SEGMENTATION AND ANALYSIS OF OSTEOSARCOMA CANCEROUS BONE MICRO ARRAY IMAGES-1

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Analysis of human bone micro array images represents a major area of research in bone biology. The normal human bone and human bone cancer 'Osteosarcoma' case has been taken up for the current study, since it is the most frequent type of bone tumor and is most common between the ages of 15 to 25. One of the biggest challenges faced in image analysis is at its gene level. In this paper, analysis of DNA microarray of normal human and osteosarcoma has been done. Using micro arrays, expression levels of thousands of genes can be measured simultaneously. In DNA Microarray experiment, the labeled nucleic acids are derived from the miRNA of a bone sample and so the microarray measures the gene expression. From this technique, the obtained microarray image consists of genes responsible for osteosarcoma case, by quantification of such genes of bone cell, able us to know the comparative study between the normal and osteosarcoma case of human bone genetic information. The digital images of DNA microarray has been analyzed and processed in this work. Analysis of digital DNA microarray bone images includes the extraction of the intensity levels of each spot in an image. Components of DNA Microarray image analysis are: Grid Alignment, Foreground Separation, Quality Assurance, and Quantification. This work provides the analysis of DNA microarray images of osteosarcoma human bone and the comparative study between the normal and osteosarcoma case of human bone samples.

Keywords: Bone Cancer Osteosarcoma, DNA Micro Array Image, Micro Array Image Analysis

1. INTRODUCTION

The Human bone cancer case 'Osteosarcoma' also called as Osteogenic sarcoma is a malignant primitive bone tumor, usually developing in children and adolescents, with a high tendency to metastasize. Metastasis is the dissemination of cancer cells [1] from the primary tumor to a distant organ and represents the most frequent cause of death for patients with cancer. This project work has two implications, First, at gene level analysis of human bone tissue of both normal and osteosarcoma case is through DNA microarray technique, in which the DNA microarray images were produced through a series of steps and procedure at DNA microarray service laboratory, and it is analyzed for intensity extraction of each miRNA detected spot in the image which corresponds to single gene expression [2]. The intensity of miRNA can be compared in both the normal and abnormal bone tissue.

The microRNA are single stranded RNA molecules of about 21 to 23 nucleotides in length which regulates gene expression, and these are transcribed from DNA but not translated into proteins. miRNA [3] Microarray is a high throughput method for studying microRNA expressions in cultured cells or tissues which facilitates the study of biological roles of microRNA.

A DNA Microarray consists of a grid of tiny spots of DNA printed on a surface, with each spot usually

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corresponding to a different gene. miRNA reflecting which genes are currently active, is extracted from cells and processed to form single stranded nucleic acids labeled with fluorescent molecules. The labeled nucleic acids bind to their corresponding microarray spot. The DNA microarray is then scanned with a laser confocal microscope (Agilent Scanner).

The analysis of digital DNA microarray images of human bone samples obtains qualitative and quantitative results. DNA Microarray qualitative image analysis includes gridding, spot finding and background correction [4]. DNA Microarray quantitative image analysis was useful in providing the intensity levels of each miRNA detected spots which correspond to the single gene expression [5]. The steps of DNA Micro array image formation from bone samples are as follows:

- RNA extraction from FFPE bone samples.
- Labeling and Hybridization - Control and test miRNA samples are processed on the same array using Cyanine-3 fluorophore.
- Scanning and feature extraction.
- miRNA profile is obtained.
- The obtained DNA Microarray image for both normal and osteosarcoma human bone samples were analyzed through Gene Spring software.

It is also analyzed through MATLAB by using Image processing Toolbox [7] wherein the images are processed and analyzed. The steps include:

- The Gridding or assigning coordinates for each spot in the image.

- Foreground separation, Segmentation of spot.

- Intensity extraction of each spot.

