Neoplasia • Vol. 6, No. 6, November/December 2004, pp. 761–767 761

www.neoplasia.com

Stability and Heterogeneity of Expression Profiles in Lung Cancer Specimens Harvested Following Surgical Resection[1]

Fiona H. Blackhall, Melania Pintilie, Dennis A. Wigle, Igor Jurisica, Ni Liu, Nikolina Radulovich, Michael R. Johnston, Shaf Keshavjee and Ming-Sound Tsao

University Health Network, Ontario Cancer Institute/Princess Margaret Hospital, Toronto General Hospital and University of Toronto, Toronto, Ontario, Canada M5G 2M9

Abstract

One of the major concerns in microarray profiling studies of clinical samples is the effect of tissue sampling and RNA extraction on data. We analyzed gene expression in lung cancer specimens that were serially harvested from tumor mass and snap-frozen at several intervals up to 120 minutes after surgical resection. Global gene expression was profiled on cDNA microarrays, and selected stress and hypoxia-activated genes were evaluated using real-time reverse transcription polymerase chain reaction (RT-PCR). Remarkably, similar gene expression profiles were obtained for the majority of samples regardless of the time that had elapsed between resection and freezing. Real-time RT-PCR studies showed significant heterogeneity in the expression levels of stress and hypoxia-activated genes in samples obtained from different areas of a tumor specimen at one time point after resection. The variations between multiple samplings were significantly greater than those of elapsed time between sampling/freezing. Overall samples snap-frozen within 30 to 60 minutes of surgical resection are acceptable for gene expression studies, thus making sampling and snap-freezing of tumor samples in a routine surgical pathology laboratory setting feasible. However, sampling and pooling from multiple sites of each tumor may be necessary for expression profiling studies to overcome the molecular heterogeneity present in tumor specimens.

Neoplasia (2004) **6**, 761–767

Keywords: Lung cancer, microarray, real-time RT-PCR, tumor heterogeneity, surgical specimen.

Introduction

Non small cell lung cancer (NSCLC) accounts for 80% of lung cancers and comprises several histologic subtypes, of which adenocarcinoma (ADC), squamous cell carcinoma (SQCC), and large cell carcinoma are the most common. Recent years have witnessed the application of cDNA and oligonucleotide microarrays to classify NSCLC at a molecular level. Studies performed in stages I to III NSCLC specimens have demonstrated gene expression profiles that distinguish types of lung cancer and subtypes of ADC with different clinical outcomes for a given tumor stage [1–3]. On this basis, a ''lung cancer chip'' has been proposed as a future clinical tool to molecularly classify and substage NSCLC specimens [1,4]. These and most other microarray studies have been done on banked frozen tissue sampled from surgically resected tumors, but the nature and timing of tissue sampling have rarely been discussed in detail. RNA is known to be highly susceptible to degradation and expression of transcripts may be altered due to acute tissue hypoxia and stress following surgical resection [5]. Because the quality of the extracted RNA could greatly influence the results of expression studies on tumor samples snap-frozen at different times following resection, these results might be influenced by changes due to surgery and sampling rather than due to the molecular phenotype of that tumor. We report here our findings on the changes of gene expression in tumor samples maintained at room temperature for different time intervals up to 120 minutes prior to snap-freezing in liquid nitrogen following surgical resection.

Materials and Methods

Tumor Samples

NSCLC specimens were obtained with informed consent according to a study protocol approved by our institutional Human Tissue Committee and Research Ethics Board. Table 1 illustrates the pathologic characteristics of these tumors. In all studies, each sample harvested was approximately 0.5 cm^3 (0.8 × 0.8 × 0.8 cm) of tumor tissue obtained from lobectomy or pneumonectomy specimens

Abbreviations: RT-PCR, reverse transcription polymerase chain reaction; NSCLC, non small cell lung cancer; ADC, adenocarcinoma; SQCC, squamous cell carcinoma; BTSVQ, binary tree–structured vector quantization; SOM, self-organizing map

Address all correspondence to: Dr. Ming-Sound Tsao, Princess Margaret Hospital, 610 University Avenue, Toronto, Ontario, Canada M5G 2M9. E-mail: Ming.Tsao@uhn.on.ca

[1]This work is supported by the Canadian Cancer Society, Inc. (NCIC grant no. 012150), Aventis Pharma, Inc. (educational fellowship to F.H.B.), the National Science and Engineering Research Council of Canada (NSERC grant no. 203833), and the Institute for Robotics and Intelligent Systems (grant to I.J.). Dr. Tsao is the M. Qasim Choksi Chair in Lung Cancer Translational Research.

