The NCI Biospecimen Research Network: The influence of warm ischemic time on gene expression profiles for colon cancer

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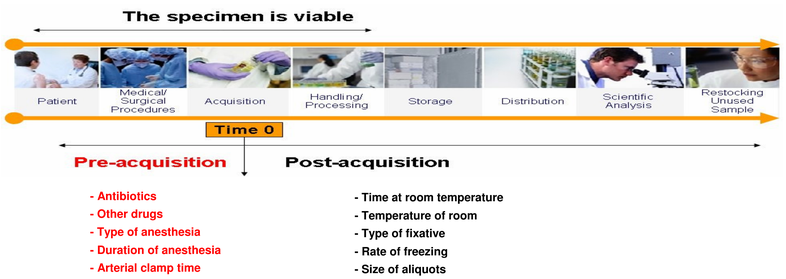
Abstract: Since access to high quality human biospecimens has been identified as a critical resource to support genomic- and proteomic-based studies, the NCI established the Office of Biorepositories and Biospecimen Research (OBBR) to coordinate biospecimen-related policies and practices for NCI-supported biorepository resources. Few studies have been published demonstrating the relationship between specimen handling, quality, and reproducibility of data in cancer research. The NCI Biospecimen Research Network (BRN), organized by the OBBR, is conducting biomolecular studies for the development of appropriate data-driven, evidence-based practices and protocols for specific specimen types and molecular analysis platforms.

The present study examines the influence of warm ischemic time on RNA quantity, quality, and gene expression profiles in colon cancer tissue. Twenty-eight cases of matched frozen colon normal and cancer tissue samples with four cases per five-minute interval from 20 to 50 minutes were microdissected using laser capture microdissection (LCM) and RNA purified. RNA was quantified by nanodrop and quality determined by Bioanalyzer. After RNA amplification and labeling, gene expression was assessed for cancer samples using Affymetrix Human Genome U133 Plus 2.0 GeneChips. Differential expression comparing the shortest ischemic time with other ischemic times was performed using principal component analysis, hierarchal clustering, and ANOVA. There was no correlation between ischemic time and RNA quantity and RNA quality. All cancer samples showed good to excellent quality RNA except one that showed poor quality. All normal samples showed poor quality RNA except five cases that showed excellent quality and therefore not subjected to gene expression analysis. For the cancer cases, three trends of gene expression changes over ischemic time were observed. Patterns include approximately 462 genes involved in cell signaling pathways such as EGFR, CDK2, and MADD, cell growth and proliferation, cell death, cell cycle, apoptosis, immune response, and cell adhesion. Selected genes will be validated using quantitative RT-PCR. This study demonstrates that warm ischemic time in colon cancer may give rise to artifactual changes in expression of genes that are significant in colon cancer.

Different morphological and biomolecule analysis techniques require different methods of biospecimen preservation



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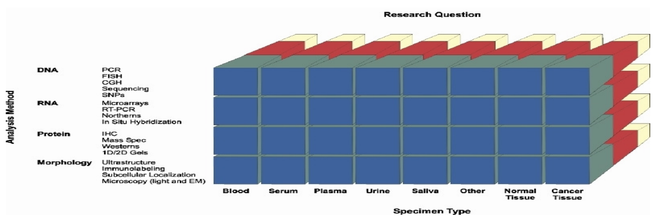


Materials and Methods

Microdissection and RNA isolation

- Total 56 colon specimens (28 matched normal/cancer)
- Tissue sections cut at 8um thick sections; Hematoxylin and Eosin stained (for optimum visualization before laser microdissection)
- Laser microdissected tumor and normal epithelium with 3000 shots (approximately 15,000 cells) in less than 45 minutes
- Isolated total RNA using PicoPure Kit with 15 minute DNAse digestion

RNA Quality and Quantity

- Bioanalyzer for quality. RNA Integrity Numbers (RIN) ≥5, 28S/18S ratios ≥ 0.8
- NanoDrop for concentration.