2. MATERIALS AND METHODS

2.1. Materials used for DNA Microarray Bone Images and DNA Microarray Imaging Technique

The normal and osteosarcoma of human bone sample tissue was obtained in the form of FFPE (formalin fixed paraffin embedded) from the Institute of Oncology. The FFPE tissues have been widely used to archive samples obtained from biopsies [6] of various human cancers and diseased tissues like linked to genetic abnormalities. The FFPE samples are then given for the RNA extraction for further process. The DNA Microarray bone image was obtained from the genetic laboratory. The following are the samples for the analysis can be seen in Fig.1.
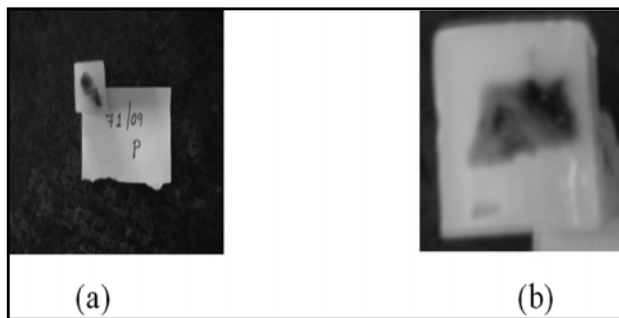

Fig.1: FFPE Samples. (a) Normal Bone Tissue.
(b) Osteosarcoma Tissue.

2.2. Methods used for DNA Microarray Imaging

DNA Micro array imaging technique for the process of obtaining the miRNA profile of Human Bone Sample and Analysis.

The following are the steps followed in the laboratory to obtain the DNA micro array bone image.

2.2.1. Extraction of RNA from FFPE Tissue (Both Normal and Osteosarcoma)

The FFPE samples were deparaffined and subjected to overnight protease digestion followed by standard RNA extraction using Tri reagent (Ambion), RNA was then quantified by nanodrop assay. RNA concentration and purity estimated using Nanodrop Spectrophotometer. The QC report of the samples has been provided.

2.2.2. Characterize the RNA Samples using Agilent 2100 Bioanalyser

To measure the total RNA integrity, purity, concentration and small RNA kit is used respectively. This lab-on-a-chip system can analyse up to 12 DNA, RNA or protein samples in a matter of minutes. Also because of the very high sensitivity, extremely small sizes are sufficient. Bioanalyser is used to perform a QC on total RNA prior to gene micro array experiments.

2.2.3. Micro Array Design, RNA Labeling and Hybridization

Human miRNA Microarray (V1) microarrays were manufactured by Agilent technologies and consisted of probes for 471 human and 64 viral miRNAs. Eight microarrays printed on a 1 inch by 3 inch glass slide. Fig.2. shows Agilent oligo Microarray (8 microarray slide format). Microarrays are printed on the slide of the glass labeled with the "Agilent" bar code (also called the "active side" or "front side").
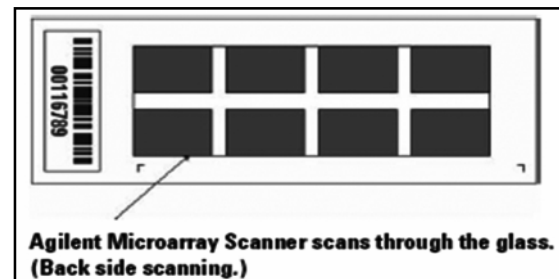

Fig.2: 8 Micro Array Slide Format

8x15K slide format printed using Agilent's 60 merSure Print technology allows as many as 8 arrays to reside on a single slide, allowing experiments to range from a genome-wide view to more focused studies. The Microarrays are manufactured using a proprietary non-contact industrial inkjet printing process, in which oligo monomers are deposited uniformly onto specially-prepared glass slides. This in situ synthesis process prints 60-mer length oligonucleotide probes, base-by-base, from digital sequence files. The precise inkjet process enables the delivery of extremely small, accurate volumes (picoliters) of the chemicals to be spotted. Fig.3.highlights four steps in the process.