Received 22 April 2004; Revised 22 June 2004; Accepted 30 June 2004.

DOI 10.1593/neo.04301

Table 1. Pathological Features of Samples Used in This Study.

Sample ID	Tumor Type	Differentiation Grade	Greatest Diameter of Tumor (cm)
P321	Squamous cell carcinoma	Moderate	3.6
UHN323	Adenocarcinoma	Moderate	2.0
P322	Adenocarcinoma	Moderate	2.0
P323	Squamous cell carcinoma	Poor	2.7
P325	Large cell carcinoma		3.5
P326	Adenocarcinoma	Well	10.0

maintained at room temperature after surgical resection. Two consecutive tumors were used in the time course experiments. In the first, tumor samples were snap-frozen in liquid nitrogen at 5, 10, 15, 20, 30, 60, and 120 minutes after resection. In time course experiment 2, samples were snap-frozen in liquid nitrogen at 10, 20, 30, 40, 50, and 60 minutes from resection. In four additional tumors, six different sites were sampled at the same time within 30 minutes of resection to examine sample heterogeneity within individual tumors. Sections corresponding to each frozen sample used for the extraction of RNA in the time course and heterogeneity studies were also stained with hematoxylin and eosin (H&E) and assessed for cellular content by light microscopy. These sections confirm that the various tumor regions sampled showed similar histologic appearances. Subsequent surgical pathologic examination of the tumors confirmed the homogeneous histopathology of these tumors.

Gene Expression Studies by cDNA Microarray

Total RNA was isolated using the guanidium thiocyanate–phenol–chloroform method [6], purified by the RNeasy Mini kit (Qiagen, Inc., Mississauga, Ontario, Canada) and the quality of RNA preparation was confirmed using an Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA). Expression profiling was performed with cDNA microarrays printed on glass slides from a 1.7K clone set (http://www.microarrays.ca) on all samples from time course experiments 1 and 2. These arrays contained 1700 probes derived from IMAGE clones. The cRNA probes were labeled and hybridization was performed as previously described [4]. All samples were referenced to an RNA stock derived from the immortalized human bronchial epithelial cell line HBE135-E6E7 [4,7]. Duplicate and reciprocally labeled arrays with Cy3 and Cy5 were performed at each time point. Microarray images were acquired using a Genepix 4000 scanner (Axon Instruments, Inc., Union City, CA) and signal intensities were quantified with Quantarray image analysis software (GSI Lumonics, Ottawa, Ontario, Canada). Quantarray data handler 2.6 software was used for data normalization. Background values were deleted, and Cy3 and Cy5 channels of each array were normalized by subarray intensity. The signals for Cy3 and Cy5 from duplicate arrays (total of four spots) were averaged and the standard deviations were calculated. Genes where the value for the standard deviation minus the mean of four spots was ≥ 0 were eliminated. The value for each gene was then expressed as a ratio that was calculated by dividing the average signal for the sample by the average signal for the reference, then converted to $\log_2$ for analysis.

Measurement of Stress and Hypoxia Pathway Genes by Real-Time Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA samples from the time course experiments ($n = 2$) and the sample heterogeneity experiments ($n = 4$) were used for these assays. Five micrograms of total cellular RNA was reverse-transcribed using Taqman Reverse Transcription reagents and Random Hexamer primer (Perkin-Elmer Applied Biosystems, Foster City, CA). A 10-ng equivalent of cDNA was used in each quantitative PCR assay using the ABI PRISM 7700 Sequence Detection System, as previously described in detail [8]. All assays were performed in duplicate. Primer sets for PCR amplifications were designed using the Primer Express software (Perkin-Elmer Applied Biosystems). The genes and corresponding forward and reverse primers (5′–3′) used were: *JNK3* (mitogen-activated protein/MAPK10, U34820), GCGGACTCCGAGCACAATAA and GCGTCGTCCACTGATATTCTTTT; *JUN-B* (JUN-B proto-oncogene, X51345), ACTCATACACAGCTACGGGATACG and AGGCTCGGTTTCAGGAGTTTG; *AP-1* (c-Jun proto-oncogene, J04111), TCCACGGCCAACATGCT and CGACGGTCTCTCTTCAAAATGTTT; *CAIX* (carbonic anhydrase IX, NM 001216), ACTTCAGCCGCTACTTCCAATATG and GAGAGGGTGTGGAGCTGCTTAG; *PRSS25* (protease, serine 25, NM 013247), AGCTTTCCCGATGTTCAGCAT and TTTGTACCATCTGCTCCCCAAT; *HHR6B* (human homologue of Rad6B or ubiquitin-conjugating enzyme E2B, M74525), CGATGGAGTCCAACATATGATG TATC and AGCTGTGCTGCCTGGCTATT. To exclude the amplification of contaminating genomic DNA sequences, primers were designed to locate on two adjacent exons, and dissociation curve and primer efficiency tests were performed as routine verification. All assays were done using duplicate samples of each RT product, and each assay was repeated twice. The C_T represented the relative abundance of a transcript and the 18*S* ribosomal RNA transcript level was used to monitor the sample-to-sample variation of RNA/cDNA quantity using the ΔC_T (C_T[gene] $-$ C_T[18*S*]) method [8]. The relative fold change in the expression of each gene was expressed as $2^{-\Delta\Delta C_T}$, where $\Delta\Delta C_T$ = the difference between the ΔC_T value of the gene for each sample and the mean ΔC_T value of the calibrator for that target. For these experiments, the calibrator was the ΔC_T measured for the gene in the first sample in the experiment (e.g., the sample at 5 minutes for time course 1).

