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Review Article

MICROARRAY APPLICATIONS IN CANCER BIOLOGY: THE CHALLENGES

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Abstract

Microarray is one of the technologies that has taken the scientific world by storm in the last decade with its high-throughput, multi-dimensional data, making an impact on every branch of life-science. The field of cancer biology is no exception and has eagerly embraced the technology with open arms, leading to vast advances in our understanding of the killer disease. Microarray analysis gives an insight into the multiple cellular processes disrupted during oncogenesis. For this reason, it has found its application in research pertaining to every stage of cancer from screening to therapeutics by way of gene expression profiling, genotyping, methylation profiling among others. Despite this, the technology is still limited by its inability to render validated information that can lead to more dramatic developments in the field of oncological practice. To achieve its full potential in cancer diagnosis and classification, microarray technology needs improvement of its ancillary technologies such as development of new microarray platforms, as well as statistical software for analysis and data mining. This will not only simplify technical and analytical procedures but will also make them more precise and cheaper. In addition, inter-laboratory cooperation for ongoing meta-profiles will help produce standardized diagnostic methods utilizing microarrays. This review is focused on challenges that need to be addressed when using microarrays in Cancer Biology. The review also mentions the aspects of oncological practice, where microarray technology has left an indelible impression.

Keywords: genotyping arrays, expression profiling, mRNA profiling, cDNA arrays

INTRODUCTION

The completion of the Human Genome Project (HGP) a decade back has been a big leap for research in life sciences, opening up the alleys for genotype-phenotype correlation studies. Microarray technology is the single largest technology to have effectively utilized the exploits of the HGP, namely, characterization of genes in a high throughput manner. Though the technology is still evolving with new variants, the most prominent application of microarray has been in gene expression profiling. The expression profile, which is representative of the mRNA population of a cell is dynamic and varies enormously among different cell types and also among the same cell types at different stages, in response to disease or therapeutics. The real power of microarray as compared to conventional genetic analysis is its ability to study the expression state of thousands of genes simultaneously in a cost-effective, time-effective manner. Microarray has been the most popular and powerful tool for molecular characterization of cells in a diseased state as compared to the normal state. The multi-dimensional nature of cancer has made it difficult to understand the disease in its entirety, as researchers have been forced with the limitation of dealing with and deriving conclusions, predominantly from one dimensional data until microarrays came in to use. The advent of the microarray technology has revolutionized the study of molecular pathways, which are responsible for the development and progression of human malignancies. It helps us to study thousands of genes that may be involved simultaneously in the mechanism of tumor progression. Though primarily used for gene expression studies, microarray has applications in Single Nucleotide Polymorphisms (SNP) identification, copy number variations, methylation profiling, micro RNA (miRNA) profiling also. The varied uses of this technology has helped oncologists in predicting tumor behavior, classification of tumors, identification of biomarkers and prognostic markers, as well as identifying genes associated with chemo resistance. Despite the versatility of the technology, microarray is not without its limitations. The 'achilles heel' of this technology is its technical limitations like the lack of rigorous standards for data collection, analysis, validation and submission. In the scenario of a heterogenous disease like cancer, it is not enough to have multi-dimensional data but also have strong significance and reproducibility across populations and across different microarray platforms. These have resulted in the inability to translate the laboratory findings using microarray into clinical use. In this review, we aim to give an account of the major areas in which microarrays have been useful in cancer research along with its limitations in those areas.

What is a Microarray?

Microarray, as the name implies, is the arrangement of many microscopic DNA spots on a solid surface. From the first cDNA microarray of 31104 clones spotted on nylon membranes with radioactively labeled probes[1] to present arrays photolithographically printed on silicon chip with fluorescent probes[2] the technology has improved by leaps and

bounds. It works on the principle of hybridization where mRNA or cDNA from the sample is labeled with a fluorescent probe and hybridized to the complementary sequence on the slide. A typical array experiment could be divided into the following steps.[3,4]

Microarray Fabrication

The most commonly used microarray for expression profiling are cDNA arrays that contain cDNA from clones and oligonucleotide arrays that contain gene-specific oligonucleotide stretches spotted on slides. Oligonucleotides are synthesized on the array in situ using photolithographic or other techniques and cDNA arrays use robotic printing of PCR products, plasmids or oligonucleotides onto nitrocellulose, nylon, plastic or glass supports at densities of the order of 100,000 oligonucleotides or 10,000 PCR products per cm^2.

Sample Processing

Cellular mRNA is extracted and labeled by the incorporation of fluorescent deoxyribonucleotides during first strand synthesis. If the RNA amount is not sufficient, it is subjected to linear amplification before labeling. Each labeled cDNA hybridizes to its complementary strand on the slide with fidelity. This hybridization is detected using confocal laser scanners that excite the fluorescent probes and collect their emission at the relevant wavelengths. The signal is obtained in the form of an array image following hybridization.