The sample can be frozen after drying and before hybridization mixture assembly.The miRNA labeling was performed as described in the Fig.4.by using Agilent miRNA labeling kit. The labeling steps are as follows:

a. Dephosphorylation: 100ng of total RNA was dephosphorylated with calf intestine alkaline phosphatase (enzyme) for 30 min at 37^0C. it is to remove the 3′ phosphate group of RNA.

b. Denaturation: Denaturation was performed by adding DMSO (acts as reducing agent and prevents hairpin loops formation of RNA) and incubating at 100^0C for 7 min and immediately transferred to ice water bath.
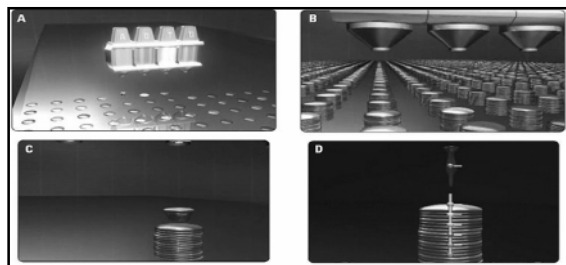

Fig.3: These Four Images Communicate the General Mechanism for Oligo Synthesis Via Inkjet Printing. (A) The First Layer of Nucleotides is Deposited on the Activated Microarray Surface. (B) Growth of the Oligos is Shown after Multiple Layers of Nucleotides have been Precisely Printed. (C) Close-up of One Oligo as a New Base is being Added to the Chain, which is Shown in Fig. D.

c. Ligation: Ligation was performed with pCp-Cy3 at 16^0C for 2h.T4 RNA ligase buffer is used for the attachment of the dye (Cy3) to the miRNA molecule. The labeled samples were dried completely in a vaccum concentrator to remove the DMSO, because the presence of DMSO or its residual will adversely affect the hybridization, and then resupended in 18ìl of nuclease free water.

d. Preparation of Hybridization Sample: The hybridization mixture [10XGE blocking agent (4.5µl), 2X Hi-RPM hybridization buffer (22.5 µl)] along with labeled miRNA sample was heated for 5 min at 100^0C and immediately cooled to 0^0C.Blocking agent was used for the attachment of the labeled miRNA sample with the probe. And the hybridization of the buffer was used to achieve the uniform rotation. Each 45ml sample was hybridized onto a micro array at 55^0C for 20hours, in a hybridization chamber.

e. Agilent's Hybridization Oven, equipped with a removal rotator rack, and designed with user settable rotator speeds and variable hybridization temperatures, is optimally suited for bubble – mixing and microarray hybridization processes used by the Agilent SureHyb microarray chambers can be seen in Fig.5.

f. Micro array wash: After hybridization, slides were washed 5 min in GE wash buffer 1 at RT and again for 5 min in GE wash buffer 2 at 37^0C, followed by an acetonitrile wash for 1 min at RT to dry the slides complete.
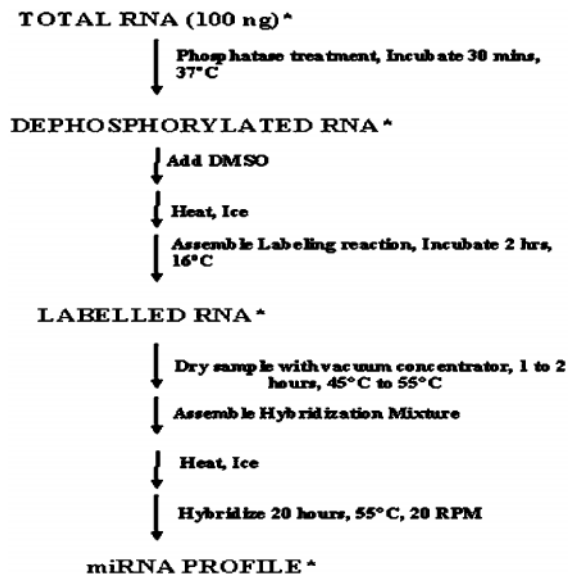

Fig.4: Workflow for Sample Preparation and Array Processing

Fig.5: Hybridization Chamber (Arrow Indicates the Gasket which Holds the Micro Array Slide and Placed in the Hybridization Chamber)

g) Scanning and Feature Extraction: Slides were scanned on an Agilent microarray scanner (model G2565BA) at 100 and 5% XDR settings. Agilent Feature Extraction software version 9.3.5 was used to extract the raw data.

h) Agilent Scanner: The Agilent DNA microarray scanner is a 48-slide scanning system enabled by Sure Scan high –resoloution Technology capable of scanning with 2,3,5 or 10 micron resolution that can read 1x3" glass slide microarrays and seamlessly feature extract them all using Agilent's feature extraction software with robust statistical analyses.

