Effects of Warm Ischemic Time on Gene Expression Profiling in Colorectal Cancer Tissues and Normal Mucosa

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Abstract

Background: Genome-wide gene expression analyses of tumors are a powerful tool to identify gene signatures associated with biologically and clinically relevant characteristics and for several tumor types are under clinical validation by prospective trials. However, handling and processing of clinical specimens may significantly affect the molecular data obtained from their analysis. We studied the effects of tissue handling time on gene expression in human normal and tumor colon tissues undergoing routine surgical procedures.

Methods: RNA extracted from specimens of 15 patients at four time points (for a total of 180 samples) after surgery was analyzed for gene expression on high-density oligonucleotide microarrays. A mixed-effects model was used to identify probes with different expression means across the four different time points. The p-values of the model were adjusted with the Bonferroni method.

Results: Thirty-two probe sets associated with tissue handling time in the tumor specimens, and thirty-one in the normal tissues, were identified. Most genes exhibited moderate changes in expression over the time points analyzed; however four of them were oncogenes, and two confirmed the effect of tissue handling by independent validation.

Conclusions: Our results suggest that a critical time point for tissue handling in colon seems to be 60 minutes at room temperature. Although the number of time-dependent genes we identified was low, the three genes that already showed changes at this time point in tumor samples were all oncogenes, hence recommending standardization of tissue-handling protocols and effort to reduce the time from specimen removal to snap freezing accounting for warm ischemia in this tumor type.

Introduction

With the introduction of new genomic technologies such as tissue-based RNA microarrays, patterns of gene expression identified by microarray analyses have been discovered that stratify tumors and predict the clinical outcomes in different cancer types [1]. Some of them have been successfully used for identifying patients that can benefit from specific treatment and FDA (Food and Drug Administration) -approved tests based on breast cancer gene signatures are now commercially available. As a consequence, collecting in tissue banks surgical specimens that can be used for these analyses has become a mandatory issue for understanding the correlative results of the majority of current clinical trials. However, the variability in tissue handling and processing of surgical specimens may affect the reproducibility and interpretation of results. Several variables, including tissue manipulation, warm ex-vivo ischemia and storage times can potentially alter mRNA expression levels and adversely affect the validity of studies that used clinical specimens [2]. All these variables need to be carefully investigated in order to set up guidelines for tissue banking [3]. The organization of qualified and certified biobanks should be the basis to guarantee networking of activities and availability of quality-certified biological material.
The purpose of this pilot study was to explore the effects of tissue handling time in precisely documented tissue samples that followed the routine processing standards in our Institution (Fondazione IRCCS Istituto Nazionale dei Tumori, INT-MI), for developing a clinically applicable method of sampling tumors in tissue banks that can be safely used for microarray analyses. We used the colorectal cancer (CRC) model. CRC is the second most frequent cause of cancer death in Western countries and despite significant advances in its management, the overall survival for advanced and metastatic disease has changed little over the last 20 years, with five years at almost 90% for early and 15% for late tumors [4]. As a consequence, there is a pressing need for new biomarkers to improve the detection and the clinical treatment of CRC. Using high-density oligonucleotide microarrays we investigated sequential effects of tissue handling in specimens obtained from 15 CRCs and in their matched normal tissue collected at our Institution and left at room temperature at different time points after surgery. The primary study outcome was to evaluate the effect of the time on tumor samples and possibly select specific genes whose expression is time-related, that could be used as detectors of tissue degradation. Additionally, to identify genes influenced by time irrespectively of the sample type (normal or tumor), we also investigated the time effect in normal tissues and compared the differential expression between the two tissue types accounting for time.

Our results show that the impact of tissue handling time on the whole gene expression profile could be considered minor in colon and that a reasonable threshold for collecting specimens could be 60 minutes after surgery, when no gene alterations were observed. Such samples may be used to generate reproducible microarray profiles to aid treatment decision making utilizing clinicogenomic models.

