lisboa and Taktak (2006). Back propagation MLP ANNs have been proposed for use in the identification of biomarkers from miRNA data by Lowery et al, 2009.

7.2.3 Linear Discriminant Analysis (LDA)

Linear discriminant analysis attempts to separate the data into two subgroups by calculating the optimal linear line that best splits the population. Calculation of this discriminating line is conducted by taking into account sample variation within similar classes, and minimizing it between classes. As a result, any additional sample has its class determined by the side of the discriminating line it falls.

LDA can outperform other linear classification methods as LDA tries to consider the variation within the sample population. Nevertheless, LDA still suffers from its linear characteristic, and often fails to accurately classify non-linear problems, which is mostly the case in biomedical sciences (Stekel et al, 2003). This is the reason why non-linear classifiers are recommended.

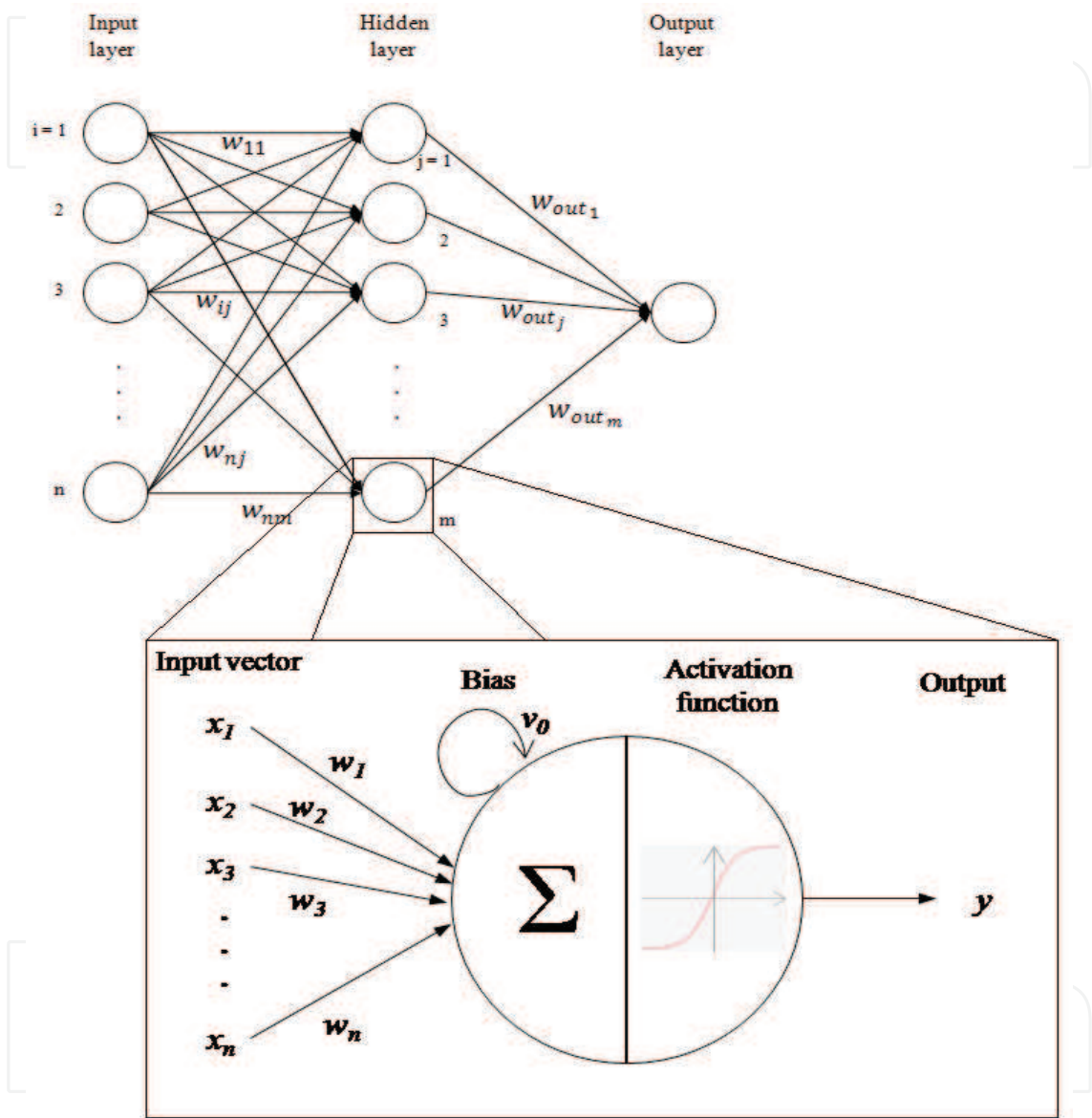


Fig. 6. Example of a classical MLP ANN topology with the details of a node (or neurone)

7.2.4 Support Vector Machines