2.2.4. Extract Data using Agilent Feature Extraction Software 9.5.3

Feature Extraction provides the information from probe features is extracted from micro array scan data, allowing researchers to measure miRNA expression in their experiments. The images obtained after scanning are subjected to image analysis using the FE. Later these are analyzed by Gene Spring GX 10, for further analysis. The

sample given was one normal and one osteosarcoma bone FFPE tissue, the replication of each sample was done for the comparison purpose. Thus generated total four images, two from control sample (Normal tissue), and two from non control sample (abnormal or osteosarcoma tissue). Microarray image of normal and osteosarcoma case can be seen in Fig.6.
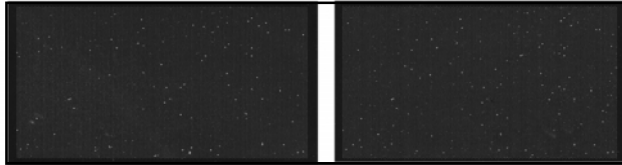

Fig.6: DNA Microarray Image. (a) Normal or Control. (b) Osteosarcoma or Non Control

2.2.5. Data Analysis by GeneSpring GX 10

The data analysis is done by using the software GeneSpring, version GX 10. Provides powerful, accessible statistical tools for fast visualization and analysis of expression data. Designed specially for the needs of biologists. GeneSpring GX offers an interactive desktop computing environment that promotes investigation and enables understanding of DNAmicro array data within a biological context. The obtained intensity values of miRNA detected in normal and abnormal were tabulated and generated cluster, scatter plots.

2.3. Methods Involved in Image Processing of DNA Microarray Image

The platform used for the software implementation is MATLAB; version 7.3.0.It is a high performance language for technical computing. Image Processing Toolbox of MATLAB is used. The images are processed and analyzed to extract intensity of spot in the micro array image. The steps include:

i) The Gridding or assigning coordinates for each spot in the image.

ii) Foreground separation, Segmentation of spot.

iii) Intensity extraction of each spot. These techniques are described in the result and discussion.

3. RESULTS AND DISCUSSION

3.1. Results from DNA Micro Array Technique

The intensities and background subtracted were taken from the raw data file generated from feature extraction. The obtained values and image from the feature extraction is further subjected to data analysis by the use of GeneSpring GX 10.The sum of background subtracted signals for each

repeated miRNA was calculated. To compare between the samples further normalization (50th percentile normalization) step was performed. (50th percentile normalization: values below 0.01 were set 0.01.Each measurement was divided by the 50th percentile of all measurements).After experiment, the total number of probes in array excluding controls is:11180.Number of miRNA's repeated more than one time with different probe ids:534.The repeated miRNA were grouped in both normal and cancer bone, as shown in the Table.1.

Table 1
miRNA Detection in Cancer and Normal Tissue

Group	miRNA Detected
Cancer tissue compared to normal bone	32
Normal bone compared to cancer tissue	75

The 'Fold change' cutoffs have been widely used in microarray assays to identify genes that are differentially expressed between query (cancerous) and references (normal) samples. More accurate measures of differential expression and data normalization strategies are required to identify high confidence sets of genes with biologically meaningful changes in transcription. The results are obtained and tabulated, then plotted for the same. The Heat map was generated for the arrays hybridized based on summed background-subtracted signals of each repeated miRNA's. The overview of all the miRNA's in the array is as shown in the form of cluster in the Fig.7.
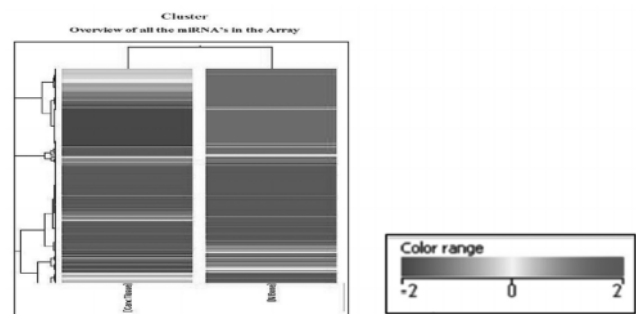

Fig.7: Overview of all the miRNA's in an Array. Yellow: Non Detected miRNA's. Green: miRNA with Low Detection Level. Red: miRNA with Low Detection Level

• Scatter plot showing the miRNA's distributed based on their expression values miRNA's in Red blocks indicate the miRNA's detected. These are shown in scatter plot can be seen in Fig.8.