Materials and Methods

1 Ethics Statement

All patients whose biological samples were included in the study signed an informed consent, approved by the Independent Ethical Committee of the INT-MI, to donate to INT-MI the leftover tissue specimens after completing diagnostic procedures for research purposes. The Independent Ethical Committee of INT-MI approved the use of the samples for this specific study in the framework of a project in biobanking quality control.

2 Study Design and Sample Handling

The tumor and the normal counter-part samples used in the experiments were prospectively collected from 15 patients who underwent surgical resection at the INT-MI and whose tumors were representative of the different pathologic stages of this tumor type. At the histological routinely examination all tumor specimens were classified moderately differentiated colonic Adenocarcinomas type. At the histological routinely examination all tumor specimens were representative of the different pathologic stages of this tumor and underwent surgical resection at the INT-MI and whose tumors were representative of the different pathologic stages of this tumor type. At the histological routinely examination all tumor specimens were classified moderately differentiated colonic Adenocarcinomas type.

Table 1. Characteristics of the cases sampled for RNA isolation.

| Case | Gender | Age  | Tumor location | Grade | Stage | Lymphonodes |
|------|--------|------|----------------|-------|-------|-------------|
| 1    | F      | 80   | sigma-rectum   | G2    | pT2   | neg         |
| 2    | F      | 45   | sigma-rectum   | G2    | pT2   | neg         |
| 3    | M      | 47   | sigma          | G2    | pT2   | neg         |
| 4    | F      | 72   | colon          | G2    | pTis  | neg         |
| 5    | M      | 67   | sigma-rectum   | G2    | pT1   | neg         |
| 6    | M      | 35   | colon          | G2    | pT2   | neg         |
| 7    | M      | 70   | sigma-rectum   | G3    | pT2   | neg         |
| 8    | F      | 76   | sigma-rectum   | G2    | pT4   | pos         |
| 9    | F      | 60   | sigma          | G2    | pT3   | pos         |
| 10   | M      | 73   | sigma-rectum   | G2    | pT2   | neg         |
| 11   | F      | 54   | sigma-rectum   | G2    | pT3   | pos         |
| 12   | M      | 32   | sigma-rectum   | G3    | pT3   | pos         |
| 13   | F      | 73   | ileum          | G2    | pT2   | neg         |
| 14   | M      | 55   | sigma-rectum   | G2    | pT4   | pos         |
| 15   | M      | 43   | sigma          | G2    | pT4   | pos         |

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Total RNA was extracted from 10–20 mg of tumor samples and from 30–40 mg of normal samples. Tissues were mechanically disrupted and simultaneously homogenized in the presence of QCazol Lysis reagent (Qiagen, Valencia, CA, USA), using a Mikrodismembrator (Braun Biotech International, Melsungen, Germany). RNA was extracted using the miRNeasy Mini kit (Qiagen) according to manufacturer’s instructions. Purification and DNase digestion were performed using two different kits: RNasey MinElute Cleanup (Qiagen) was employed for up to 45 μg of RNA while RNasey Mini kit (Qiagen) was used for RNA ranging between 45 and 100 μg. RNA concentrations were measured with the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) while RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano kit (Agilent Technologies). The RNA Integrity Number (RIN) [5] was determined using the software provided by the manufacturer.

3 RNA Extraction and Evaluation

3.1 RNA extraction

Total RNA was extracted from 10–20 mg of tumor samples and from 30–40 mg of normal samples. Tissues were mechanically disrupted and simultaneously homogenized in the presence of QCazol Lysis reagent (Qiagen, Valencia, CA, USA), using a Mikrodismembrator (Braun Biotech International, Melsungen, Germany). RNA was extracted using the miRNeasy Mini kit (Qiagen) according to manufacturer’s instructions. Purification and DNase digestion were performed using two different kits: RNasey MinElute Cleanup (Qiagen) was employed for up to 45 μg of RNA while RNasey Mini kit (Qiagen) was used for RNA ranging between 45 and 100 μg. RNA concentrations were measured with the NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, DE) while RNA quality was assessed with the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA) using the RNA 6000 Nano kit (Agilent Technologies). The RNA Integrity Number (RIN) [5] was determined using the software provided by the manufacturer.