Microarray Data Analysis

The aim of this analysis was to investigate the possible general trends over time in gene expression following surgical resection. Therefore, all genes for which there was no sample:reference ratio (i.e., missing data) at one or more

time points were excluded. First, all summary statistics of the data at each time point were calculated and examined for a general trend. Second, a line was fitted for each gene to examine the possible alterations of some genes. For this part of the analysis, the significance analysis of microarray (SAM) technique was applied [9]. A binary tree–structured vector quantization (BTSVQ) (http://www.cs.toronto.edu/~juris/btsvq/downloads.html) algorithm was used to cluster the samples in an unsupervised way, using all expression profiles [10]. BTSVQ combines a partitive *k*-means clustering and a self-organizing map (SOM) algorithm in a complementary way to achieve unsupervised clustering of both samples and genes. Partitive clustering results were cross-verified with the clusters generated by SOMs. Each component plane of SOMs represents a single microarray experiment. Visualization is achieved by assigning a color to each group of genes from a spectrum to render low expression (blue) to high expression (red). Thus, finding a visually similar pattern across multiple samples indicates that the same set of genes is similarly expressed.

Real-Time RT-PCR Data Analysis

The $\Delta\Delta C_T$ values were compared using line graphs and scatter plots. This analysis was performed for two tumors over time, and for four tumors on samples taken from six different parts of each tumor at the same time following resection. The data were plotted and summary statistics were calculated to investigate the variation due to time and that due to heterogeneity within each tumor. The variance of expression of each gene within the tumors due to the time between resection and freezing as well as due to the heterogeneity of expression within the tumors was calculated using variance component analysis.

Results

Changes in Gene Expression with Time after Resection

Two NSCLC specimens were obtained from lobectomy/pneumonectomy specimens and maintained at room temperature, and consecutive samples were snap-frozen at various time intervals after resection. Evaluation of the RNA samples extracted from these tissues showed no deterioration or trend toward poorer quality in samples obtained at later time points (Table 2). Microarray data at all time points were available for a total of 623 and 300 genes for time course experiments 1 and 2, respectively. First, we obtained a measure of the global trend in gene expression with time. The $\log_2$ gene expression ratios were averaged and standard deviations were calculated for each time point. The result demonstrates that the mean and median gene expression ratio varied among the time points, but there was no clear monotonic relationship with time (Table 2). We then examined the possibility that individual genes may either increase or decrease with time using the SAM technique [9]. Only two genes (CACNB3, IMAGE clone H08978; LAMP1, IMAGE clone R89708) in time course 1 were identified to have a possible linear relationship with time. In time course 2, there were no genes that demonstrated a linear relationship with time. We next examined the patterns of relative gene expression with time using the BTSVQ clustering algorithm as previously reported [10], and the expression profiles were illustrated using component planes of SOMs (Figure 1). In time course 1, the gene expression profiles at 5, 10, 30, 60, and 120 minutes were most alike and clustered separately (cluster 1) from those obtained at the 15- and 20-minute time points (cluster 2). In time course 2, the gene expression profiles at 10, 20, 40, and 50 minutes clustered separately (cluster 1) from those at 30 and 60 (cluster 2) minutes. The results demonstrate striking similarity between five of seven, and four of six gene expression profiles for the two time course experiments, respectively. A clear pattern of change in relative gene expression with time did not emerge from these data.

Table 2. Changes in RNA Quality and Gene Expression Ratios Due to the Time Elapsed between Resection and Freezing of Tissue Samples.

