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ORIGINAL PAPER

Universal Reference RNA is Not a Representative Normal Sample for Oligonucleotide Microarray Studies

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Abstract Translational research has been defined as the scientific study using human material that will ultimately generate patient specific data. A major caveat in human directed study is the availability of high quality and quantities of patient derived homogeneous cells for analysis. Whereas there exist sources for which tumor tissue and blood samples can be made available, the same cannot be said for normal tissue. The absence of normal control tissue has led to the creation of pooled cell lines and tissues for purchase known as "reference RNA". Although initially created for purposes of standardization, the difficulty associated with acquiring normal tissue has led some investigators to use sources of universal pooled RNA for comparative analysis with clinical tissue specimens. In order to study the effects of using Universal Reference RNA on expression profiling experiments we have evaluated the performance of universal RNA compared to RNA obtained from a purified population of colon epithelial cells in defining a set of altered transcripts in colon cancer.

Keywords Reference RNA · Oligonucleotide expression microarray · Experimental bias

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Introduction

The identification of genetic abnormalities in cancer cells has been at the center of efforts to understand the fundamental causes of cancer. Over the past decade, the development of microarray technologies has been one of the most enabling developments fueling this effort, by allowing comprehensive analyses of genetic changes at high resolution. The application of these technologies have clearly been facilitating in respect to the understanding of the sum of genetic changes that occur during the complex series of events leading to the initiation, development and progression of cancer cells. One issue that consistently plagues these studies however, is obtaining adequate matching normal cells of the same lineage as the tumor cell being studied. The most well designed experiments involve analysis of the differences in expression levels of genes between normal cells and their malignant counterparts. The lack of adequate normal cells and tissues however, have resulted in a search for alternative sources designed to serve as control RNA. To provide reproducibility in control samples, several investigators have used "reference RNA", a commercially available product as a normal control sample [1]. Reference RNA was originally developed as a control for many molecular biological procedures and has been used extensively as a baseline for two-color fluorescence cDNA arrays [2, 3]. The utility of these reference RNA products was originally suggested for the standardization, cross-referencing and data comparison between intra and inter-laboratory microarray experiments [4]. Specifically, the variability in array designs of oligonucleotide-based arrays, and the very diverse protocols for labeling, hybridization, washing, staining, imaging and normalization conditions required to achieve optimum performance from each array design, have caused many to

champion the use of reference RNA in order to be able to directly compare expression profiling data across multiple array types. These products are derived from a variety of animal or even human tissue or cell lines, but are generally distributed as a collection from pooled samples. However, because of the lack of availability of normal tissues or cells and a paucity of adequate material for reference material, some, even as recent as 2007, have adopted their use for comparisons with experimental tumor data [5–8]. Here we describe experiments designed to discern the implications of using universal RNA as a control to compare with tumor cells and suggest that skewed expression array data that may not necessarily be biologically informative will result. We have recently described a cell procurement technique capable of enriching for epithelial cells and using this technology, we were able to obtain matched samples consisting of non-neoplastic and neoplastic epithelial cells from the same colonic resection specimen [9]. Microarray analysis was performed on the RNA isolated from each sample as well as on a commercially available reference RNA. The expression profile for the "normal" sample was compared to the reference RNA sample to emphasize the inherent differences between the two. To demonstrate the importance of using matched normal cells as the control for tumor cells in microarray experiments, differences in the data resulting from the comparison of the tumor vs. normal (T–N) and tumor vs. reference (T–R) were noted. Our findings demonstrate the potential to generate biased data if matching normal cells are not used as the control sample.

Materials and Methods

Reference RNA Reference total RNA was purchased from a commercially available source (Universal Human Reference RNA, Stratagene, CA,USA). This particular product was chosen because of its availability at the time of the experiment and because it is the most widely used reference RNA for molecular biological studies. Other types of reference RNA have since entered the market. This particular reference RNA is a pooled composite from the following 10 different cancer cell lines, four of ectodermal origin: adenocarcinoma of the breast, cervix, glioblastoma multiforme and melanoma; one of endodermal origin: hepatoblastoma of the liver; and five of mesodermal origin: embryonal carcinoma of the testis, liposarcoma, Hodgkin's lymphoma, plasmacytoma and T-cell lymphoblastic lymphoma. The product was received on dry ice and kept in a −80°C deep freezer until use.