• Line Plot: Line plot is created for the values of both normal and cancerous bone and also only for cancer bone can be seen in Fig.9.The plot provides the comparison between normal and abnormal tissues. Plot in blue color indicates the cancer and red plot indicates the normal fold values.
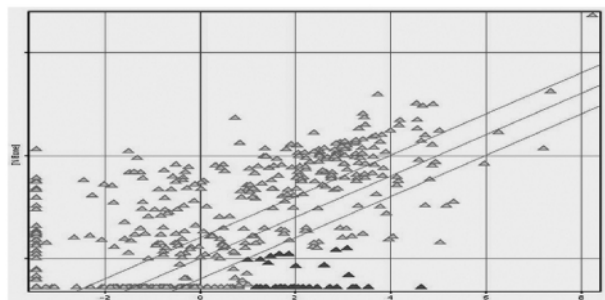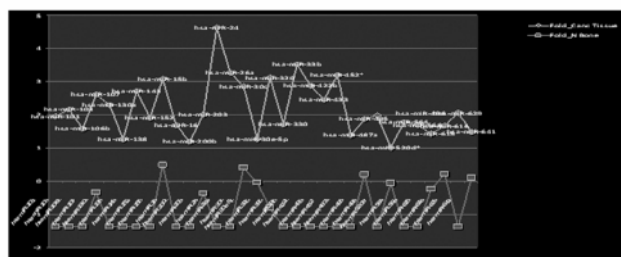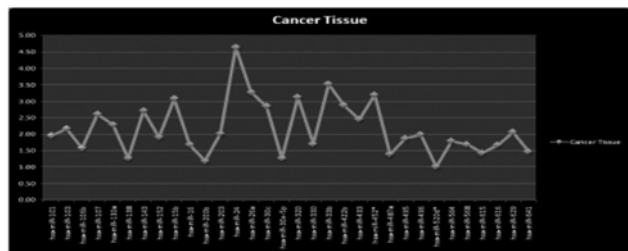
First obtained input image is in the form of JPEG. The image cropping operation is done to extract a region of interest in the input image. Convert RGB image to Grayscale image for spot finding, it is also used to enhance the spots position color, can be seen in Fig.10.
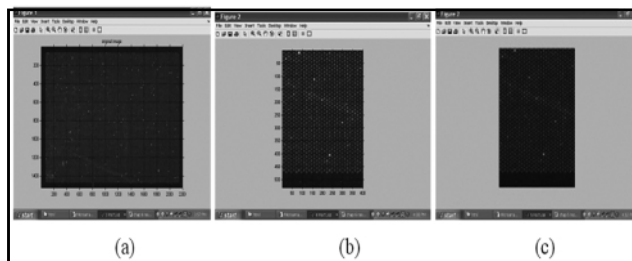

Fig.10: (a) Input Image (b) Cropped Image c) Grayscale Image

To create a regular grid of spots, the mean intensity of each column of image is calculated. The centre of the spots and the gaps between the spots can be found from this step. Autocorrelation is used to enhance the cell similarity of profile and to avoid irregular profile. This results in peak finding and estimation of spot spacing. The spacing estimates are used to design a filter to remove the background noise from intensity profile thus enhances horizontal profile, as shown in Fig.11.
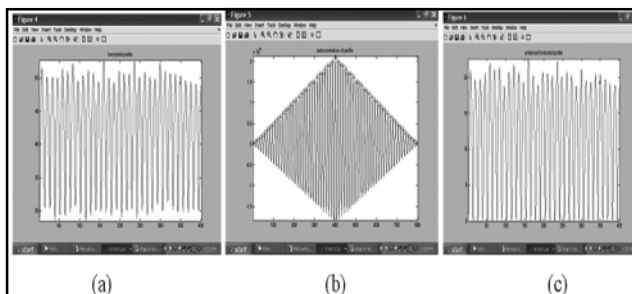