4 Gene Expression Profiling

Gene expression profiles to aid treatment decision making utilizing clinicogenomic models.
each with a unique sequence derived from human genes in the National Centre for Biotechnology Information Reference Sequence and UniGene database. Array chips were washed with manufacturer’s EIBC solution, stained with 1 μg/ml Cy3-streptavidine (Amersham Biosciences; GE Healthcare, Piscataway, NJ, USA) and eventually scanned with Illumina BeadArray Reader.

5 Real Time PCR

Taqman® gene assays were used for validation of ABL1 (Hs00245443), FOSB (Hs01547109), JUN (Hs00277190) genes in the Tumor samples and HIST1H1D (Hs002771167), HIST1H1E (Hs00271195), HIST1H4E (Hs003743461), HIST4H4 (Hs00545522) in both Tumor and Normal samples. All genes were normalized to 18S (HS03003631), while the three Tumor-associated genes were also normalized to ACTB (HS03023942) and GAPDH (Hs00266705). Briefly, cDNA was synthesized in duplicate for the validation of ABL1, FOSB and JUN and in a single reaction for the validation of histone genes from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Real-Time qPCR was performed using the FAST chemistry (Applied Biosystems) with the gene-specific assays in ABI PRISM 7900 HT Real-Time PCR system (Applied Biosystems) using 10 ng of cDNA.

6 Data Analysis

6.1 Microarray data pre-processing. Raw data were obtained from scanned images using the Illumina BeadStudio software (version 3.3.8) and pre-processed using the lumi package [6] of the Bioconductor project [7]. The signal mean, the detection rate and the between array distances were evaluated in the quality control step. Two of the 90 profiles from tumor samples and two from the 90 profiles from normal samples did not pass quality controls and were discarded in subsequent analysis. Data were normalized using the Robust Spline Normalization method and probes with a detection p-value <0.01 in less than 10% of samples were filtered out. All microarray data are MIAME compliant and the raw data were deposited into the NCBI’s Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/ projects/geo/) with accession number GSE37182. The association of baseline gene profiles and clinic-pathological features was analyzed by One-way ANOVA.

6.2 Time course analyses. In order to identify probes in tumor samples differently expressed across the four different time points (time course expression analysis) a mixed-effects model was implemented on the microarray data by considering the factor Time (T0, T1, T2, T3) as fixed and the factor Patient as random [8]. The model was implemented by considering as dependent variables the log (base 2) expression value of each probe. The p-values of the model were adjusted with the Bonferroni method [9].

For the validation study the same approach was applied by considering as dependent variables the –ΔΔCt values obtained from qPCR performed on the genes of interest. Statistical analysis was performed implementing a specific code developed in SAS® software v. 9.2 (SAS Institute Inc. Cary, NC). Superimposable results were produced by using the programming language R v. 2.12.0 [10] as well as by using the freely distributed and open-source EDGE software package [11]. An identical statistical procedure was subsequently applied in processing the data from Normal tissue. RIN values corresponding to both normal and tumor samples were analyzed following the same approach used for microarray data by also considering the factor Type (normal or tumor) as fixed (time course RIN analysis).

6.3 Gene set analysis. Gene set functional analysis using the Gene Ontology (2010) [12,13] databases was performed using the Bioconductor packages GOstats [14] (v. 2.16.0) and Category (v. 2.16.0) with default parameters (over-representation with p-value <0.01).