Support Vector Machines (SVMs) are another popular form of machine learning algorithms in the field of analyzing MA data for non-linear modeling (Vapnik and Lerner, 1963). They are an evolution of LDA in the sense that they work by separating the data into 2 sub-groups. They work by separating the data into two regions by constructing a straight line or hyper plane that best separates between classes (figure 7). In the common example of a two-class classification problem, SVMs attempt to find a linear “maximal margin hyperplane”

able to accurately discriminate the classes (Dreiseitler et al, 2001), similarly to what does Linear Discriminant Analysis. If no such linear hyperplane can be found, usually due to the inherent non-linearity of the dataset, the data are mapped into a high-dimensional feature space using a kernel function (for example polynomial or radial basis functions) in which the two classes can now be separated by a hyperplane which corresponds to a non-linear classifier (Furey et al, 2000). The class of the unknown sample is then determined by the side of the “maximal marginal hyper plane” on which it lies. SVMs have been used to analyse miRNA data by Xue et al, 2005.

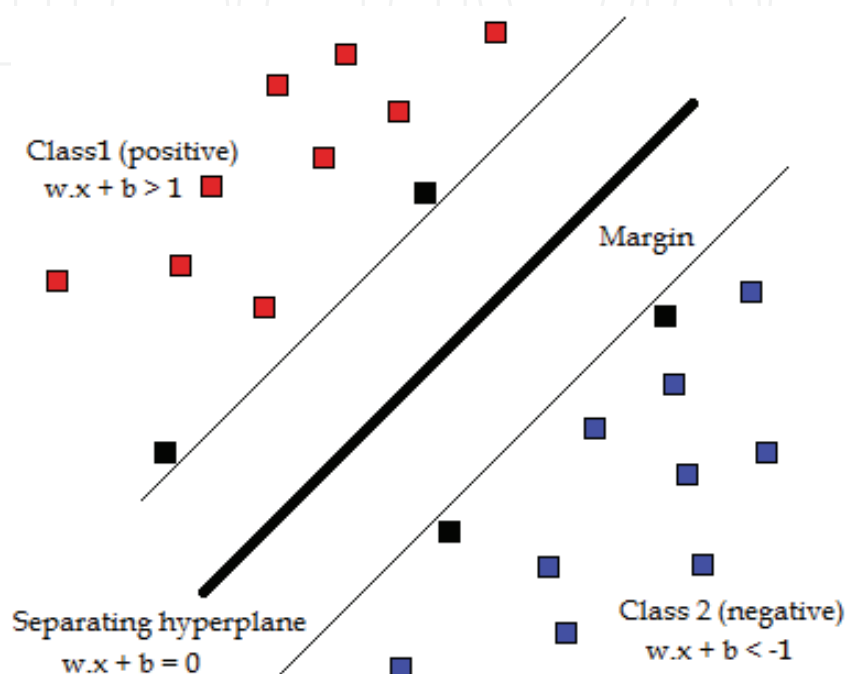


Fig. 7. Schematic representation of the principle of SVM. SVM tries to maximise the margin from the hyperplane in order to best separate the two classes (red positives from blue negatives).