Data Mining

The raw expression profiling data is subjected to a series of steps including, identification of spots, calculating background and hybridization signal using algorithms designed for it and calculation of local background in vicinity of each spot. This data is subjected to normalization and the genes that do not show any difference in expression from the reference or control sample is filtered out. The differentially expressed genes thus obtained are subjected to further analysis. There are different computer algorithms and different methods available for the sophisticated analysis and identification of non-random groups of genes associated with particular biological events.

Applications of Microarray in Cancer Biology

Early Detection of Cancers

Early detection strategies are an important area of research in cancer biology as it is difficult to treat the disease in advanced stages. To prolong the survival rate, emphasis is laid on the early detection of cancer among the high risk groups and this involves identification of highly sensitive and specific early markers of cancer. miRNA microarrays have been widely used to detect early markers in various cancers.[5-10] Yang *et al*., studied differentially expressed miRNAs in tumor tissue and adjacent normal tissue and identified that aberrant expression of hsa-miR-338-3p, hsa-miR-139-5p, hsa-miR-574-5p and hsa-miR-601 increased the risk of esophageal squamous cell carcinoma.[11] Sharma *et al*., analyzed the expression pattern of 1,368 genes in peripheral blood cells of 24 women with breast cancer and 32 women with no signs of this disease and identified a set of 37 genes that correctly predicted the diagnostic class in at least 82 % of the samples. These results showed that peripheral blood-based gene-expression test could be used to detect breast cancer early in asymptomatic patients.[12] Recent Evidences show that microarray based Methylation CpG island recovery assay (MIRA) can be used for screening of significantly hypermethylated genes as early detection markers for breast cancer.[13]

Diagnosis

The conventional method of cancer diagnosis uses a combination of clinical and histopathological data but, this may not be precise due to atypical clinical or histopathological information. Microarray technology aids in tumor diagnosis on a molecular basis. The demonstration of use of cDNA microarrays to elucidate tumor-specific gene expression profiles in human cancers has paved the way for their use as a diagnostic tool[14]. The landmark study in cancer diagnosis by Golub *et al*., showed class prediction study on 38 bone marrow samples from acute leukemia patients to differentiate between the two subtypes of leukemia, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL). It is a known fact that, a multitude of tests is required to distinguish between the two sub types and still the diagnosis could go wrong. With this study, the authors identified a set of 50 genes differentially expressed among the AML and ALL training samples and tested them on a training set of 34 samples. 29 of the samples were classified correctly using this class prediction study.[15] The benefit of using microarray for diagnosis is that they can distinctly differentiate cancers that are known to be histologically similar, thus helping in better treatment. Gordon *et al*., used gene expression analysis by microarray to distinguish between malignant pleural mesothelioma and adenocarcinoma of the lung.[16] Another approach to microarray in diagnosis is screening the expression profiles of genes among normal, premalignant and malignant tissues from the same organ to identify genes to be used as diagnostic markers. A large number of markers identified by this method are non-invasive, using body fluids like blood or saliva for the test. Li *et al*. demonstrated the utility of salivary transcriptome diagnostics by microarray to detect oral cancer. They identified potential salivary biomarkers namely, IL8, IL1B, DUSP1, HA3, OAZ1, S100P, and SAT that can distinguish Oral Squamous Cell Carcinoma with high sensitivity (91 %) and specificity (91 %).[17] A blood-based five gene biomarker set for colorectal cancer was identified by Han *et al*., by expression analysis of RNA from 211 blood samples.[18] Serum miRNAs can also be used to differentiate cancer patient sera from normal donor sera, by a simple microarray assay.[19] By microarray gene expression analysis of 218 tumor samples comprising of 14 common tumor types and 90 normal tissue samples, Ramaswamy *et al*., compiled a multiclass cancer diagnostic signature.[20] The field of cancer diagnosis has benefitted immensely from microarray technology, with an exponential increase in the number of plausible diagnostic markers that could be used.

Prognosis

Microarray studies can identify a set of up-regulated and down-regulated genes and this information can be used to characterize a particular tumor thus helping in its precise classification and clinical behavior. In the last decade, many groups have identified gene-signatures or a group of genes that could be used as a prognostic classifier. There are over 4500 publications currently available on PubMed about

