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RT-PCR and DNA microarray measurement of mRNA cell proliferation

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Abstract**—** *For mRNA quantification, RT-PCR and DNA microarrays have been compared in few studies (RT-PCR). Healing callus of adult and juvenile rats after femur injury was found to be rich in mRNA at various stages of the healing process. We used both methods to examine ten samples and a total of 26 genes. Internal DNA probes tagged with 32P were employed in reverse transcription-polymerase chain reaction (RT-PCR) to identify genes (RT-PCR). Ten Affymetrix® Rat U34A cRNA microarrays were hybridized with biotin-labeled cRNA generated from mRNA. There was a wide range of correlation coefficients (r) between RT-PCR and microarray data for each gene. Meaning became genetically unique because of this diversity. Relatively lowly expressed genes had the highest r values. The distance between PCR primers and microarray probes was found to be higher than previously assumed, leading to a drop in agreement between microarray calls and PCR outcomes. Microarray research showed that RT-PCR expression levels for two genes had a "floor effect." As a result, PCR primers and microarray probes that overlap in mRNA expression levels can provide good agreement between these two techniques.*

Keywords**— mRNA, DNA, RT-PCR, DNA Microarray.**

I. INTRODUCTION

Many methods exist for determining the amount of messenger RNA (mRNA) (Lung, et al., 2017). reverse transcription PCR (RTPCR) has been a common technique for genes with low expression levels (RT-PCR). For each experiment, this semiquantitative approach takes a lot of time and provides data for only one or a few genes (Kiyama, 2017). For this reason, scientists are now looking to develop more effective ways for measuring the quantities of mRNA in several genes simultaneously. Gene expression levels can be measured simultaneously using DNA microarrays since a single hybridization results in an assessment of all genes on the array simultaneously (Narancio, et al., 2018). Many interesting gene transcripts can be studied this way, and new genes implicated in the system under research can be discovered. Few studies have been done to compare the results of DNA microarrays with those of older, more established technologies (Shekha, et al., 2013), despite their enormous potential. Insufficient research has been done on the factors that influence microar- rays' ability to accurately measure mRNA levels. For a long time now, our group has investigated the healing of fractures in a rat model (Xiang, et al., 2021). The mRNA expression of multiple genes in the fracture callus of adult and juvenile rats following a simple transverse diaphyseal femoral fracture has recently been measured by RT-PCR (Pala, et al., 2021). This approach was chosen because of the speed with which it can look at a large number of gene transcripts.

Affymetrix GeneChip DNA microarray technology was used to reexamine a subset of these samples after an Affymetrix DNA microarray facility (Rashid & Saler, 2018) became available (Sultankulova, et al., 2017). As a result, both methodologies were used to examine the same data set. According to our findings in this research, we compared the RT-PCR results to the DNA microarray results for identical specimens.

II. MATERIALS AND METHODS

In a nutshell, an intramedullary rod was inserted retrogradely into the left femur of anesthetized rats (Rashid, 2021). Inducing a closed simple transverse mid-diaphyseal fracture of the femur was done. The femora were quickly extracted and one-third of the femoral length, focused on the fracture site, was collected and frozen in liquid nitrogen at time points of 0 (no fracture), 1, 2, 4, and 6 weeks after fracture. For each time point, two samples were taken from each age group.

DNA Microarray

Each microarray sample used RNA from two rats of the same age and stage. In order to synthesize double-stranded cDNA from 30 g of RNA, we used a SuperscriptTM Double Stranded cDNA Synthesis Kit (GIBCO/Invitrogen) An Enzo® RNA Transcript Labeling Kit was used to produce biotin-labelled cRNA by in vitro transcription, and cRNA fragments were isolated from this product. Each sample was tainted with a combination of bioB, bioC, bioD and cre. Ten Rat U34A microarrays were hybridized with the cRNA (one for each sample). Fluorescent biotin labels were attached to hybridized arrays using the Affymetrix Fluidics Station 400, followed by a second biotin-labeled antibody, and then a final staining of the biotin.

RT-PCR Gene Expression

Genes each have a P-labeled internal oligonucleotide (probe) that was employed for hybridization of the blots (Vina-Rodriguez, et al., 2017). Southern blot radioactivity was measured using the Fujifilm Bio-Imaging Analyzer BAS-1500 and MacBAS Ver.2.5 software. In pilot studies for each gene, the number of PCR cycles was varied to determine the frequency response of the result.

III. FINDINGS AND DISCUSSION

Each microarray sample contained RNA from two rats. Gene expression data from these two rats was averaged to provide a single gene expression value. RT-PCR and microarrays were used to generate a data set with 10 matched values for each gene. Linear regression, linear correlation, and t tests were used to compare these results (de Souza, et al., 2017).