Results

1 RIN Analysis

The RNA Integrity Number (RIN) of all samples over all time points had a median value of 5.8 with an inter-quantile range of 1.95. Tumor samples had on average a higher RIN value than the normal samples (median of 6.4 and 5.35 respectively), especially at the first time point T0. Similar distributions of RIN values could be observed across the different time points and considerable variation was observed for each sample within the first time point T0 (RIN variance median = 1.668, IQR = 2.084) which was more pronounced in the normal samples. The time course RIN analysis showed that RIN values had a tendency to decrease over time (overall p-value for Time = 0.0565 and for single contrasts compared to T0, T1 = 0.0214, T2 = 0.1705 and T3 = 0.0529). The distribution of RIN values differ significantly (p-value = 0.0008) in Tumor samples with respect to the Normal ones (Fig. 1).

By considering all the four time points we identified a subset of 6 normal and 7 tumor cases the RIN values of which was ever higher than five and time course expression analysis was performed also for this subset of samples. It should be stressed
that no association was observed between gene profiles on tumor samples at T0 and stage (p-value = 0.59) or location (p-value = 0.93) of the disease.

2 Time Course Expression Analysis

After the quality control of microarray data, the expression profiles of 4 samples (2 profiles from tumor and 2 from normal samples) were removed. These outliers belonged to different time points of two patients in the case of normal samples and to the same patient for tumor samples. Due to the nature of the study design it was crucial to have for each patients a full pictures across all the time points and thus all profiles associated with those patients were not take into consideration in the time course analysis. As a consequence, a total of 13 patients were used in the Normal dataset which contained 17895 probes and 14 in the Tumor dataset containing 19007 probes. Both datasets shared 16698 common probes.

The probes identified by the stringent Bonferroni correction (p-value < 0.05) method in the Time factor or in at least one of the time contrasts (T0 baseline) in the Normal and Tumor datasets are listed in Table 2 (N = 32) and Table 3 (N = 31) respectively. In these tables, the rows have been ordered according to the raw p-values deriving from the Time factor and a star (*) under the columns ‘Time’, ‘T1’, ‘T2’, or ‘T3’ indicates from which contrast the probe was selected. Heatmaps in the panel A and B of Figure S1 graphically represent the direction of the expression alteration at each time point in the Tumor and Normal datasets, respectively.

Five probes (genes) exhibiting different means of expression across the four time points were identified both in the Normal and the Tumor datasets (HIST1H1D, HIST1H1E, HIST1H4E, HIST4H4 and 5960086). The majority of the variability observed derives from the last time point T3 at 360 minutes when comparing to the baseline T0. This is especially true in the Tumor dataset whereas some probes deriving from the T2 contrast emerge in the Normal dataset. All genes identified in the Tumor dataset are consistently up-regulated over time; the same applies to the probes deriving from the T2 contrast in the Normal dataset and 6 in the Tumor dataset, dataset probably because of the reduced sample size. No common probes were found in Tumor dataset.

Four of them were also time-dependent in the tumor samples, together with other 27 genes with various functions involved in cancer, including oncogenes, such as JUN, FOSB, ABL1 and EGR1. Another gene commonly modulated in Normal and Tumor tissues was RNU7I1, a small nuclear RNA.

An identical analysis was performed using the subset of samples with a RIN higher than five which identified only 6 probes in the Normal dataset and 6 in the Tumor dataset, dataset probably because of the reduced sample size. No common probes were found in the two lists. Five of 6 probes (HIST1H1E, HIST1H4B, HIST1H4E, HIST4H4, 5960086) in the Normal dataset were also present in the analysis using all samples and 2 (HSPA1A, IERS) were in common in Tumor dataset.

