UNIVERSITY OF VIRGINIA
HEALTH SYSTEM
Department of Pathology

qRT-PCR as a pre-analytic test for transcript microarray analysis of formalin-fixed paraffin-embedded tissues

Patcharin Pramoonjago, Paul D. Williams, Jae K. Lee, Dan Theodoresu, Christopher A. Moskaluk

Introduction

Microarray gene expression profiling has been used to classify tumors(1,2), predict prognosis(3,4), and to predict response to chemotherapy(5,6). The majority of these reports have used fresh or flash frozen tumor specimens as sources of RNA transcript. As the indications for microarray profiling have grown in translational research and clinical medicine, it is the availability of fresh or frozen tumor tissue that has proved to be limiting. The majority of clinical tissue specimens are fixed in formaldehyde and embedded in paraffin (FFPE) for histologic examination. There are several reports of the successful use of FFPE tissue sample in microarray profiling(7-10), but it is still not well established how many archival FFPE clinical tissue samples yield satisfactory results. There have been recent advances in the extraction of RNA from FFPE samples, and in the preparation of labeled probes from FFPE tissue. Here we present the results of our recent experience in isolating RNA from FFPE samples, creating representative probes of transcripts using cDNA isothermal amplification, and in hybridization to Affymetrix oligonucleotide microarrays and comparing the results with those of RNA isolated from matched frozen tissue samples.

Sample code	Tissue type	Years in storage
1	Serous papillary carcinoma of ovary	0
2	Papillary thyroid carcinoma	0
3	Endometriod adenocarcinoma of uterus	1
4	Wilm's tumor of kidney	1
5	Transitional cell carcinoma of bladder	4
6	Transitional cell carcinoma of bladder	5
7	Transitional cell carcinoma of bladder	1
8	Basal cell carcinoma of skin	1
9	Transitional cell carcinoma of bladder	2
10	Leiomyosarcoma of uterus	2
11	Squamous carcinoma of skin	2
12	Hepatocellular carcinoma of liver	3
13	Squamous carcinoma of lung	0
14	Transitional cell carcinoma of bladder	4
15	Transitional cell carcinoma of bladder	6
16	Transitional cell carcinoma of bladder	6

Table 1: **Characteristics of samples used in study**

PCR designation	Target gene	Forward	Reverse	Product size(bp)
B2M-106	Beta-2-microglobulin	5'-ATTCACCCCCACTGAAAAAG-3'	5'-TCCATGATGCTGCTTACATG-3'	106
B2M-154	Beta-2-microglobulin	5'-ATTCACCCCCACTGAAAAAG	5'GCAGCTTTATGATATGACTAC-3'	154
RPL13a	Ribosomal Protein L13a	5'-GTACGCTGTGAAGGCATCAA-3'	5'-GTTGGTGTTCATCCGCTTG-3'	90
ACTB-67	Beta-actin	5'-TCCCCCAACTTGAGATGTATGAA	5'-AACTGGTCTCAAGTCAGTGTACAGG-3	67
ACTB-117	Beta-actin	5'-ATCCCCCAAAGTTCACAATG-3'	5'-GTGGCTTTTAGGATGGCAAG-3'	117
HPRT	Hypoxanthine Guanine	5'-TGACACTGGCAAAACAATGCA-3'	5'-GGTCCTTTTCACCAGCAAGCT-3'	94

Table 2: **RT-PCR assays used in this study**

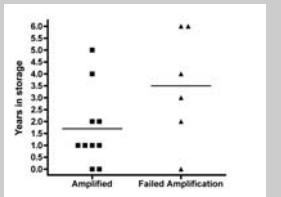


Figure 1: **Effect of sample age on cDNA amplification.** Although the mean age of the FFPE samples that failed amplification is greater than the samples that were successfully amplified, this difference is not statistically significant (P = 0.909).