Time from Resection (min)	RNA Quality 28*S*:18*S*	Mean Gene Expression Ratio ($\log_2$)	SD	Median Gene Expression Ratio ($\log_2$)
Time course 1 (number of genes = 623)				
5	2.27	−0.01	0.42	−0.09
10	2.06	0.08	0.32	0.04
15	2.46	−0.16	0.76	−0.34
20	2.16	−0.08	0.38	−0.14
30	2.44	0.09	0.35	0.04
60	2.09	0.01	0.37	−0.06
120	2.05	−0.14	0.58	−0.28
Time course 2 (number of genes = 300)				
10	1.94	−0.04	0.39	−0.05
20	2.00	−0.03	0.36	−0.06
30	1.91	−0.06	0.49	−0.05
40	2.11	0.01	0.33	0
50	2.03	−0.15	0.46	−0.16
60	2.56	−0.32	0.47	−0.41

Expression Changes of Stress and Hypoxia-Activated Genes

Real-time RT-PCR was used to measure the expression of six genes in both time course experiments and in the experiments designed to assess intratumoral heterogeneity of samples harvested simultaneously from six different parts of tumors within 30 minutes of resection. Examination of the H&E-stained sections confirmed the presence of $\geq$ 60% cancer cells for each sample, with the majority of samples demonstrating $\geq$ 80% cancer cells. Figure 2 shows that the levels of expression of these genes varied over the time period examined, but the relationship with time from resection was not linear. Furthermore, the sample heterogeneity experiments also demonstrated a variation of gene expression levels among the samples harvested from six different sites in all four tumors studied (Figure 3). When we compared the variance in expression of these genes among samples in the time course *versus* the samples from the heterogeneity experiments, all but one gene (*HHR6B*) revealed a greater variation in samples from different sites of the tumor than samples harvested at different time intervals

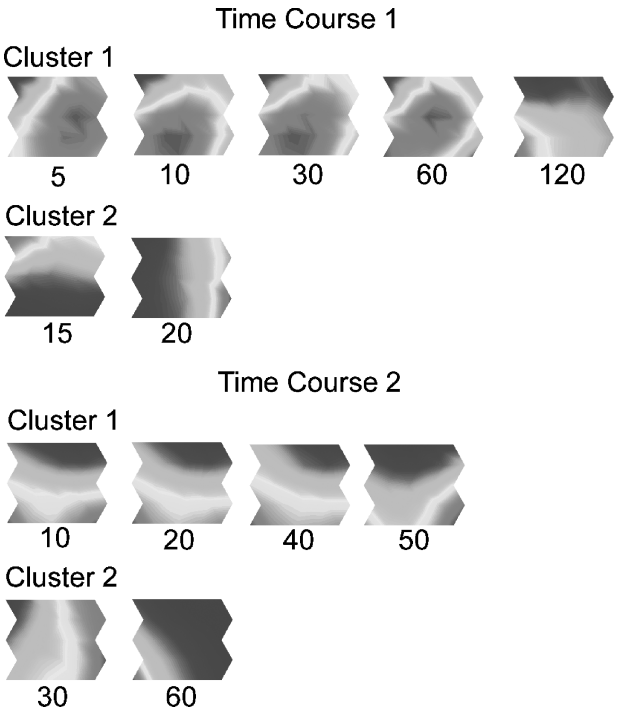

Figure 1. *Unsupervised clustering analysis by the BTSVQ algorithm. Component planes of SOMs illustrate the gene expression, based on data from two arrays, at each time point. Finding a visually similar pattern across multiple samples indicates that the same set of genes is similarly expressed.*

following resection (Table 3). Furthermore, the expression heterogeneity was not related to whether the sample was harvested from the central or peripheral parts of the tumors (Figure 3).

Discussion

The aim of this study was to examine the effect of time following surgical resection and sampling on expression studies in NSCLC specimens. We demonstrate that when different samples of a tumor are snap-frozen at increasing time intervals following surgical resection, they can have similar gene expression patterns. In samples taken between 5 and 120 minutes in time course 1, and between 10 and 60 minutes in time course 2, the quality of RNA extracted did not deteriorate as evaluated by the Agilent Bioanalyzer, nor was there a global decline in mean gene expression with time. Only two genes were identified to have a linear relationship with time, and cluster analysis revealed a high degree of similarity in more than two thirds of these samples. With one exception, the genes specific to stress and hypoxia pathways that were measured using real-time RT-PCR demonstrated greater variation among several samples harvested simultaneously from various parts of the tumor, than in samples of a tumor taken at different times up to 120 minutes after resection.