Sample procurement of normal and neoplastic colon cells Approval for the procurement of cells from human tissue was obtained from the Institutional Review Board at the State University of New York at Buffalo. A hemicolectomy specimen, excised for curative purposes, was obtained in the operating suite at the time of extirpation. The specimen was transported unopened on ice to the Department of Pathology in less than 5 min where it was opened and briefly rinsed in 0.9% normal saline. At a distance greater than 5 cm from the grossly obvious tumor mass, non-neoplastic colonic epithelial cells were manually exfoliated as previously described (Fig. 1) [9]. This was accomplished by applying the edge of a glass slide to the exposed surface of the opened colonic resection specimen and gently scraping it. The exfoliated cells were then deposited into a microcentrifuge tube containing PBS and immunomagnetic beads embedded with the ber-Ep4 antibody (Dynal Epithelial Enrich, Invitrogen) for positive cell enrichment. A colon specimen obtained from a patient with a colon tumor was utilized in this experiment because (1) both normal and neoplastic colonic cells strongly and uniformly express the antigens recognized by the ber-Ep4 antibody conjugated to the magnetic bead, (2) there is no intermingling between normal and neoplastic colonic epithelial cells in the invasive areas of colonic adenocarcinoma and (3) surgical excision of the colon for tumor typically is performed with ample amounts of uninvolved colonic tissue, an approach done in order to ensure negative involvement of the surgical margins by tumor. The exfoliated cells and immunomagnetic beads were allowed to equilibrate through gentle rocking at 4°C for 20 min. Unbound cells were then washed off after a magnet was used to immobilize and retain the magnetic beads and bound cells. This wash step was performed twice.

Tumor cells were similarly procured by scraping the cut surface of the tumor after the interior of the mass was opened by a scalpel blade. The exfoliated tumor cells were enriched in parallel with the "normal" colonic epithelial cells. This method of exfoliation and enrichment for selecting specific cell types was previously shown to be advantageous to procurement from fixed tissue and a viable alternative to procurement from frozen tissue [10].

RNA isolation The enriched cells from both the normal and tumor samples, from the same patient, were lysed using a micropestle. Cells were lysed in the presence of Trizol (Invitrogen) and RNA isolation performed in accordance with the manufacturer's recommendations. Recovered RNA was further purified using Qiagen spin columns.

Assessment of RNA integrity RNA integrity was assessed through capillary electrophoresis using the RNA 6000 PicoLabChip kit on the Agilent 2100 BioAnalyzer following the manufacturer's recommendations, specifically we expect discreet 18S and 28S peaks with no DNA