3 RT-PCR Validation

The three genes that were significant for ‘Time’, ‘T2’ and the ‘T3’ contrasts in the Tumor samples (JUN, FOSB and ABL1) were selected for technical validation with Real Time PCR (RT-PCR) analysis in the same Tumor tissues belonging to the 14 different patients analyzed by microarrays. Two of them (JUN and FOSB) confirmed the results from the arrays. Figure 2 reports the expression dynamic over time of the genes, normalized to 18S. Normalization using GAPDH and ACTB housekeeping genes confirmed these results (data not shown). ABL1 was not validated as ischemia associated gene likely due to the weak level of association between microarray and RT-PCR data. This can be partially explained by the limited expression variability of this gene in our samples compared with JUN and FOSB (Fig. S2). Four genes commonly modulated in normal and tumor tissue, were also analyzed (HIST1H1D, HIST1H1E, HIST1H4E and HIST4H4); only one of them, HIST1H4E, confirmed the microarray data.

Discussion

We studied the effect of tissue handling time on global gene expression using CRC specimens sub-sampled and snap-frozen at different time points post-surgery, following routine tissue handling protocols of a Pathology Unit. RNA quality assessed by the RNA Integrity Number (RIN) showed a slight trend of decrease...
Table 3. Top candidates identified in the Tumor dataset (Bonferroni P<0.05).

| ProbeID | ILMN | Symbol | P-value | Time | T1 | T2 | T3 |
|---------|------|--------|---------|------|----|----|----|
| 4590440 | ILMN_1708922 | ABL1 | 4.00E-11 | * | * | * | * |
| 6380717 | ILMN_1789074 | HSFA1A | 8.57E-10 | * | * | * | * |
| 1340600 | ILMN_1659936 | PPP1R15A | 1.14E-09 | * | * | * | * |
| 1030017 | ILMN_1746435 | HIST1H1E | 1.73E-09 | * | * | * | * |
| 7160239 | ILMN_1751607 | FOSB | 1.98E-09 | * | * | * | * |
| 6510367 | ILMN_1806023 | JUN | 7.87E-09 | * | * | * | * |
| 2260309 | ILMN_1704056 | RPPH1 | 9.68E-09 | * | * | * | * |
| 4920110 | ILMN_1718977 | GADD45B | 1.20E-08 | * | * | * | * |
| 4490520 | ILMN_1798706 | EB2 | 2.21E-08 | * | * | * | * |
| 2230619 | ILMN_1681542 | HIST1H4E | 2.75E-08 | * | * | * | * |
| 2680450 | ILMN_1749789 | HIST1H1D | 3.02E-08 | * | * | * | * |
| 4180360 | ILMN_2053992 | HIST4H4 | 2.56E-07 | * | * | * | * |
| 4280113 | ILMN_1773154 | NFKBIA | 3.16E-07 | * | * | * | * |
| 5960866 | ILMN_1901419 | EGR1 | 4.18E-07 | * | * | * | * |
| 870338 | ILMN_1762899 | TAGAP | 6.31E-07 | * | * | * | * |
| 5890739 | ILMN_1733965 | STX11 | 1.18E-06 | * | * | * | * |
| 60470 | ILMN_1720771 | STX11 | 1.40E-06 | * | * | * | * |
| 6860377 | ILMN_1781285 | DUSP1 | 1.47E-06 | * | * | * | * |
| 3800168 | ILMN_1775708 | SLC2A3 | 2.07E-06 | * | * | * | * |
| 830619 | ILMN_1676984 | DDIT3 | 2.25E-06 | * | * | * | * |
| 3400332 | ILMN_1802205 | RHOB | 2.54E-06 | * | * | * | * |
| 650241 | ILMN_1721833 | IER5 | 2.58E-06 | * | * | * | * |
| 7570411 | ILMN_1761314 | NFKBIA | 2.87E-06 | * | * | * | * |
| 5050162 | ILMN_1780582 | CD83 | 2.92E-06 | * | * | * | * |
| 6020470 | ILMN_1756937 | ST8SIA4 | 4.26E-06 | * | * | * | * |
| 1940047 | ILMN_1703538 | AIF1 | 4.36E-06 | * | * | * | * |
| 7550484 | ILMN_1760347 | SRGN | 4.93E-06 | * | * | * | * |
| 4490176 | ILMN_1656011 | RGS1 | 9.37E-06 | * | * | * | * |
| 5700670 | ILMN_1668417 | WASPPIP | 9.49E-06 | * | * | * | * |
| 3170128 | ILMN_2353732 | CD8A | 1.04E-05 | * | * | * | * |
| 620717 | ILMN_1773352 | CCL5 | 1.08E-05 | * | * | * | * |