Materials and Methods

Tissue samples

Tissue samples were obtained from surgical resection specimens at The University of Virginia Health System by the UVA Biorepository and Tissue Research Facility (BTRF) (http://www.healthsystem.virginia.edu/internet/tissueprocure/) with IRB approval. Frozen tissue samples were withdrawn from the Biorepository that matched formalin-fixed paraffin-embedded samples in the archives of the Pathology Department at the UVA Health System. Frozen tissue was subjected to cryostat sectioning, with tumor areas corresponding to >70% cellularity dissected from the frozen tissue using frozen histologic sections as guides. FFPE tissues were sectioned at 8 micron thickness onto glass slides which were then deparaffinized with xylene. Using cover-slipped slides stained with hematoxylin and eosin as guides, tumor areas corresponding to >70% cellularity were manually scraped from the deparaffinized slides. The tissue samples are described in Table 1.

RNA isolation

RNA was isolated from FFPE tissue using the RNeasy® FFPE kit (Qiagen) using the reagents and conditions supplied by the manufacturer, except for increasing the 55º C incubation time from 15 minutes to overnight. RNA was isolated from frozen tissue using the RNeasy® kit (Qiagen). RNA concentration was determined by absorbance at 260 nM. RNA and cDNA were also analyzed by microcapillary electrophoresis (Bioanalyzer, Agilent). Sample pairs were rejected if the microcapillary electrophoresis profiles of the RNA from the frozen tissue showed significant RNA degradation.

Quantitative reverse transcription and polymerase chain reaction (qRT-PCR)

cDNA was made from 1 ug of RNA using Superscriptase II (INVITROGEN). qPCR was performed on a Biorad iCYCLER Thermal cycler using iQ SYBR Green supermix. See Table 2 for PCR designation and primer pairs. Threshold cycle number (C_t) was determined for each study sample (S) and for a reference sample (R) of intact RNA (Universal Human Reference RNA, Catalog #740000, Stratagene). The assays were performed in triplicate and the results averaged. To control for run to run variability, the results are reported as ΔC_t, where $\Delta Ct = (C_t^S - C_t^R)$

Reverse transcription, cDNA isothermal amplification, probe labeling

Reverse transcription and whole transcriptome cDNA isothermal amplification was performed using the WT-Ovation™ FFPE RNA Amplification System Version 2 kit (NuGEN Technologies, Inc.). 50 ng of input RNA was used from frozen samples and 100 ng of input RNA was used for FFPE samples. cDNA probe biotin labeling was performed using the FL-Ovation™ cDNA Biotin Module Version 2 kit (NuGEN Technologies, Inc.).

Oligonucleotide microarray hybridization

Biotin-labeled cDNA probes were hybridized to U133 Plus 2.0 GeneChips (Affymetrix) using reagents, equipment and protocols provided by the manufacturer to a core facility (UVA Biomolecular Research Facility, http://www.healthsystem.virginia.edu/internet/biomolec/home.cfm).

Data Analysis

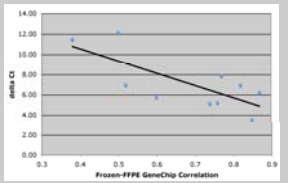
The Robust Multichip Average technique was used to preprocess the Affymetrix GeneChip data, yielding background adjusted, quantile normalized, and summarized expression values . To determine whether there was a linear relationship between the expression values for each pair of chips, the Spearman rank correlation coefficients were calculated and the expression values for each probe set for each sample were plotted against each other in XY scatter plots. Comparison of results between sample sets was analyzed for significance using the 2 tailed t-test.

Sample	Yield of amplified cDNA (ug)	Fr-FFPE GeneChip Correlation	ΔCt of RT-PCR B2M-106	B2M-154	RPL13a	HPRT	ACTB-67	ACTB-117
1	6.12	0.87	6.23	6.00	3.60	5.37	3.33	4.77
2	5.62	0.76	5.27	5.07	5.00	6.20	4.40	4.90
3	10.12	0.60	5.80	1.60	3.70	5.97	2.57	3.80
4	9.75	0.74	5.17	1.63	2.70	3.83	2.27	2.90
5	4.58	0.77	7.87	5.80	6.90	7.87	5.00	6.30
6	4.51	0.82	6.97	5.17	7.50	8.43	6.37	5.70
7	5.66	0.85	3.57	2.30	7.07	4.20	4.10	3.00
8	10.48	0.52	7.00	1.67	4.43	5.67	3.47	4.13
9	4.43	0.50	12.17	4.27	7.90	10.50	7.40	8.20
10	5.90	0.38	11.50	3.87	9.83	9.53	9.50	6.77
11	2.08		10.80	9.80	11.20	11.73	11.37	7.80
12	3.80		7.87	8.40	9.33	8.80	7.13	7.83
13	3.20		9.73	7.77	9.97	10.57	11.53	7.07
14	3.16		12.37	7.23	10.00	12.33	7.70	7.87
15	3.80		6.17	4.63	8.03	7.40	5.13	4.87
16	2.91		12.07	7.63	11.47	11.80	9.90	8.00