A total of 34 gene transcripts were detected in our previous study of mRNA gene expression during fracture healing (Pala, et al, 2021). The Rat U34A array failed to locate five of these genes. mRNA for two more genes, VEGF and IL-1, were found in several isoforms. IL-1 and VEGF were both compared to their single gene counterparts on the U34A array, and one of each was found to be significantly different from the other two. RT-PCR and microarray analysis were able to identify a total of 26 genes for comparison.

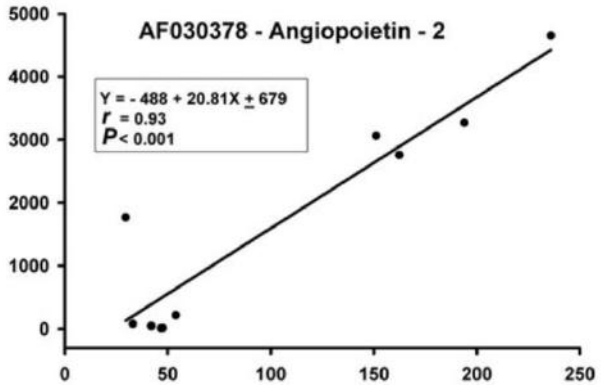

Fig.1: Correlation test for Angiopoietin

The expression of mRNA genes changed over time following a fracture (Shen, et al., 2019). Typical outcomes included the highest mRNA expression of a gene must be induced before its transcript can no longer be detected in undamaged bone fracture, and subsequently return to undetectable levels at 2 weeks and 6 weeks after the fracture (Bulut, et al., 2020). A wide variety of expression levels across the 10 samples analyzed for each gene was obtained, with several samples being undetectable early and late in the healing process.

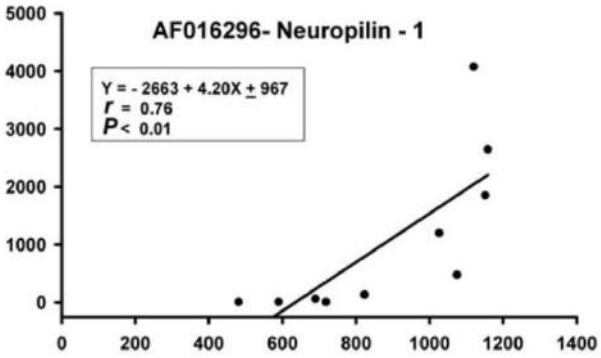

Fig.2 : Correlation test for Neuropillin

For these 10 samples, RT-PCR results were associated with microarray data (one measurement per point). A total of 26 correlation coefficient values were generated, ranging from -0.48 to +1.93, for the 26 genes in the study. Some genes, such as angiopoietin-2, showed high connections between the two techniques. Osteocalcin, for example, has a lot of discrepancies in the data.

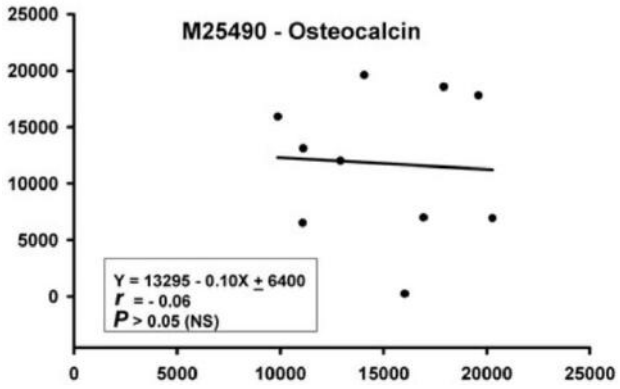

Fig.3 : Correlation test for Osteocalcin

When data were selected from a bivariate normal distribution, the mean correlation coefficient (r) was 0.28 with a 95 percent confidence interval (CI) of -0.42 to +0.77. A significant (P = 0.05) departure from the expected distribution of r values was found, however. In other words, this showed that the observed variation was more than what would be predicted by chance alone, and that some genes were better correlated than others between the two techniques.

There were 271 and 225 microarray values for the tested gene transcripts, respectively. Overall, the global mean of 500 genes on microarrays differed considerably from the microarray median of 225 (P 0.05). Studying genes associated with fracture repair has been a focus of this data set (Cao, et al., 2018). The majority of cytokine genes are inactive. As a result, the average signal value fell below the average of all genes on the array. A different set of data with more consistent expression levels and less fluctuation for each gene transcript may provide more generally good agreement. Moreover, for genes with modest levels of expression, newly developed software techniques may produce more useable data (Rashid, 2017).

Table 1: Correlation Test

Nucleotide Separation	Correlation value	Absent calls	Correlation value
< 410 nucleotides	>1000 nucleotides	<4 calls	>5calls
0.69 ± 0.05 0.11. = 0.07	0.51 ±	0.62 ± 0.05 0.11±0.11 = 0.05	

RT-PCR revealed a floor effect for the mRNA of two genes (FGF receptor-1 and neuropilin-1). RT-PCR was unable to detect many spots on the microarray for each of these genes, resulting in a non-linear graph for that gene. The lowest mRNA expression levels of three additional genes (osteocalcin, type I collagen, and bone sialoprotein) with high expression levels were undetected by RT-PCR but discernible by microarray, including osteocalcin.