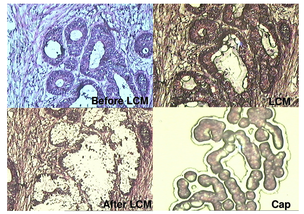
Amplification, Hybridization

- Two rounds amplification in vitro transcription biotin incorporation and fragmented
- Streptavidin-phycoerthythin Staining
- Hybridizations using Affymetrix U133 plus 2.0 Genechip in duplicate

Analysis

- Data analysis in GCOS (Affymetrix provided). Signal intensities normalized using Z-transformation and Quantile transformation
- Principal component analysis, hierarchal clustering, ANOVA and gene ontology and pathway analysis using Ingenuity Pathway Analysis

1A



1B

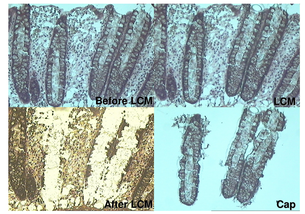


Figure 1 LCM of colon tissue (A) Cancer and (B) Normal.

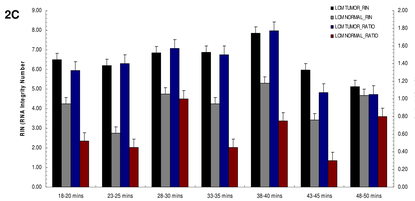
2A

Time	Case ID	Con. (Ng/ul)	RIN	Ratio	Quality
16-20	A114 TN 14	16.8	2.4	0	GOOD
	A243 TN 15	9.7	8.5	1.5	EX
	A548 TN 14	5.8	2.4	0	POOR
	A622 TN 13	11	7.2	0.6	POOR
23-25	A156 TN 16	5.21	4.1	0.7	POOR
	A238 TN 15	24.24	0	0.2	POOR
	A511 TN 15	13.87	2.7	0.3	POOR
	A165 TN 13	13.57	4.2	0.6	POOR
28-30	A161 TN 14	3.36	4.4	0.7	POOR
	A182 TN 13	2.06	6.3	1.8	POOR
	A725 TN 14	1.25	4.7	0.9	POOR
	A101 TN 14	5.39	3.6	0.6	POOR
33-35	A251 TN 13	8.4	3.9	0.4	POOR
	B128 TN 16	2.6	4.9	0.5	POOR
	B329 TN 16	25.9	4	0.3	POOR
	A76 TN 16	7.3	4.2	0.6	POOR
38-40	A11 TN 14	17.1	5.7	1.1	GOOD
	A197 TN 16	4.9	6.2	0.8	EX
	A423 TN 15	7.8	5.2	0.8	GOOD
	A151 TN 16	7.07	4.1	0.3	GOOD
43-45	A24 TN 13	11.93	3.8	0.6	POOR
	A201 TN 16	73.4	2.3	0	POOR
	A304 TN 16	10.07	4.3	0.6	POOR
	A546 TN 15	12.25	3.3	0	POOR
48-50	A387 TN 14	8.4	3.1	0.2	POOR
	B153 TN 16	24.9	3.6	0.1	POOR
	B222 TN 16	11.9	6.9	1.7	EX
	A61 TN 16	22.6	5.1	1.2	EX

2B

Time	Case ID	Con. (Ng/ul)	RIN	Ratio	Quality
16-20	A114 TP 1	12.1	6.9	1.6	EX
	A243 TC 7	26.2	4.8	1.3	EX
	A548 TC 9	9.6	6.7	1.2	GOOD
	A622 TP 2	11.92	7.2	1.2	EX
23-25	A156 TP 2	20.46	6.5	1	EX
	A238 TP 2	9.91	6.6	1.6	EX
	A511 TP 3	22.35	7.7	1.6	EX
	A165 TP 3	7.04	5.2	1.4	EX
28-30	A161 TP 2	14.9	5	1.5	GOOD
	A182 TC 7	5.4	7.1	1.7	EX
	A725 TP 4	15.7	7.6	2	EX
	A101 TP 3	15.9	7.7	1.1	EX
33-35	A251 TC 7	15.1	7.2	3	EX
	B128 TP 3	9.03	6.6	0.9	EX
	B329 TC 10	7.41	7.1	1.1	EX
	A76 TC 7	8.49	6.6	1	EX
38-40	A11 TP 1	19.6	7.8	1.7	EX
	A197 TP 2	11.81	7.9	1.7	EX
	A423 TC 7	12.4	7.8	1.9	EX
	A151 TP 2	9.9	7.9	1.8	EX
43-45	A24 TP 4	9.25	5.9	0.9	GOOD
	A201 TC 9	12.23	5.5	1.2	GOOD
	A304 TC 8	7.01	6.5	1.2	EX
	A546 TC 10	8.69	6	1	EX
48-50	A387 TC 10	7.3	4.8	1.4	GOOD
	B153 TC 7	12.8	3	0	POOR
	B222 TP 3	24.7	7.9	1.8	EX
	B61 TC 7	9.54	4.8	1	GOOD