prognostic markers in cancer identified using microarray. The first prognostic gene signature was identified from an extensive breast cancer study that analyzed 5000 differentially expressed genes and defined a 70 gene-signature that identified a group of good prognosis patients with minimal risk of development of distant metastasis within 5 years in patients who were systemic therapy-naïve.[21] The same group showed using 295 cases, that this 70 gene signature predicts outcome independent of current clinic-pathological prognostic markers. The gene signature also classified greater than 95 % of ER negative cancers as poor prognosis and that there is a strong correlation between 70-gene signature-defined poor prognosis and high histological grade.[22] The conclusions defining the chemotherapy use, led to the development of commercially available prognostic test, MammaPrint with the 70 gene signature.[23] There are other studies that show over-expression of a particular gene indicating poor prognosis for specific types of cancer. Some examples include, CD88 in non-small-cell lung cancer,[24] Cyclin D1 in oropharyngeal cancer,[25] TM4SF5 in oesopharyngeal cancer,[26] Cullin1 in breast cancer,[27] KPNA2 in multiple cancers.[28] Most of these prognostic markers could also be targeted for treatment. A number of retrospective studies conducted on pre-treatment tissue samples have strongly indicated the efficacy of gene expression profiles in the prognostic classification of solid tumors.[29-32] Metastasis markers have also been identified by microarrays. Low expression of Galectin-2 has been associated with lymph node metastasis in gastric cancer indicating the aggressive behavior of tumors and the need for the treatment plan to be devised accordingly.[33] ITGA3 has been identified as a potential marker for aggressive cancer phenotype using microarray based analysis.[34] In an interesting approach, methylated DNA immunoprecipitation-CpG Island microarray has been effectively used by Gyobu *et al*., to identify 2 CpG Islands significantly associated with the presence of lymph node metastasis in esophageal squamous cell carcinoma.[35] Apart from identifying metastatic markers, it is also possible to identifying the site of origin of the tumor using microarray. Azueta *et al*. showed a microarray-based gene expression approach to determine the tumor site of origin in a series of metastatic tumors.[36]

Therapeutics

Cancer therapeutics is important in the form of customized, personalized medicine. Molecular markers have been identified for gauging treatment response and for designing tailor-made treatment for a patient based on his molecular profile. Rouzier *et al*., showed the different molecular subtypes of breast cancer responding differently to preoperative chemotherapy by studying the gene expression profile of 82 breast cancer samples after treatment with 4 commonly used chemotherapeutic drugs.[37] In a similar study, increased expression of MAD1L1 was found to be insensitive to Taxol treatment in breast cancer.[38] Taxol being one of the most commonly used drug in breast cancer treatment, it will be useful to check a patient for MAD1L1 expression before administering the drug. A patient with high MAD1L1 could be suggested alternative treatment to avoid chemo resistance. Similar studies have demonstrated strong correlations between gene expression patterns and chemo sensitivity profiles from a panel of NCI60 cancer cell lines to hundreds or thousands of tested chemical compounds.[39,40] Apart from this, many of the prognostic markers identified could also be direct therapeutic targets to treat cancer. Microarray based methods are also used to find new drug targets that could treat the cancer with more specificity and less side-effects. A computational tool based on microarray data sets could help in the identification of cancer specific drugs and in the drug discovery process.[41] A microarray based gene expression profiling experiment carried out on drug treated human liver cancer cell lines indicated that a novel combination of sorafenib and celecoxib provided a synergistic anti-proliferative and pro-apoptotic effects.[42] Some studies have thrown light on modifying the expression of particular genes to increase the efficiency of the treatment. For example, PLCgamma1 inhibition has a therapeutic potential in the treatment of metastasis dissemination.[43]

Challenges of the Microarray Technology

Technical Challenges

In spite of being used for numerous experiments by researchers all over the world and leading to some path-breaking discoveries, microarray is not without its limitations. The most common technical limitations of microarray include high turnaround time, unequal labeling efficiency of fluorescent dyes, leading to data variability, especially for genes with very low expression. These can however be alleviated by running replicate arrays of each sample reducing false positives but, this is not a feasible option for cancer samples wherein the availability of tissue is limited. Additionally, microarrays cannot give a panaromic view of the subject under study. Since it is imperative to take all the genes into consideration for a complex disease like cancer, it results in increasing the cost, computational complexity and dimensionality of the study. With the increase in dimension, there is also an increase in irrelevant variables, normally screened out during analysis. The analysis is only done by a set of computer algorithms and so, we never know if relevant information of great value was lost during data mining, as the analysis programs are standard and not specific for each experiment. Due to the multitude of microarray platforms being in use, it becomes difficult to compare and reproduce results across platforms. Classification accuracy in microarray analysis is complicated as there are many more parameters (genes) than number of cases (samples) that we are trying to classify. Many methods are available to simplify sample classification like clustering methods, compound covariate prediction, fuzzy logic, sunken centroid gene list filtering, and neural networks. The onus is therefore on the researcher to identify the method that best suits his purpose of the study and this requires a sound knowledge of these methods. If not used wisely, even though a result may be statistically significant, it may not be significant biologically and vice versa. The association of a genetic signature with the disease outcome has been found to be significantly lesser in subsequent validation than in the preliminary study.[44] Cross validation could reduce but not rule-out such false results. Hence studies done with large sample size become crucial to understand the degree of biological variation and also get a more statistically significant, biologically relevant and clinically applicable result.[45] Clinical trials are essential to determine how best to integrate genomics-based diagnostics into standard patient care. While there has been no dearth of pilot projects using microarray, very few have been rigorously validated enough