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Effects of Warm Ischemic Time on Gene Expression

associated to time with tumor samples exhibiting higher and less variable RIN values at T0 compared to the normal samples. The presence of degradation at T0 could indicate the variability of the procedure or a specific sensitivity of RNA from colon tissue. Results obtained for tumor samples are in agreement with what found by Bray et al. [15]. All the samples were used for microarray analysis irrespective of their RIN number to understand if the microarray platform could highlight better proxies of quality for analysis irrespective of their RIN number to understand if the microarray platform could highlight better proxies of quality for

The present finding is in agreement with what found by Dumur et al. [16] on ovary cancer cases and by Bray et al. [15], in CRC, where the number of probes with increased expression augmented with time. As discussed by the authors of the two studies, this is opposite to what expected since handling time should favor degradation of RNA transcripts and may in part at least reflect an active modulation of gene expression. Comparison between GO classification of differentially expressed genes in our (FDR adjusted p-values<0.05) and Bray’s analyses showed few common enriched terms (‘protein dimerization activity’ and ‘transcription factor activity’). Possible explanations for these results could be the different time points evaluated (up to 360 minutes in our study and up to 120 minutes in Bray’s study), different procedures followed for sample collection (surgical specimens that followed the routine processing standard vs tumor biopsies) and different microarray platforms used for expression analyses (Illumina vs Affymetrix).

Two of the three genes selected for the validation in tumor tissues (JUN and FOSB) confirmed the array results. These genes were also among the mRNAs most significantly affected by time to freezing in a breast cancer study [17]. As reported by these authors, JUN and FOSB are stress induced immediate transcription factors which are components of AP-1 dimers, and these dimers have been found altered by ischemia in different tissues including...
prostate and colon cancer. In ischemic and reperfused cells the two genes induce proliferation and apoptosis and could be closely related to attempts for degradation or regeneration of injured tissues [18]. Five probes were shared among normal and tumor specimens. They identified genes from the histone family (HIST1H1D, HIST1H1E, HIST1H4E and HIST4H4) involved in DNA organization, and a small nuclear RNA (RNU11). Only one of these genes (HIST1H4E) validated the microarray data. The high sequence similarity of the histones could explain the high number of modulated histones identified, that could be due to partially aspecific hybridization and could be the reason for the lack of validation with an independent technique.

In this paper we have described for the first time the effect over time on handling up to 6 hours of normal colorectal tissue, which is frequently used as control in genome wide analyses. Interestingly, normal tissue showed less degradation than its corresponding tumor specimen, both in terms of RNA quality (RIN value) and of modulated genes that were mainly histones. Our results favor the storage of normal tissue, in addition to the tumoral.

The overall changes in gene expression seen in the specimens analyzed in the current study, where more than 16000 probes were investigated, do not seem to correlate with a global transcriptome event that one might expect, at least during the time frame analyzed in this study (<20 minutes, 60 minutes, 180 minutes and 360 minutes). Considering the very low number of affected genes found, the impact of tissue handling time on the overall gene expression profiling, at least in CRC, could be considered minor and would not be expected to play a major role in gene expression-based tumor stratification.