2C



2D

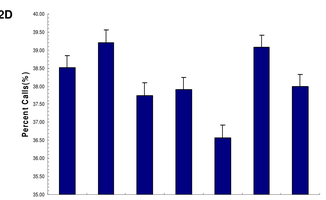


Figure 2. Recovery and quality of RNA from microdissected (A) colon cancer and (B) normal colonic epithelium. (C) Quantity and quality for both normal and cancer. (D) Percent probe sets called present for cancer samples.

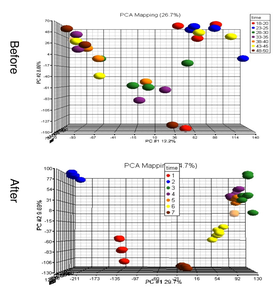


Figure 3. Principal Component Analysis (PCA) for the different ischemic times for colon cancer. These data sets are analyzed such that the greatest variance by any projection of the data comes to lie on the first coordinate (called the first principal component), the second greatest variance on the second coordinate, and so on.

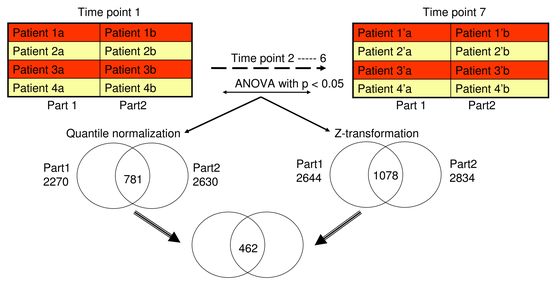


Figure 4. Gene expression analysis using Z-transformation and Quantile Normalization to find overlap candidate genes

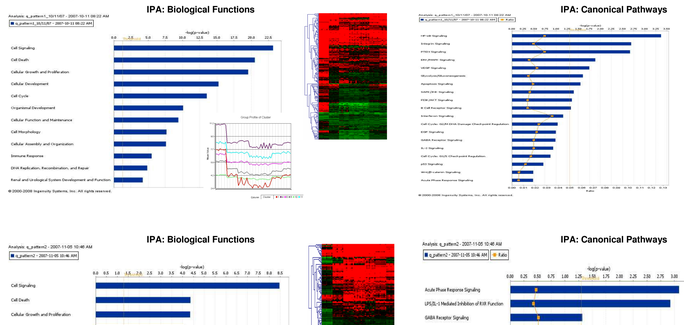


Figure 5. Gene expression profile, biological gene ontology and pathways in 291genes found in pattern 1; U,D .

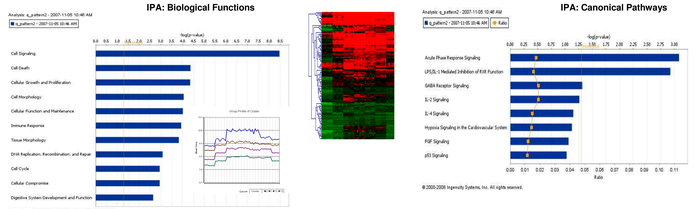


Figure 6. Gene expression profile and gene ontology of 138 candidate genes found in pattern 2; D,U,D.

