Identification of Differentially Expressed Isoforms Associated with Chemotherapy Resistance in Colon Cancer Cell Lines

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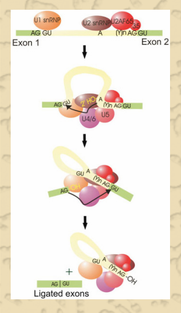
1. Abstract

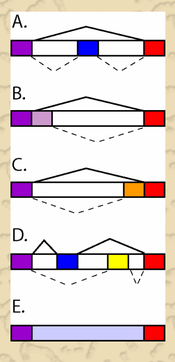
Objective: To investigate the prevalence of differentially expressed alternatively spliced (AS) transcripts associated with the transition from chemotherapy sensitivity to resistance in colorectal cancer cell lines. Such transcripts may provide new therapeutic targets or biomarkers for disease progression.
DESIGN: Exon tiling microarrays were used, in multiple replicates, to profile the expression of ~1.4 million exon regions in RNA samples isolated from chemotherapy sensitive and resistant colorectal cancer cell lines.
Methods: DNase I-treated total RNA was isolated, in triplicate using Trizol, from a clonally derived colorectal cancer cell line (MIP101) and a chemotherapy resistant derivative established by long-term exposure to 5-FU (Tai et al. 2005. J. Clin. Invest.). The presence of rRNA was reduced using Invitrogen's RiboMinus kit. Samples were further processed and hybridized to Affymetrix's Human Exon 1.0 ST Array according to the manufacturer's instructions. Raw probe intensity values were pre-processed with Affymetrix's 'ExACT' software, the mean of triplicate values for each probe was calculated, and these values were processed further by custom Perl and R scripts. Splicing index (SI) values were calculated for all exons to provide a measure of the change in expression of each exon after normalization to account for changes in expression at the gene level (Clark et al. 2002, Science). Probe sequences were mapped to the EnsEMBL gene set and custom UCSC tracks were generated to allow visualization of raw intensities and SI values at each locus and to facilitate comparisons to publicly available expression data.
Results: In the comparison between 5-FU sensitive and resistant states, approximately 100 genes had SI values indicative of at least a 4-fold difference in the abundance of at least one exon. Such high SI values indicated cases where specific exonic regions, corresponding to alternate 3' or 5' exons or skipped exons had changed in expression but the overall level of gene expression had not changed. Several of these differentially expressed (DE) exons corresponded to known alternate isoforms of genes encoding hedgehog interacting protein, laminin 3 and G-protein, gamma.
Conclusion: Exon tiling microarray data indicated the presence of differentially expressed alternatively spliced isoforms associated with the transition from chemotherapy sensitivity to resistance in colon cancer cell lines. Cross-platform hybridizations as wells as RT-PCR and cloning experiments will be conducted to validate candidate isoforms and assess the sensitivity and specificity of this approach. Confirming the relevance of differentially expressed isoforms to the resistance phenotype will require further functional validation.

Basic Splicing Machinery



RNA splicing is accomplished by the 'spliceosome' which consists of a complex of several nuclear components including a number of snRNPs. Through the action of RNA-RNA, RNA-protein and protein-protein interactions, this complex recognizes a pair of exon-intron boundaries and catalyzes sequential trans-esterification reactions to remove an intron and join two exons (Kalnina et al. 2005 Gen. Chrs. Cancer).