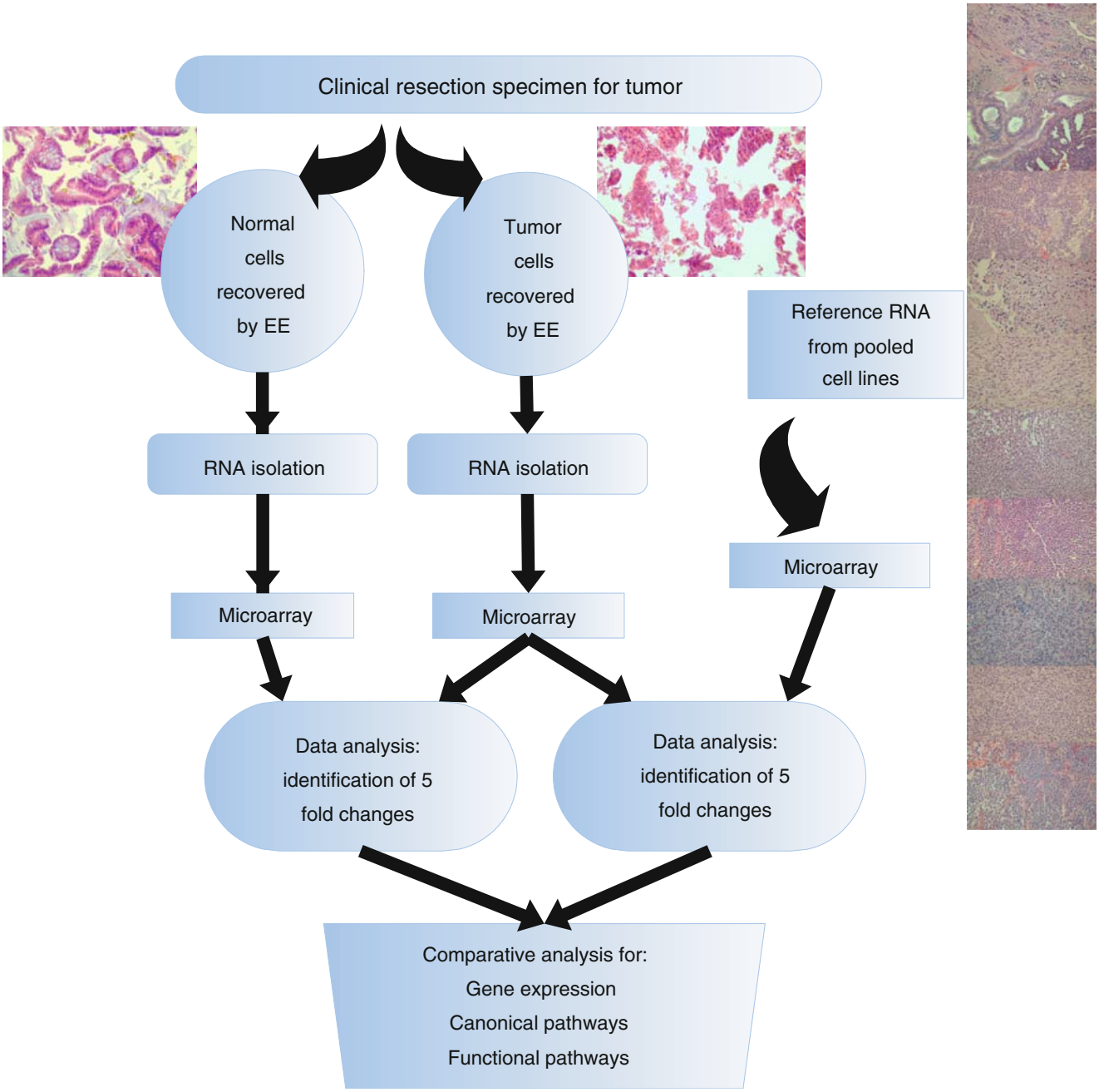

Fig. 1 Overview of experimental design. Enriched cell populations of normal and tumor were procured by the technique of exfoliation and enrichment (*EE*) from a colonic tumor resection specimen. Cytologic examination of both samples confirmed successful enrichment for either non-neoplastic colonic epithelial or tumor cells (*insets*). A commercially available reference RNA was purchased and found to consist of 10 pooled cell lines. A morphologic representation of each tumor type is demonstrated along the right border. See text for details on the composition of the reference RNA. General observations of the resulting microarray data as well as more detailed and in depth analysis using IPA software was performed on the resulting data

contamination. RNA integrity was assessed for all three samples: enriched normal colonic epithelial cells, enriched tumor colonic epithelial cells and reference RNA.

Microarray Description Equimolar quantities of RNA from the three separate samples were prepared for amplification as previously described [11]. Total RNA was isolated and purified using RNAeasy columns. Total RNA double stranded cDNA was synthesized using the Superscript Choice System. A T-7 (d24) primer was used to prime the first strand cDNA synthesis. An in vitro transcription reaction was then followed by a second round of amplification. The final *in vitro* transcription reaction was performed in order to further amplify and biotinylate the samples. The full-length cRNAs were then fragmented to 20–200 base pairs. Labeled cRNA was then fragmented and

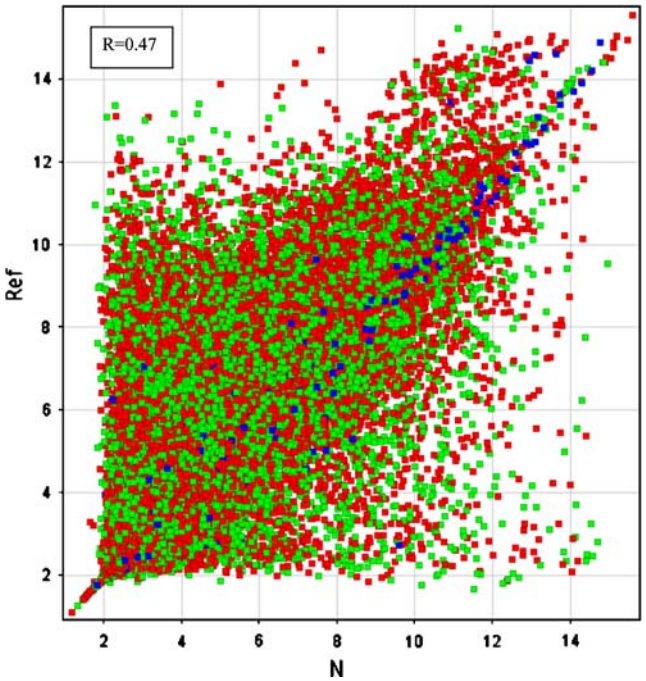

Fig. 2 Correlation between normal and reference RNA. In order to demonstrate where potential bias in data can originate from, a Pearson correlation was performed on the microarray data between the RNA from the normal colon cells and the reference RNA. The resulting score was 0.47, indicating that these two separate sources may lead to widely divergent results when individually used as a control against a tumor sample

hybridized unto Affymetrix HGU 133 Plus 2.0 chips, which are arrayed with sequence specific oligonucleotides representing 54,000 genes.