Fig.11: (a) Horizontal Profile (b) Autocorrelation (c) Enhanced Profile

Segment peaks or labeled regions are numbered with 'bwlabel' command. The centres or centroids are located. The mid-points between the adjacent peaks provide grid point location. Put bounding boxes around each spot. Transpose and repeating of all the steps used to obtain horizontal spacing, and thus found rectangular grid, can be seen in Fig.12 (a) and (b).To segment spots from background by thresholding apply a single threshold level to the whole image so all spots detected equally, but it presents large differences in spot brightness. To equalize large variations in magnitude intensity values are transformed to logarithmic space but spots are still missed. The bonding boxes can be used to determine local thresholding. Local and global threshold are combined by using the logical operation on the binary masks to get better spot segmentation results.

Fig.8: Scatter Plot Showing the miRNA's Distributed based on their Expression Values miRNA's in Red Blocks Indicates the miRNA's Detected in Cancer Bone (fold>1) Compared to Normal Bone (fold<0.5). All the Red Blocks Fall under Positive Values. Note: X Axis: Cancer Bone and Y Axis: Normal Bone

Fig.9: Line Plot. (a) Expression Level of Detected miRNA's in Normal Bone. (b) Line Graph of Cancer Bone. All the miRNA's Expression Level are >1. The Graph Represents the Fold Values of the Detected miRNA's in Cancer Bone. X-axis-miRNA's, Y-axis: Fold Value

3.2. MATLAB Results of DNA Microarray Image Processing

The control and noncontrol sample is stained using single color dye, Cy3 (green), consists of A rows and B columns of genes. The controlled microarray image obtained is the normal bone microarray image and noncontrol microarray image obtained is the osteosarcoma microarray image. The results are shown for one control and one noncontrol microarray image. The qualitative analysis includes gridding, spot finding and image segmentation. Quantitative analysis gives us the intensity of genes based on their expression levels.
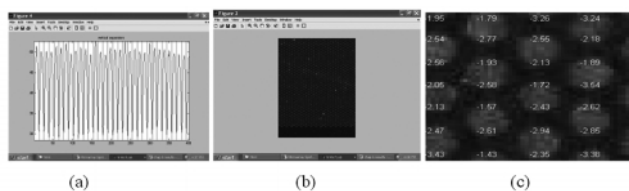
Fig.12: (a) Vertical Grid (b) Gridding (c) The Nominal Intensity Over the Spot is Displayed

The silhouettes of some spots still contained pinholes. The whole image could be filled using a single call to 'imfill'. Extract first spot for measurement and its intensity. The nominal intensity over the spot is displayed as shown in Fig.12(c).

4. Conclusion

The work presented in this project shows some very interesting results in a field where very little research has been done on DNA microarray bone image analysis. The Human Bone samples are obtained from Institute of Oncology, of both normal and osteosarcoma case has been taken up and they are analyzed at genetic level. This research work is funded in part from the Department of Science and Technology, Govt. of India, New Delhi.

The Emerging DNA Microarray Technology has been utilized to get the microarray images of human bone samples for both normal and osteosarcoma case, and intensity of each detected miRNA in both normal and osteosarcoma case has been obtained. The miRNA quantification is achieved from this powerful technique.

The DNA microarray image analysis of bone in MATLAB provides a way to extract the intensity of gene expression or which corresponds to each spot in the image. In the plots it is clearly shown the expressed and suppressed genes & miRNA's for further research.

The detected miRNA expression for the human bone sample from the microarray technique, are associated with clinical variables of cancers, so the miRNA's can be readily used for cancer diagnosis and prognosis. Each detected miRNA in cancer can be studied for further research and drug development.

The major roles of miRNA's in regulating genetic regulatory circuit and also in the response of immune system give us a clue of the impact miRNA would have on vaccine research and development.

5. Future Enhancements

During processing of many DNA microarray images in MATLAB, it was realized that a GUI based, user interface is needed to change the image file to be analyzed. So that user can simply select the file to be analyzed. This technique makes more automatic image analysis.

Further developments can made in future to achieve better gridding techniques for the microarray image using MATLAB. The microarray images of other cancers case can be analyzed.

Acknowledgement

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