Other laboratories have looked at the linearity of the PCR at constant numbers of PCR cycles, and the results imply a linear relationship between concentration and amplimer signal levels (Uno, et al., 2021). According to research on mRNA quantified by microarray, there is a linear correlation between the micro-array signal and mRNA concentrations (Rashid & Basusta, 2021). When developing our PCRs, we made every effort to ensure that the peak levels of each gene's expression were within the lin-ear range of amplification (Shifman, et al., 2019). RT-PCR may have missed low expression levels because of this. For genes with moderate expression levels, the dynamic range of microarrays may be superior to that of PCR. For several genes, there was a lack of agreement between the two techniques, and two hypotheses were tested to see if they might explain the discrepancies.

Clearly this is the case. Primers for PCR were used that overlapped the locations of the microarray probe sets in order to see if increasing the number of absence calls would have any influence on gene expression levels in 16 genes with moderate levels of expression. The correlation coefficient for these genes was significantly (P 0.05) lowered by an increase in the number of absent calls (Comtet-Marre, et al., 2018) among the 10 data points for each gene (Table 2).

The position of the probe was also tested. Probes for PCR and microar- rays tend to be located closer to the 3' and 5' ends of the DNA sequence, respectively. It was found that just three genes had microarray probes in the 3′ untranslated region. Genes were identified by their PCR primers and micro-array probes and their nucleotide segregation was assessed. We focused on 11 genes with low expression levels and a small number of microarrays absent call calls. The correlation coefficient (Table 2) for these genes dropped close to significance (P = 0.06) when a distance of more than 1000 nucleotides separated the PCR primer position from the microarray probe set location. Nucleotide separations ranging from 400 to 1000 nucleotides were not included in this study. Measuring a protein synthe- sis-supporting intact mRNA would appear to produce similar results regardless of the location tested. It is possible that some sequences are more prevalent than others because of cellular mRNA fragmentation or alternative splicing. It's unclear if this explains the discrepancy in the data.

Data from the 26 genes were correlated with the average microarray values, as seen in the graph below. A wide nucleotide separation between the PCR and microarray probe sets, or both, can result in missing call signals. There was poor agreement between the two approaches for all

genes with low mean microarray fluorescence. Genes with mean fluorescence intensities between 100 and 4000 were found to have the best agreement. Some genes' correlation coefficients were reduced because of an increase in absent calls or probe separation; however this was not the case for all of them. Gene-specific interpretation is made possible by this variation. The data from some genes can be used to make valid physiological inferences despite the low signal levels; however, the data from other genes may be more difficult to interpret. Despite the fact that each gene is assigned a signal value by the microarray scanner and related software, the absence of a call indicates that the data may not accurately reflect mRNA transcriptional activity.

IV. CONCLUSION

Inadequate concordance for osteocalcin had no evident cause. The PCR primers and microarray probes for this gene overlapped, hence no missing calls were found. But there was an extremely high signal strength. There is no upper limit to fluorescence measurements, as genes typically reach 4,000 units in intensity. The detector was not saturated based on histograms of the data distribution, as there was no evidence of truncation at the upper end of the data. This gene's probe sets on the a-ray may be saturated, according to one theory. Rather of a fixed microarray signal value, biotin tagging of the cRNA may determine saturation. Because of the reduced biotin labeling, a gene with lower biotin labeling may oversaturate its probe sets at lower fluorescence levels.

Only a small number of research have compared microarray results to those from other approaches for assessing mRNA levels. While both commercial and custom-made microarrays were found to be in agreement, for genes that grew in abundance, both micro-arrays assessed smaller increases in mRNA levels than those determined by real-time polymerase chain reaction (PCR) (Bulut & Rashid, 2020). Findings from Northern analysis and spotted microarrays have identical sensitivity levels when compared, indicating good agreement between the two approaches (Taguchi, et al., 2021). This research was carried out by Rashid, et al., (2018), who investigated gene expression in the suprachiasmatic nuclei of mice. Quantitative PCR confirmed the diurnal cycles observed on oligonucleotide arrays for several genes (Cho, et al., 2019). It has also been shown that serial gene expression analysis ranks genes in the same order as oligonucleotide microarray, which has also been compared (Um, et al., 2017).

Finally, RT-PCR and DNA microarray measurements of mRNA gene expression were shown to be in good agreement for genes with moderate levels of expression and PCR primers positioned close to microarray probes. A lack of agreement between the PCR primers and microarray probes of genes with high or low levels of expression, or those with a considerable distance between the two locations, was common.

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