Data analysis In order to emphasize the inherent differences between the matched normal cell profile and the reference RNA profile, a scatterplot was generated and exported to excel to allow for the generation of a Pearson correlation ratio (Fig. 2). A linear correlation with a ratio greater than 0.7 would indicate a strong positive correlation between the samples. A low correlation ratio would indicate the presence of a number of differences in the gene probe set hybridization values between the RNA from the matched normal cells and the reference RNA.

Quantile normalization between all arrays and probe level signal estimation was done using the GCRMA method. This method is similar to the RMA algorithm [12], but with the added feature of correcting for intensity bias due to G-C content. GCRMA uses only the perfect match (PM) and ignores the mismatch (MM) which by doing so has been shown to reduce variation in the data. The normalized PM values are then log transformed and a Tukey's median polishing procedure is applied to each probe set resulting in a final intensity value.

Using Genetraffic software from Stratagene the normalized data was then analyzed using a head to head three way comparison between the normal (N), tumor (T) and reference (R) samples using $p=0.05$. Because only one sample was obtained from each source, we concentrated our initial analysis to the gene probe sets that expressed a five-fold difference between the N and T samples. The three-way head to head analysis was performed on these gene probe sets found to demonstrate a five-fold difference between these samples. The data set was queried for biased results by looking for gene probes that were up-regulated when N was used as the control and compared to T, but down-regulated when R was used as the control and compared to T (Fig. 3A). The complete opposite results, down-regulated and up-regulated gene probes when N was compared to T, or when R was compared to T, respectively, were identified from the data (Fig. 3B).

The gene expression data was then partitioned into two separate data sets, one composed of the tumor RNA relative to the matched normal cell RNA (T–N), and the other composed of the tumor RNA data relative to the reference RNA (T–R). In gene expression studies, one major goal is in the determination of biological significance once a statistically validated list of differentially expressed genes has been obtained. Some of the most common questions are

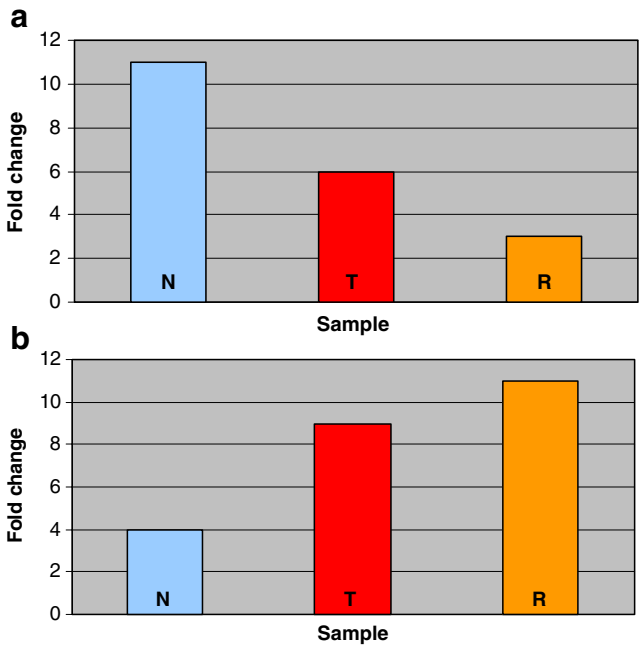

Fig. 3 General observations using a three way comparison of the microarray data. A head to head three way comparison between the normal (*N*), tumor (*T*) and reference RNA (*R*) samples demonstrating the potential to generate biased results when R is used in lieu of N as a control against a T sample. A total of 598 gene probe sets were found to demonstrate T values down-regulated five-fold or greater when compared to N, but up-regulated when compared to R (**a**). A total of 1,241 gene probe sets were found to exhibit tumor values up-regulated 5 fold or greater when compared to N, but down-regulated when compared to R (**b**)