In theory, hypoxia and stress due to disruption of the circulation could lead to rapid changes in the cellular microenvironment and, therefore, gene expression. Consistent with the data in the present study, D'Orazio et al. [11] demonstrated stable expression levels of glyceraldehyde-6-phosphate dehydrogenase (GAPDH), β-actin, c-*myc*, and *Fra-1*, which is upregulated in response to oxidative stress, for 4 hours using Taqman quantitative RT-PCR. Dash et al. [12] demonstrated little change in relative gene expression up to 5 hours after resection using cDNA microarray in prostate cancer specimens. However, an increase in the expression of a minor proportion (< 0.6%) of the genes at 1 hour following resection was demonstrated [12]. These genes included *jun B* proto-oncogene, which did not change significantly in the present study (Figure 3); thus, there may be tissue-specific differences in the stability of gene expression following surgical resection. We suggest that although it is prudent to snap-freeze tumor samples as soon as possible after resection, tissue sampled anytime within 30 to 60 minutes from resection appears to be suitable for expression studies including genomewide microarray analysis.

We were intrigued to detect greater intratumoral heterogeneity than variation due to the time from resection of five of six stress- or hypoxia-associated genes studied. These genes included *HHR6B* (or ubiquitin-conjugating enzyme E2B), which is induced in response to cellular stress [13]

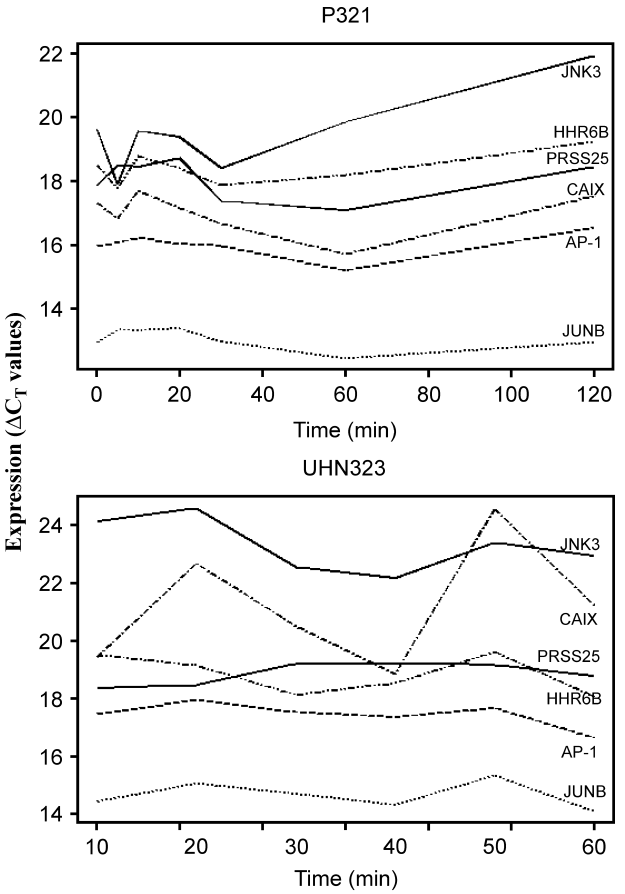

Figure 2. *The effect of time from surgical resection on the expression of selected stress/hypoxia genes measured by real-time RT-PCR.*