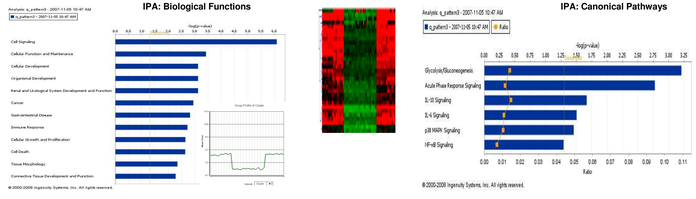


Figure 7. Gene expression profile and gene ontology of 31 candidate genes found in pattern 3.

Gene	Description	Type	Therapeutic Drugs Associated with Gene	Gene Exp
CAT2	Carbonic anhydrase XII	enzyme	Methazolamide, hydrochlorothiazide, acetazolamide, trichloromethiazide, chlorothiazide, chlorthalidone, benzthiazide, sulfacetamide, topiramate	Pattern 1; U,D
MAOB	Monoamine oxidase B	enzyme	Safinamide, ladostigil, rasagiline, selegiline, dextroamphetamine, procainamide, tranylcypromine, phenelzine, isocarboxazid, benzphetamine	Pattern 1; U,D
BCR	Breakpoint cluster region	kinase	Imatinib	Pattern 1; U,D
CDK2	Cyclin-dependent kinase 2	kinase	BMS-387032 , flavopiridol	Pattern 1; U,D
EGFR	Epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	kinase	Cetuximab, AEE788 , panitumumab, BMS-599626 , AERY-334643 , XL647 , canertinib , gefitinib , HKI-272 , PD 153035 , lapatinib , vandetanib , erlotinib	Pattern 1; U,D
LCK	Lymphocyte-specific protein tyrosine kinase	kinase	Dasatinib	Pattern 1; U,D
PPP3CA	Protein phosphatase 3 (formerly 2B), catalytic subunit, alpha isoform	phosphatase	ISAtx-247 , tacrolimus , pimecrolimus , cyclosporin A	Pattern 1; U,D
PML	Promyelocytic leukemia	transcription regulator	Arsenic trioxide	Pattern 1; U,D
IL2RG	Interleukin 2 receptor, gamma (severe combined immunodeficiency)	transmembrane receptor	Aldesleukin, denileukin diftitox	Pattern 1; U,D
FGB	Fibrinogen beta chain	Other	Thrombin	Pattern 2; D,U,D
GABRB3	gamma-aminobutyric acid (GABA) A receptor, beta 3	ion channel	Methohexital, aspirin/butalbital/caffeine, aspirin/butalbital/caffeine/codeine, pagoclone, alphadolone, SEP 174559, acetaminophen/butalbital/caffeine, sevoflurane, isoflurane, gaboxadol, isoniazid, felbamate, etomidate, muscimol, halothane, fluoxetine/olanzapine, amobarbital, atropine/hyoscyamine/phenobarbital/scopolamine, acetaminophen/butalbital, eszopiclone, mephobarbital, hyoscyamine/phenobarbital, acetaminophen/butalbital/caffeine/codeine, butabarbital, temazepam, zolpidem, lorazepam, olanzapine, clonazepam, zaleplon, secobarbital, butalbital, phenobarbital, pentobarbital, thiopental, D 23129, desflurane, methoxyflurane, enflurane, pregnenolone	Pattern 3; U,D,U
GABRP	gamma-aminobutyric acid (GABA) A receptor, pi	ion channel	Alphadolone, sevoflurane, isoflurane, isoniazid, felbamate, etomidate, halothane, fluoxetine/olanzapine, eszopiclone, zolpidem, lorazepam, olanzapine, zaleplon, secobarbital, phenobarbital, pentobarbital, desflurane, methoxyflurane, enflurane	Pattern 3; U,D,U

Table. Statistically significant genes of clinical significance that are affected by intraoperative ischemia.

Conclusions and Future Experiments

- No correlation between RNA quality and quantity from samples
- Colon cancer showed good to excellent quality RNA
- Normal colon showed essentially poor quality RNA
- With respect to ischemia, there are 3 patterns of gene expression changes and include clinically significant genes
- Further validation using RT-PCR of 10-15 key candidate genes