(1) which pathways and (2) which biological processes and molecular functions are highly associated with the differentially expressed genes. In order to determine whether there were any biological themes over or under-represented when reference RNA, instead of matched patient and cell lineage RNA was used, we performed a series of pathway analyses using Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com). Canonical pathway analysis identified biologic themes from the transcripts that demonstrated altered gene expression profiles between the two comparison data sets. Canonical pathways in Ingenuity Pathways Assist (IPA) are well-documented metabolic and signaling pathways. A *p*-value is calculated using the right tailed fisher's exact test to determine how closely the experimental data correlates with each canonical pathway. The results are then plotted on a bar graph in decreasing order from the *y*-axis starting with the most significant pathway. A secondary line graph details the ratio of experimental genes present in that particular pathway versus the total number of genes in that same pathway. In addition, to further understand the biological and molecular functions represented by these transcripts, an IPA Functional analysis was performed. Functional pathway *p*-values in IPA are calculated and graphed in the same manner as canonical pathways. However, as the name implies, it calculates p-values based on the number of genes in the dataset that share a common function. A line graph for ratio is not present in this case since the total number of genes for each individual function is not defined. Therefore, the number of genes in the dataset with a common function has nothing to be compared to in order to calculate a ratio. The top 10 canonical and top 10 functional pathways based on *p*-value were identified between each comparison set (Figs. 4 and 5). The presence of significant differences between the resulting comparative data highlights our hypothesis that using reference RNA instead of the RNA from patient and cell lineage matched cells results in biased data.

Results

Amount of RNA recovered and RNA integrity The concentration of the reference RNA was 2,311 ng/µl. The concentration and amount of RNA recovered from the normal colon cells was 4,254 ng/µl and 51,048 ng. For the RNA recovered from the tumor cells, the concentration and amount of RNA was 921 ng/µl and 11,052 ng. Quality control indicators of the initial quality from each sample were satisfactory. The A260/280 ratios for the normal RNA and tumor RNA and reference RNA was 1.84, 1.92, and 2.07 respectively. Distinct peaks for the 18S and 28S ribosomal fractions were present in each electropherogram. Yields for the first and second rounds of amplification and the quality of the amplified second round RNA were satisfactory. The 3′/5′ ratios for the housekeeping genes β-*actin* and *GAPDH* from the microarray data were satisfactory. Ratios for β-actin for N were 2.6, 2.8 for T, and 2.17 for R. Ratios for GAPDH for N were 1.4, 1.5 for T, and 1.0 for the R.

Correlation Plot

The Pearson correlation between the RNA from the normal colon cells and the reference RNA was 0.47 meaning there was minimal concordance in the number of genes with similar hybridization signals.

Microarray Analysis

Gene traffic software identified 3,102 transcripts as being up or down regulated equal or greater than five fold when tumor sample was compared to the normal sample. From this list of genes, 598 were found to demonstrate down-regulation when T was compared to N but up-regulation when T was compared to the R data. An additional 1,241 gene probes were found to demonstrate up-regulation when T was compared to N, but down-regulation when T was compared to R. These biased results accounted for 59% of the gene probes initially identified to exhibit a five-fold difference between the tumor and normal sample.

Similarities and differences were then noted between the comparison sets T–N and T–R. Of the 3102 transcripts in the T–N data, 2311 transcripts were up regulated and 791 were down regulated. For the T–R set, 2793 transcripts were identified as being up or down regulated equal or greater than five fold. These numbers can be categorized into 1,157 transcripts that were up-regulated and 1,636 down-regulated. Further analysis found that only 581 transcripts were similarly up regulated in both comparison sets while only 38 transcripts were similarly down regulated in both. This translates to only 25% of the up-regulated genes and only 5% of the down-regulated genes in the T–N comparison set being present in the T–R set. Alternatively, 50% of the up-regulated genes and 2% of the down-regulated genes in the T–R comparison set exhibited similar findings in the T–N set.