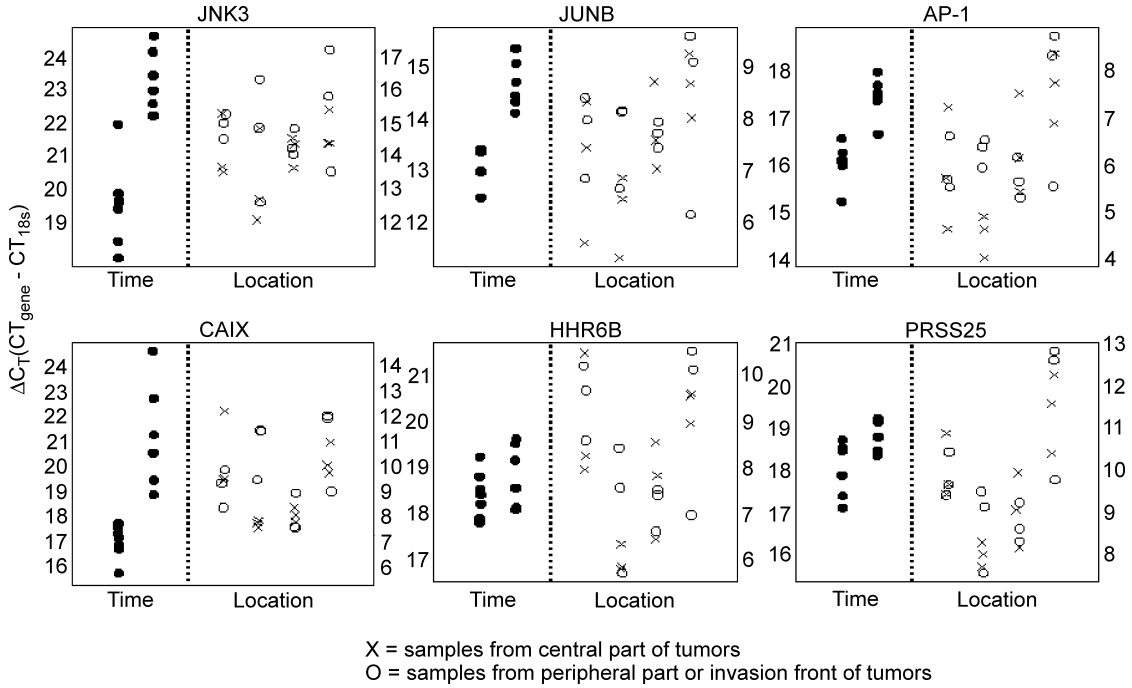

Figure 3. *Expression of stress/hypoxia genes by real-time RT-PCR. For each gene, the measurements for two time course studies at each time point (left) are compared to the gene measurements for four tumors sampled from six different sites at the same time point from resection (right). The Y-axis values represent the ΔC_T (C_T[gene] − C_T[18S]). The numbers on the left side are for the two longitudinal time course specimens, whereas the numbers on the right are for the four multiple sampling specimens. The differences in the ΔC_T values of the two groups of specimens were due to different dilutions of cDNA used to perform 18S real-time RT-PCR measurements. X, central samples; O, peripheral samples.*

and also demonstrated a high variation in microarray experiments independent of time (data not shown); two genes activated in response to hypoxia, *AP-1* [14] and *CA-IX* [15]; and the stress-activated genes *JNK3* [16], *JUN-B* [17], and *PRSS25* [18]. Gene expression data are usually based on a single extraction of RNA. Vasselli et al. [19] reported on the reproducibility of microarray-derived gene expression data for renal cell cancer. When replicate arrays were performed using the same RNA samples, there was a Pearson correlation among log ratio expression profiles of > 0.5 for 20 of 22 patients. In contrast, when RNA was isolated from three separate areas of the same tumor, samples from only two of six patients had correlations > 0.5.

Table 3. Variance of Gene Expression According to Time Course *Versus* Sampling.

Gene	Gene Name (Synonyms)	Intratumoral Variance/ Total Variance (%)	
		Time Course Experiments (n = 2)	Heterogeneity Experiments (n = 4)
JNK3	JNK3 α1 protein kinase (MAPK10)	16	97
JUN-B	JUN-B proto-oncogene	12	81
AP-1	c-Jun proto-oncogene	15	57
CAIX	Carbonic anhydrase IX	21	66
HHR6B	Human homologue of Rad6 (UBE2B, ubiquitin-conjugating enzyme E2B)	89	42
PRSS25	Protease, serine, 25	16	28

The problem of intratumoral heterogeneity is frequently encountered in studies that use immunohistochemical methodology. For example, mixed reports with respect to the utility of *CA-IX* as a prognostic marker for cervical cancer may be attributable to sampling error [20,21]. Hedley et al. [21] measured *CA-IX* in multiple biopsies using a semiautomated fluorescence image analysis technique, and observed intratumoral heterogeneity to account for 41% of the variance in the data set. The authors estimated the probability of failing to detect the true underlying association between *CA-IX* and outcome to be 28% when only one biopsy was obtained from a heterogeneous tumor compared to 13% in the ideal situation with no heterogeneity. Intratumoral heterogeneity of gene expression in part arises from different cellular populations (i.e., stromal cell *versus* cancer cell). Sugiyama et al. [22] noted markedly different results when gene expression profiles of microdissected cancer cells were compared to those of nondissected cancer tissues. Although laser capture microdissection offers a means to circumvent the potential confounding effect of different cell types in a sample, cluster analysis of pyramidal neurons has also revealed that even a single cell type *in vivo* is not a homogeneous population at the gene expression level [23]. The distribution of a gene may depend on the cellular location. Expression of *CA-IX* is most marked around necrotic central areas of tumors [24]. However, it is interesting that