Our results agree with that of Dumor et al. [16] and of Micke et al. [19] that showed no relevant changes in ovary cancer and with what Hatzis et al. found in breast cancer [20]. Other studies have shown significant alterations in RNA after 30 minutes of tissue extirpation[15;21;22]. Since our design did not include early times, we cannot exclude that relevant changes have already

Figure 2. Time course for selected genes. RT-PCR values of FOSB, JUN and ABL1 (−ΔCt) normalized to the house-keeping gene 18S.
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occurred before our first time point (20 minutes). However, the aforementioned studies typically have very low sample sizes and would need further validation.

Significant alteration of gene expression profile were observed in our previous study on breast cancer specimens where tissue handling time altered the expression of genes included in the commonest breast cancer predictive gene signatures [23]. These data were in agreement with Borgan et al. [17], that found miRNA (microRNA) and mRNA expression altered in breast cancer with ischemia time up to six hours. Indeed, Hatzis and colleagues [20] in breast cancer showed no relevant changes in expression levels of single genes and multigene signatures, probably because they considered 40 minutes as longest time point at room temperature or up to 190 minutes but using the RNA-later to preserve RNA integrity.

Despite partial disagreement on its extent, time of tissue handling can have an effect on gene expression, and probably might vary with tissue and with tumor type, including those that could exhibit greater sensitivity to hypoxia effects, such as brain tumors [24].

Conclusions

The findings that four of the few genes significantly different among the timepoints analyzed in the Tumor samples were oncogenes, hints that analysis of their expression in tumor specimens could lead to misleading results. Even if tissue handling time has a weak impact on the overall gene expression profiling, the deregulation of genes directly involved in tumor processes implies that tissues should be stored at early times after surgery (in our context no more than one hour) and strongly support its introduction as guideline for tissue repositories.

Supporting Information

Figure S1 Expression alteration at each time point in the Tumor (A) and Normal (B) datasets

Figure S2 Scatter plots of RT-PCR ΔCt values (x-axis) versus log2 microarray intensity values(y-axes) for ABL1, JUN and FOSB genes. In each graph the Pearson correlation coefficient (R) was used to measure the strength of association.

Author Contributions

Conceived and designed the experiments: PV SP MC VM MGD MG MM MAP. Performed the experiments: VM MG MM LDC. Analyzed the data: MH SM SV PV SP JFR MC. Contributed reagents/materials/analysis tools: MM SV MC JFR PV SP. Wrote the paper: MGD MAP MG MC VM JFR.

References

1. McHugh SM, O'Donnell J, Gillen P (2009) Genomic and oncoproteomic advances in detection and treatment of colorectal cancer. World J Surg Oncol 7: 36.
2. Erickson HS, Josephson JW, Vira M, Albert PS, Gillespie JW, et al. (2010) Influence of hypoxia induced by minimally invasive prostatectomy on gene expression: implications for biomarker analysis. Am J Transl Res 2: 210–22.
3. Bell WC, Sexton KC, Grizzle WE (2010) Organizational issues in providing high-quality human tissues and clinical information for the support of biomedical research. Methods Mol Biol 576: 1–30.
4. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, et al. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893–917.
5. Schroeder A, Mueller O, Stocker S, Salosky R, Leiber M, et al. (2006) The RIN: an RNA integrity number for assigning integrity values to RNA measurements. BMC Mol Biol 7: 3.
6. Do P, Klibbe WA, Lin SM (2008) hmi: a pipeline for processing Illumina microarrays. Bioinformatics 24: 1547–8.
7. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, et al. (2004) Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 5: R80.
8. Mc Culloc C, Searle S (2001) Generalized Linear, and mixed models. New York: John Wiley & Sons.
9. Miller RG (1981) Simultaneous statistical inference. Second ed. Springer Verlag.
10. R Development Core Team (2010) R: A Language and Environment for Statistical Computing. Vienna: R Foundation for Statistical Computing.
11. Leck JT, Macaskill E, Dabney AR, Storey JD (2006) EDGE: extraction and analysis of differential gene expression. Bioinformatics 22: 307–8.
12. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 23–9.
13. Kanchisa M, Goto S (2000) KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res 28: 27–30.