IPA Canonical Pathway Analysis revealed that only two of the top 10 pathways were similar between the T–N and T–R data sets (Fig. 4). One pathway, p53 signaling, was present in the T–N set and not in the T–R set. This is expected, since the T–N set compared tumor cells to matched normal cells, whereas the T–R set compared tumor cells to cultured, but similar, in the sense they are

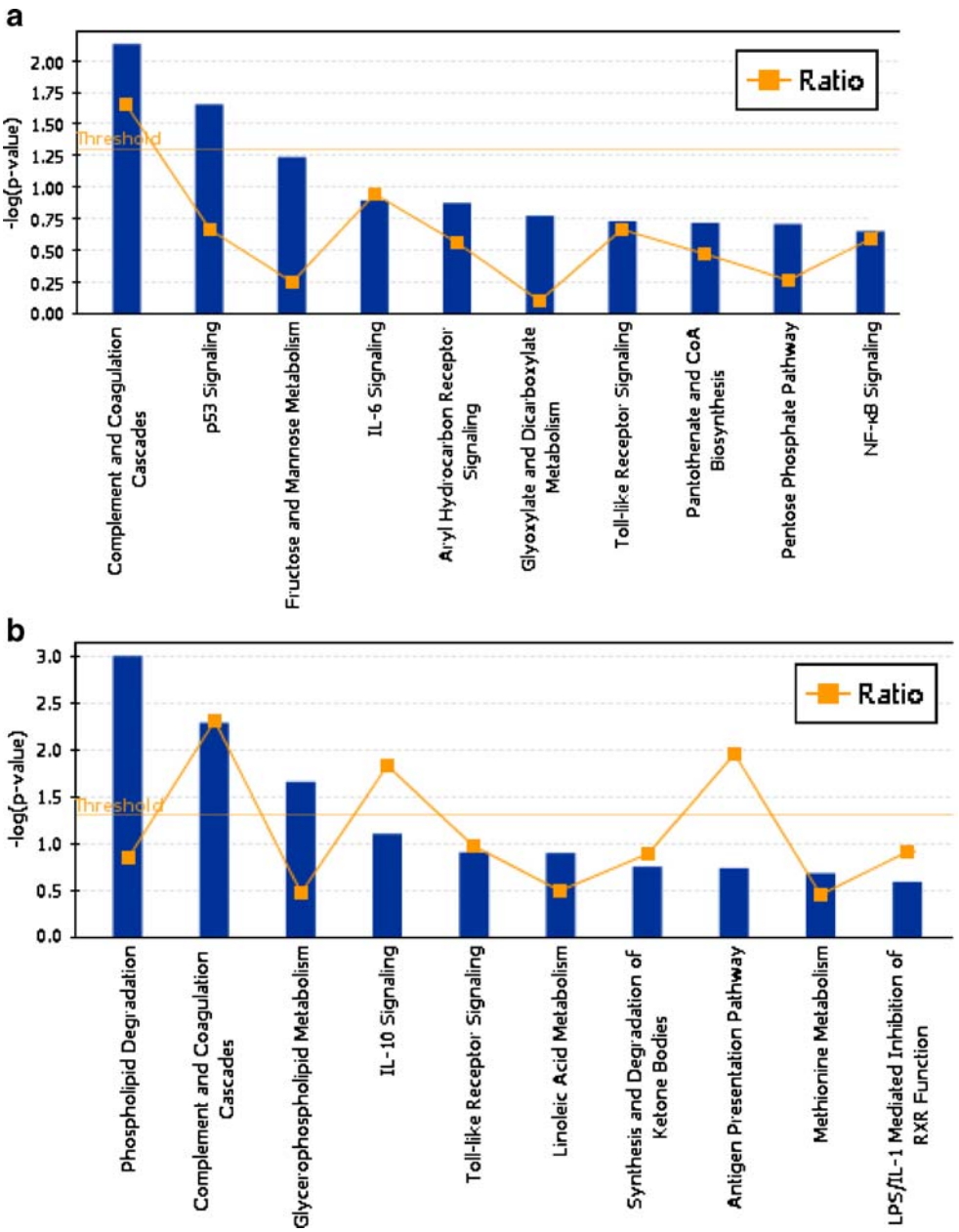

Fig. 4 IPA canonical pathway analysis. Demonstration of the top 10 canonical pathways in the T–N (**a**) and T–R (**b**) data sets

neoplastic, cells. IPA Functional Pathway analysis showed that the transcripts involved in five of the top 10 functional pathways were dissimilar between the two data sets (Fig. 5). Prominently evident in the T–N set but absent in the T–R set were genes associated with cancer and cellular development. Connective tissue development and function were notable in the T–N data set relative to the T–R set. One functional pathway that related to gastrointestinal disease appeared in the T–R but not T–N pathway list. Again, this was to be expected since there were no intestinal derived cells for comparison in the pooled reference RNA sample.