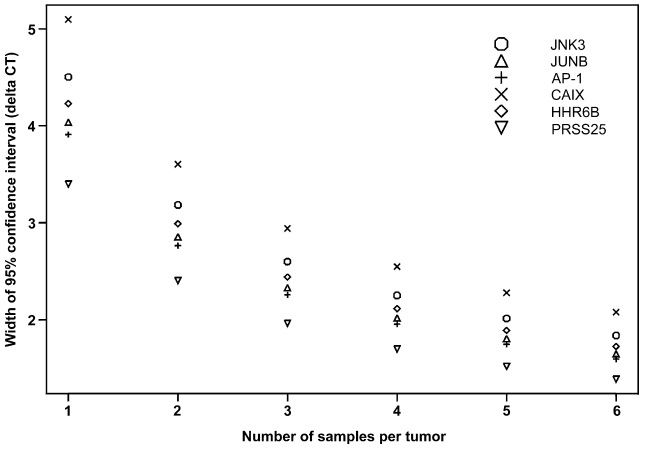

Figure 4. *The effect of increasing the number of samples per tumor on the width of the 95% confidence interval for expression of selected stress/hypoxia genes.*

the heterogeneity of the genes we examined in the present study does not appear to relate to whether the sample was derived from the center or the periphery of the tumor. With this in mind, it is notable that we obtained a similar result in greater than two thirds of samples taken for the time course experiments. Therefore, the chance of getting a ''representative'' sample may be estimated at 60%. Analysis of the width of 95% confidence intervals of ΔC_T for the six stress/hypoxia–related genes when one to six samples per tumor were used demonstrated a range from 1.39 to 5.10 (Figure 4), with the means for four and five samples per tumor being 2.10 and 1.87, respectively. Because C_T is the $\log_2$ of changes in expression levels, it can be estimated that four samples would allow two-fold variation on either side of the mean expression level to be within the range of heterogeneity. Thus, we would recommend that pooling samples from four different areas of a tumor should be sufficient to minimize artifacts from tumor heterogeneity.

In conclusion, we have investigated the potential confounding effects of sampling procedure on gene expression profiling studies using surgically resected tumor specimens. We suggest that samples snap-frozen within 30 to 60 minutes from resection are acceptable for such analyses and this should be feasible in a routine surgical setting. Molecular heterogeneity within tumors should also be considered as a confounding factor; thus, sampling and pooling of tissue/RNA from multiple sites of a tumor are advisable. In studies that correlate clinical outcomes with gene expression, a large number of cases might possibly offset the effect of heterogeneity within single samples.

Acknowledgements

We thank Frances Shepherd for her continuing support of the thoracic oncology translational research program at the PMH, and Fannong Meng for assistance in the harvesting of tumor samples.

References

[1] Beer DG, Kardia SL, Huang CC, Giordano TJ, Levin AM, Misek DE, Lin L, Chen G, Gharib TG, Thomas DG, Lizyness ML, Kuick R, Hayasaka S, Taylor JM, Iannettoni MD, Orringer MB, and Hanash S (2002). Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat Med* **8,** 816–824.

[2] Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P, Ladd C, Beheshti J, Bueno R, Gillette M, Loda M, Weber G, Mark EJ, Lander ES, Wong W, Johnson BE, Golub TR, Sugarbaker DJ, and Meyerson M (2001). Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci USA* **98,** 13790–13795.

[3] Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacyna-Gengelbach M, van de Rijn M, Rosen GD, Perou CM, Whyte RI, Altman RB, Brown PO, Botstein D, and Petersen I (2001). Diversity of gene expression in adenocarcinoma of the lung. *Proc Natl Acad Sci USA* **98,** 13784–13789.

[4] Wigle DA, Jurisica I, Radulovich N, Pintilie M, Rossant J, Liu N, Lu C, Woodgett J, Seiden I, Johnston M, Keshavjee S, Darling G, Winton T, Breitkreutz BJ, Jorgenson P, Tyers M, Shepherd FA, and Tsao MS (2002). Molecular profiling of non-small cell lung cancer and correlation with disease-free survival. *Cancer Res* **62,** 3005–3008.

[5] Mizuno T, Nagamura H, Iwamoto KS, Ito T, Fukuhara T, Tokunaga M, Tokuoka S, Mabuchi K, and Seyama T (1998). RNA from decades-old archival tissue blocks for retrospective studies. *Diagn Mol Pathol* **7,** 202–208.

[6] Chomczynski P and Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162,** 156–159.

[7] Tsao MS, Zhu H, and Viallet J (1996). Autocrine growth loop of the epidermal growth factor receptor in normal and immortalized human bronchial epithelial cells. *Exp Cell Res* **223,** 268–273.