8. Conclusion

The capability of microarray to simultaneously analyse expression patterns of thousands of DNA sequences, mRNA or miRNA transcripts has the potential to provide a unique insight into the molecular biology of malignancy. However, the clinical relevance and value of microarray data is highly dependent on a number of crucial factors including appropriate experimental design and suitable bioinformatic analysis. Breast cancer is a heterogeneous disease with many biological variables which need to be considered to generate meaningful results. Cohort selection is critical and sufficient biological and technical replicates must be included as part of microarray study design. Experimental protocols should be appropriate to the research question. The research community have enthusiastically applied high throughput technologies to the study of breast cancer. Class prediction, class comparison and class discovery studies have been undertaken in an attempt to unlock the heterogeneity of breast cancer and identify novel biomarkers. Molecular signatures have been generated which attempt to outperform current histopathological parameters at prognostication and

prediction of response to therapy. Two clinical tests based on gene expression profiling (Oncotype DX and Mammaprint) are already in clinical use and being evaluated in multicentre international trials. It is essential that the potential of microarray signatures is carefully validated before they are adopted as prognostic tools in the clinical setting. Standards have been set for the reporting of microarray data (MIAME) and such data is publically available to facilitate external validation and meta-analysis. It is imperative that the data is integrated with knowledge normally processed in the clinical setting if we are to overcome the difficulties in reproducibility, standardization and lack of proof of significance beyond traditional clinicopathological tools that are limiting the incorporation of microarray based tools into today's standard of care.

Deriving biologically and clinically relevant results from microarray data is highly dependent on bioinformatic analysis. Microarray data is limited by inherent characteristics that render traditional statistical approaches less effective. These include high dimensionality, false discovery rates, noise, complexity, non-normality and limited reproducibility. High dimensionality remains one of the most critical challenges in the analysis of microarray data. Hierarchical clustering approaches, which have been widely used in the analysis of breast cancer microarray data, do not cope well with dimensionality. In overcoming this challenge supervised machine learning techniques have been adapted to the clinical setting to complement the existing statistical methods. The majority of machine learning techniques originated in weak-theory domains such as business and marketing. However, these approaches including Artificial Neural Networks and Support Vector Machines have been successfully applied to the analysis of miRNA microarray data in the context of clinical prognostication and prediction.

It is clear that the goal of translating microarray technology to the clinical setting requires close collaboration between the involved scientific disciplines. If the current momentum in microarray-based miRNA and mRNA translational research can be maintained this will add an exciting new dimension to the field of diagnostics and prognostics and will bring us closer to the ideal of individualized care for breast cancer patients.

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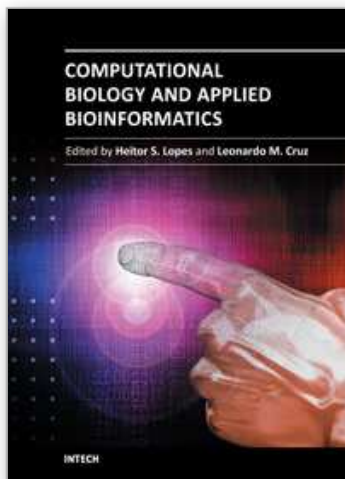
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Nowadays it is difficult to imagine an area of knowledge that can continue developing without the use of computers and informatics. It is not different with biology, that has seen an unpredictable growth in recent decades, with the rise of a new discipline, bioinformatics, bringing together molecular biology, biotechnology and information technology. More recently, the development of high throughput techniques, such as microarray, mass spectrometry and DNA sequencing, has increased the need of computational support to collect, store, retrieve, analyze, and correlate huge data sets of complex information. On the other hand, the growth of the computational power for processing and storage has also increased the necessity for deeper knowledge in the field. The development of bioinformatics has allowed now the emergence of systems biology, the study of the interactions between the components of a biological system, and how these interactions give rise to the function and behavior of a living being. This book presents some theoretical issues, reviews, and a variety of bioinformatics applications. For better understanding, the chapters were grouped in two parts. In Part I, the chapters are more oriented towards literature review and theoretical issues. Part II consists of application-oriented chapters that report case studies in which a specific biological problem is treated with bioinformatics tools.

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