Discussion

Microarray technology holds the potential to advance the scientific communities' knowledge in human disease processes. This is particularly true in regards to cancer, where previous microarray studies have already helped subcategorize and better biologically define hematologic neoplasms [13]. However, attempts at carrying these studies over to the examination of solid tissue tumors have run into several difficulties. One persistent problem is in the acquisition of appropriate normal cells. Since the significance of the data from microarrays studies lies largely on

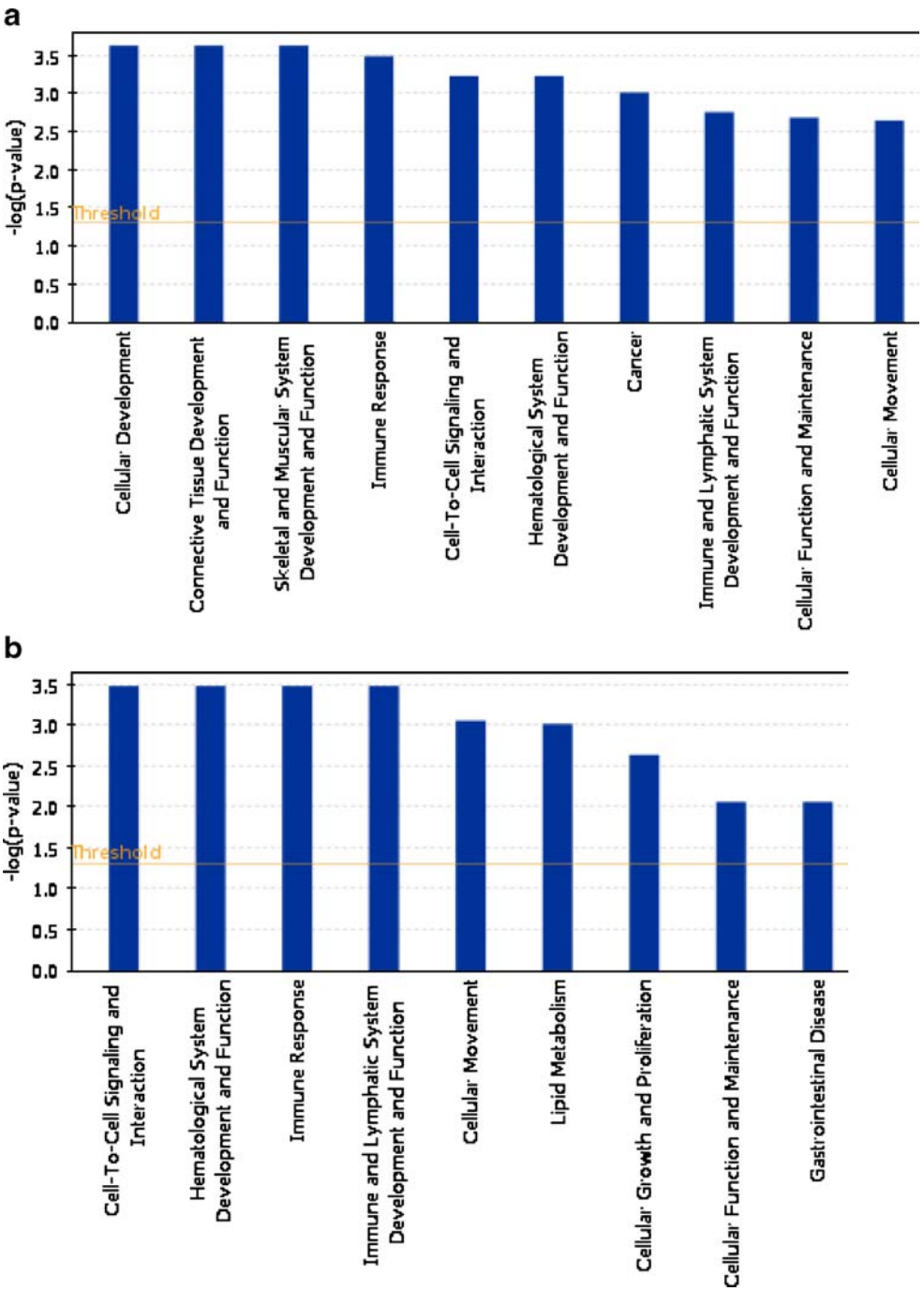

Fig. 5 IPA functional pathway analysis. Demonstration of the top 10 functional pathways in the T–N (**a**) and T–R (**b**) data sets

the differences in gene expression levels, it is scientifically imperative that comparisons be made between tumor cells and their matched normal counterpart. The merit of this requirement is based on the development of cancer along the normal—dysplasia—cancer paradigm developed by Bert Vogelstein. Since "normal" cells for scientific study are scarce, and even if procured, difficult to culture, alternatives have been proposed to enable continued scientific investigation [14, 15]. Industry has contributed through the creation of human reference RNA, a product whose purpose is to be a reference source for the analysis of expression studies. Specifically, it is intended to provide a source that is capable of providing the broadest coverage of as many genes as possible for quality assessment [4]. Deviation from this intended purpose may lead to misdirected findings.