Table 3: **Performance of FFPE samples in cDNA amplification, GeneChip assay and qRT-PCR.** Samples shown in green achieved good correlation with their matched frozen tissue in GeneChip assays (7/16, 44%). Samples shown in orange yielded adequate cDNA, but did not correlate well with their matched frozen tissue sample in GeneChip assays (3/16, 19%). Samples shown in red did not yield sufficient cDNA in isothermal CDNA amplification to perform a GeneChip assay (6/19, 38%).

	RT-PCR B2M-106	B2M-154	RPL13a	HPRT	ACTB-67	ACTB-117
Av. ΔCt Amplified samples	7.15	3.74	5.86	6.78	4.84	5.05
Av. ΔCt Failed samples	9.83	7.58	10.00	10.44	8.79	7.24
t-test P value	0.0655	0.0012	0.0004	0.0049	0.0114	0.0099
Av. ΔCt of GeneChip Corr. Samples	5.84	3.94	5.21	5.98	4.00	4.48
Av. ΔCt of Non-corr. Samples	9.96	6.14	9.13	9.84	8.13	6.95
t-test P value	0.0008	0.0780	0.0019	0.0014	0.0020	0.0033
Correlation of ΔCt to GeneChip Corr.	-0.74	0.34	-0.38	-0.56	-0.52	-0.45

Table 4: **Correlation of qRT-PCR with cDNA amplification and GeneChip results.** While several of the qRT-PCR assays predicted adequate CDNA amplification of the FFPE samples, the B2M-106 assay was best correlated with FFPE samples that matched their frozen tissue counterparts (smallest negative number is best).



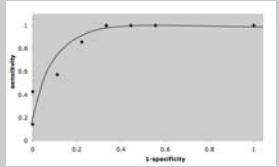


Figure 3: **The B2M-106 qRT-PCR assay predicts FFPE GeneChip performance.** Panel A: ΔCt plotted vs. Spearman rank correlation coefficient of GeneChip comparisons across the cohort. Panel B: ROC curve

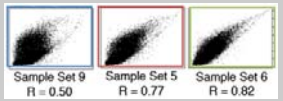


Figure 2: **Scatter plots of matched fluorescence intensity values for U133 Plus 2.0 GeneChip probe sets from selected pairs of FFPE & frozen tissue.**

Results

Of the 16 case sets whose frozen tissue samples had high quality RNA as determined by microcapillary electrophoresis (indicating initial integrity of the procured specimen) 10 had RNA from the FFPE tissue that yielded sufficient amplified cDNA (> 5 ug) to continue to microarray hybridization. While there was a trend for older cases to fail reverse transcription and isothermal DNA amplification (Fig. 1), there was not a statistically significant difference in age of the FFPE blocks between those cases that amplified and those that did not (P = 0.909). The age of the blocks ranged from less than 1 year old to 6 years of age, While both 6 year old cases failed amplification, a 5 year old case performed adequately, and at least one block less than one year old did not yield RNA of sufficient quality to perform GeneChip hybridization.

Table 3 contains the results of the correlation of GeneChip probe sets between matched FFPE and frozen tissue samples, while Fig. 2 shows a graphical comparison of the correlation for a subset of the matched cases. 7 of the 10 matched sample sets (70%) had correlation coefficients of 0.6 or higher, which are deemed similar enough for the FFPE results to produce gene expression signatures that are reflective of the frozen samples. Thus, from our cohort of 16 matched samples, and using these techniques, RNA from 7 FFPE samples (44%) yielded gene expression signatures nearly equivalent to good quality RNA obtained from frozen tissue.