[8] Wang KK, Liu N, Radulovich N, Wigle DA, Johnston MR, Shepherd FA, Minden MD, and Tsao MS (2002). Novel candidate tumor marker genes for lung adenocarcinoma. *Oncogene* **21,** 7598–7604.

[9] Tusher VG, Tibshirani R, and Chu G (2001). Significance analysis of microarrays applied to the ionizing radiation response. *Proc Natl Acad Sci USA* **98,** 5116–5121.

[10] Sultan M, Wigle DA, Cumbaa CA, Maziarz M, Glasgow J, Tsao MS, and Jurisica I (2002). Binary tree–structured vector quantization approach to clustering and visualizing microarray data. *Bioinformatics* **18,** S111–S119.

[11] D'Orazio D, Stumm M, and Sieber C (2002). Accurate gene expression measurement in formalin-fixed and paraffin-embedded tumor tissue. *Am J Pathol* **160,** 383–384.

[12] Dash A, Maine IP, Varambally S, Shen R, Chinnaiyan AM, and Rubin MA (2002). Changes in differential gene expression because of warm ischemia time of radical prostatectomy specimens. *Am J Pathol* **161,** 1743–1748.

[13] Koken MH, Reynolds P, Jaspers-Dekker I, Prakash L, Prakash S, Bootsma D, and Hoeijmakers JH (1991). Structural and functional conservation of two human homologs of the yeast DNA repair gene *RAD6*. *Proc Natl Acad Sci USA* **88,** 8865–8869.

[14] Premkumar DR, Adhikary G, Overholt JL, Simonson MS, Cherniack NS, and Prabhakar NR (2000). Intracellular pathways linking hypoxia to activation of c-*fos* and *AP-1*. *Adv Exp Med Biol* **475,** 101–109.

[15] Potter C and Harris AL (2004). Hypoxia inducible carbonic IX anhydrase, marker of tumour hypoxia, survival pathway and therapy target. *Cell Cycle* **3,** 164–167.

[16] Hu MC, Qiu WR, and Wang YP (1997). JNK1, JNK2 and JNK3 are p53 N-terminal serine 34 kinases. *Oncogene* **15,** 2277–2287.

[17] Hara T, Mies G, Hata R, and Hossmann KA (2001). Gene expressions after thrombolytic treatment of middle cerebral artery clot embolism in mice. *Stroke* **32,** 1912–1919.

[18] Jones JM, Datta P, Srinivasula SM, Ji W, Gupta S, Zhang Z, Davies E, Hajnoczky G, Saunders TL, VanKeuren ML, Fernandes-Alnemri T, Meisler MH, and Alnemri ES (2003). Loss of Omi mitochondrial protease activity causes the neuromuscular disorder of *mnd2* mutant mice. *Nature* **425,** 721–727.

[19] Vasselli JR, Shih JH, Iyengar SR, Maranchie J, Riss J, Worrell R, Torres-Cabala C, Tabios R, Mariotti A, Stearman R, Merino M, Walther MM, Simon R, Klausner RD, and Linehan WM (2003). Predicting survival in patients with metastatic kidney cancer by

gene-expression profiling in the primary tumor. *Proc Natl Acad Sci USA* **100,** 6958–6963.

[20] Loncaster JA, Harris AL, Davidson SE, Logue JP, Hunter RD, Wykoff CC, Pastorek J, Ratcliffe PJ, Stratford IJ, and West CM (2001). Carbonic anhydrase (*CA IX*) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. *Cancer Res* **61,** 6394–6399.

[21] Hedley D, Pintilie M, Woo J, Morrison A, Birle D, Fyles A, Milosevic M, and Hill R (2003). Carbonic anhydrase IX expression, hypoxia, and prognosis in patients with uterine cervical carcinomas. *Clin Cancer Res* **9,** 5666–5674.

[22] Sugiyama Y, Sugiyama K, Hirai Y, Akiyama F, and Hasumi K (2002). Microdissection is essential for gene expression profiling of clinically resected cancer tissues. *Am J Clin Pathol* **117,** 109–116.

[23] Kamme F, Salunga R, Yu J, Tran DT, Zhu J, Luo L, Bittner A, Guo HQ, Miller N, Wan J, and Erlander M (2003). Single-cell microarray analysis in hippocampus CA1: demonstration and validation of cellular heterogeneity. *J Neurosci* **23,** 3607–3615.

[24] Giatromanolaki A, Koukourakis MI, Sivridis E, Pastorek J, Wykoff CC, Gatter KC, and Harris AL (2001). Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. *Cancer Res* **61,** 7992–7998.