Because genotype determines phenotype, using a cell of a different lineage than the tumor cell being studied may result in skewed data from what it would have been between normal and tumor cells of the same lineage. Complicating data analysis further would be the use of pooled cell lines. This point is evident from the disparate

findings from two recent gene expression studies involving synovial sarcoma. Using synovial sarcoma as the tumor being queried, one study utilized an osteosarcoma cell as a control while the other independent study used a pool of 11 cell lines [16, 17]. As expected, there was minimal overlap in the gene cluster deemed characteristic for synovial sarcoma between the two studies. Although the use of matching normal cells as the control for tumor cells is intuitive, the use of reference RNA as the control persists [5–8].

There are additional concerns about the use of cell lines as reference controls. As is becoming evident, the origin, cell type and even species for existing cell lines may not be truly indicative of that particular cell line [18]. Human errors in communication or labeling may be the cause, but the impact is significant [19]. The artificial environment these cells are grown in may lead to alterations in gene expression due to selection or adaption [20]. Therefore the population of cells and their clones may be skewed towards only a subpopulation of the original tissue source. Finally, differences between individuals and age may account for some heterogeneity in gene expression [21]. These findings support our contention that one of the most important decisions in experimental design is procurement of patient matched, non-neoplastic cells of the same lineage as the tumor cells being tested.

Our findings demonstrate that the use of reference RNA from pooled cell lines in lieu of matched normal cells of the same lineage as the tumor cells being queried will yield discrepant results at multiple levels. The most obvious difference is phenotype. Since genotype determines phenotype, differences in the basic histology of different types of tumors will lead to differences in gene expression profiles. This is evident when the histologic or cytologic appearance of normal cells are compared to representative histologic sections from the tumors present in the pooled reference RNA sample (Fig. 1). The inherent differences in the comparison between normal and pooled tumor cell lines becomes more evident when a Pearson correlation plot is generated from the data between these two samples (Fig. 2). A Pearson correlation of 0.47 indicates little if any similarities between the gene expression profiles between these two samples. The degree of bias becomes more evident when the expression profiles of these genes are compared at the individual transcript level between all three samples (Fig. 3). When T is compared to N, 3,102 transcripts are noted to be differentially expressed five-fold or greater. When the same transcripts from R are introduced for comparison, over half of the data will be biased based solely on the use of R as the control instead of N. A total of 598 gene probe sets will yield completely different data if R is used instead of N for comparison with T. The gene probe data would demonstrate down-regulation of these genes when compared to N, but up-regulation of these same genes when T is compared to R. A total of 1,241 gene probe sets would also yield discrepant results, with genes from T regarded as being up-regulated when compared to N, but down-regulated when compared to R. The biological processes attributable to these biased results included important functions such as DNA replication, DNA repair, transcription, protein biosynthesis, protein amino acid phosphorylation, proteolysis and regulation of cell growth.

The use of sophisticated software programs only compounded the bias. A comparison between the T–N and T–R data using canonical and functional pathway analysis yielded a significant number of discrepant findings (Figs. 4 and 5). The canonical pathway analysis in the T–N data set demonstrated a large number of altered gene transcript levels, as should be expected when neoplastic is compared to non-neoplastic. The most notable differences in the functional pathways between these two sets was the up-regulation of genes involved in cancer and connective tissue development and function in the T–N data set, and genes involved in gastrointestinal disease in the T–R data set.

Our findings indicate that one of the most important factors in deriving useful translational data from clinical tumor material is in the choice of the control tissue. Because expression microarray studies require significant financial resources, adequate preparation should be made in all aspects of experimental planning. Our experiment was not designed to definitively identify and confirm the altered gene transcript levels between a normal and reference sample, but only to survey and highlight that significant differences do exist. Our findings have implications for all discovery-based studies. Using patient and lineage matched cells instead of reference RNA will lead to higher confidence levels in determining which gene should be targeted for further study. In this study, multiple levels of analysis, at the gene probe set hybridization level, at data analysis of the biologic function for these genes, and at the pathway analysis these genes are involved in, the use of a reference RNA in lieu of matched normal cells led to significantly biased data.

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