To determine if pre-analytical qRT-PCR assays can predict if the RNA in FFPE samples will yield adequate GeneChip results, the RNA from the FFPE tissue samples was subjected to reverse transcription and subsequent quantitative PCR assays of six different areas present in 4 housekeeping genes. The assay results are shown in Table 3 and the correlation of the assay results to amplified cDNA yield and GeneChip correlation are shown in Table 4. In general the ΔC_t values are negatively correlated with the degree of similarity of the GeneChip expression profile between the matched FFPE and frozen tissue. That is, the smaller the degree of difference between the FFPE samples and a high quality reference RNA sample in the qRT-PCR samples, the greater the correlation of signal intensity across the spectrum of probe sets in GeneChip assays. The RT-PCR assay with the greatest correlation between and GeneChip correlation is B2M-106 (Fig. 3A), which in our preliminary data has good receiver operator characteristics that should allow for discrimination of FFPE samples that will perform well in GeneChip assays (Fig. 3B).

Conclusions

- Slightly less than half of FFPE samples may be amenable to microarray gene expression profiling
- Pre-analytic RT-PCR can be used to triage FFPE samples that will not perform adequately

Next Steps

- Expand cohort to obtain a more reliable assessment of pre-analytic RT-PCR test results and to obtain a greater sampling of matched probe set comparisons between FFPE and frozen tissue samples
- Determine if specific probe sets are more reliable than others in querying gene expression in FFPE samples

References

1. Ramaswamy S, Tamayo P, Rifkin R, Mukherjee S, Yeang CH, Angelo M, et al. Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci U S A. 2001;98(26):15149-54.
2. Su AI, Welsh JB, Sapinoso LM, Kern SG, Dimitrov P, Lapp H, et al. Molecular classification of human carcinomas by use of gene expression signatures. Cancer Res. 2001;61(20):7388-93.
3. Raponi M, Zhang Y, Yu J, Chen G, Lee G, Taylor JM, et al. Gene expression signatures for predicting prognosis of squamous cell and adenocarcinomas of the lung. Cancer Res. 2006 Aug 1;66(15):7466-72.
4. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med. 2002;347(25):1999-2009.
5. Potti A, Dressman HK, Bild A, Riedel RF, Chan G, Sayer R, et al. Genomic signatures to guide the use of chemotherapeutics. Nat Med. 2006 Nov;12(11):1294-300.
6. Lee JK, Havaleshko DM, Cho H, Weinstein JN, Kaldjian EP, Karpovich J, et al. A strategy for predicting the chemosensitivity of human cancers and its application to drug discovery. Proc Natl Acad Sci U S A. 2007 Aug 7;104(32):13086-91.
7. Frank M, Doring C, Metzler D, Eckerle S, Hansmann ML. Global gene expression profiling of formalin-fixed paraffin-embedded tumor samples: a comparison to snap-frozen material using oligonucleotide microarrays. Virchows Arch. 2007 Jun;450(6):699-711.
8. Scicchitano MS, Dalmas DA, Bertiaux MA, Anderson SM, Turner LR, Thomas RA, et al. Preliminary comparison of quantity, quality, and microarray performance of RNA extracted from formalin-fixed, paraffin-embedded, and unfixed frozen tissue samples. J Histochem Cytochem. 2006 Nov;54(11):1229-37.
9. Haque T, Faury D, Albrecht S, Lopez-Aguilar E, Hauser P, Garami M, et al. Gene expression profiling from formalin-fixed paraffin-embedded tumors of pediatric glioblastoma. Clin Cancer Res. 2007 Nov 1;13(21):6284-92.
10. Penland SK, Keku TO, Torrice C, He X, Krishnamurthy J, Hoadley KA, et al. RNA expression analysis of formalin-fixed paraffin-embedded tumors. Lab Invest. 2007 Apr;87(4):383-91.
11. Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP. Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res. 2003 Feb 15;31(4):e15.
12. Bolstad BM, Irizarry RA, Astrand M, Speed TP. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics. 2003 Jan 22;19(2):185-93.