to take it to the level of clinical trials. There is huge amount of microarray data available in the various microarray data repositories like GEO, Express DB, GXD and Array Express. But often, crucial details that may be necessary for a researcher may be missing especially, for data generated and stored before 2002. This is because of the absence of a standard format for submission, which was later rectified by Minimal Information about a Microarray Experiment (MIAME) guidelines.[46] However, there is still no unified expression archive for microarray data comparable to Gen Bank, EMBL, DDBJ databases and hence exploring microarray data remains tedious. Microarray experiments include multiple steps of chip production, probe hybridization, image quantification, normalization and finally data interpretation. Variability can be introduced to the results in any of the steps and hence it is difficult to compare two microarray experiments done at different times in the same lab unless a standardized procedure covering all the above mentioned steps is made.[47]

Biological Challenges

Source of sample is the most predominant challenge faced while using microarrays for cancer research. Most of the experiments conducted from clinical samples comprise of dead cells. Most often tumors will be having a lot of normal cells interspersed and therefore it is important to choose a sample with more than 70 % tumor cells for the sake of representation. This contributes to the heterogeneity of the clinical tumor with variable numbers of fibroblasts, inflammatory cells and epithelial cells, apart from the tumor cells[48] leading to masking of signal from the tumor cell in an expression analysis. The complexity of this heterogenous mass increases even among different patients having the same tumor. In such a scenario, it is necessary to do a laser-capture micro dissection of cellular subtypes of interest. Microarray expression profiling assays are based on mRNA that can get degraded easily. In small samples, the amount of mRNA will be insufficient for the experiment and will have to be amplified to produce antisense RNA (aRNA), leading to possible amplification bias. Also, due to the fragile nature of mRNA, it is necessary to handle the samples with utmost care as even minor variations in handling or surgical manipulation or RNA extraction methods could lead to degradation, leading to spurious results and conclusions. Unlike certain other techniques, microarray is not a stand-alone technique and always requires further validation. The difference in mRNA levels as measured by gene expression profiling, does not always correlate with a corresponding increase in protein level due to post-transcriptional regulation. The maximum number of genes that can be studied simultaneously using microarray at present is only 20,000 and the present arrays do not include the different isoforms of transcripts that occur as a result of splicing. Hence it becomes mandatory to study the physiological relevance of a microarray result by supplementing it with other focused experiments. Microarrays can be used only to identify target genes for diagnosis or prognosis. Clinically, it becomes important that they are validated by more specific experiments like Immunohistochemisty or PCR. Similarly, class discovery studies are able to uncover diagnostic classes of tumor even in cases when morphological or phenotypical tests are still not available. Diagnostic and prognostic marker predictions are often done with a small sample size due to the difficulty in procurement of samples, proper age-matched, sex-matched controls and high cost of microarray. It is essential to conduct additional studies with a larger sample size to negate the variance that gets introduced in various stages of the experiment. In spite of the voluminous amount of microarray data available on diagnostic and prognostic markers, very few have seen the light of day in terms of clinical applications. No information is available on the use of these new biomarkers from prospective randomized trials in a healthy, asymptomatic population. One reason for this is the poor designing of experiments by researchers. For screening tests, a prospective-specimen collection and a retrospective-blinded-evaluation study design has to be used. It has been shown that that poorly differentiated tumor cells have fundamentally distinct gene expression patterns and hence the markers found by expression arrays may not apply to them.[20]

Perspectives

Considering all the above mentioned limitations, it is absolutely essential to have a healthy collaboration between surgeons, pathologists, molecular biologists and bioinformaticians to execute a meaningful microarray experiment on cancer. To achieve its full potential in cancer diagnosis and classification, microarray technology needs improvement of its ancillary technologies such as development of new microarray platforms, as well as statistical software's for analysis and data mining. This will not only simplify technical and analytical procedures but will also make them more precise and cheaper. In addition, inter-laboratory cooperation for ongoing meta-profiles will help produce standardized diagnostic methods utilizing microarrays. Conventional microarray technology heavily relies on the transcriptome while cancer phenotype is not completely defined by its transcriptomes alone. So, while microarray experiments definitely provide the leads, the results obtained from them are not categorical. Newer techniques like the Next Generation Sequencing with its variants is fast overtaking microarrays as the preferred choice of experiment for transcriptomics as it gives much more information than a standard microarray. Never the less, microarray has given us a wealth of information with respect to cancer biology and it remains to be seen if microarray will adapt to the changing scenario and come up with more precise and simple versions that could be used in applied research in cancer biology.

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