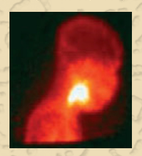
Types of Alternative Splicing



A.) Exon skipping/inclusion
B.) Alternate 3' splice site usage
C.) Alternate 5' splice site usage
D.) Mutually exclusive exons
E.) Intron retention

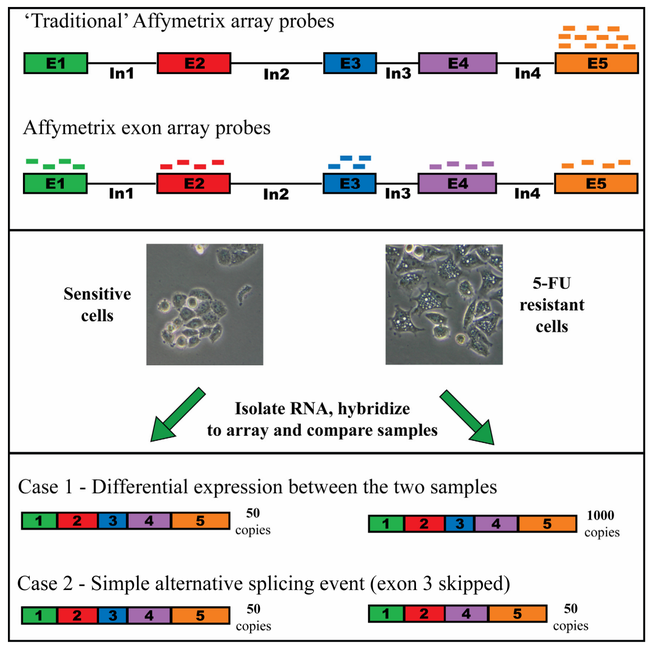
Alternative Transcription and Cancer

Alternative transcription (initiation, splicing and polyadenylation) generates multiple isoforms for most human genes. Specific isoforms may represent useful therapeutic targets or diagnostic markers. The image below shows a throat and neck tumor which has been targeted by a therapeutic antibody specific to a single alternate CD44 exon which is aberrantly expressed in the cancer (Venables, 2006).

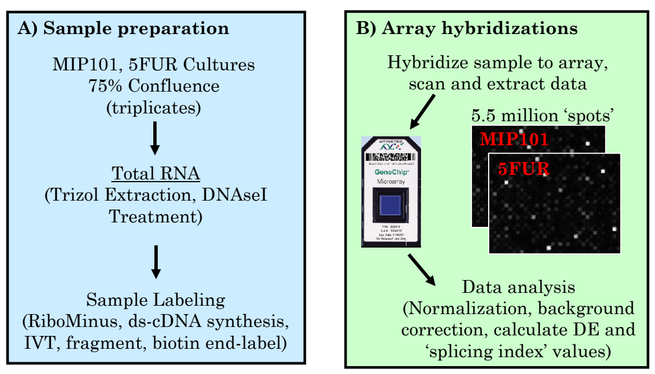


2. Methods

Array design strategy



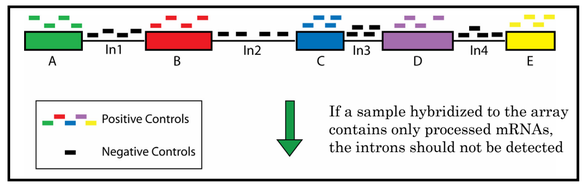
Experimental overview



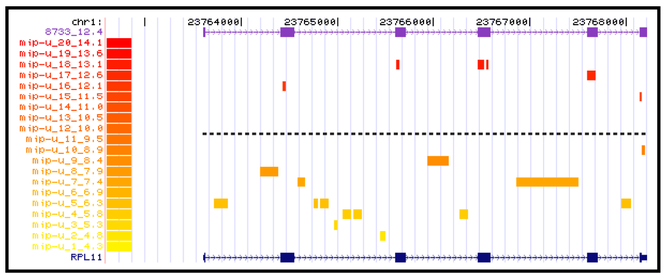
3. Results

Assessing array performance with control genes

The performance of each hybridization was assessed by examining the expression estimates from positive control probes (exonic) and negative control probes (intronic) for 100 housekeeping genes. The mean expression value for all exon probes was 12.78 +/- 0.74 fold higher than for intron probes. The following figures show the arrangement of control probes and the log2 expression values for exon and intron probes for a single housekeeping gene.



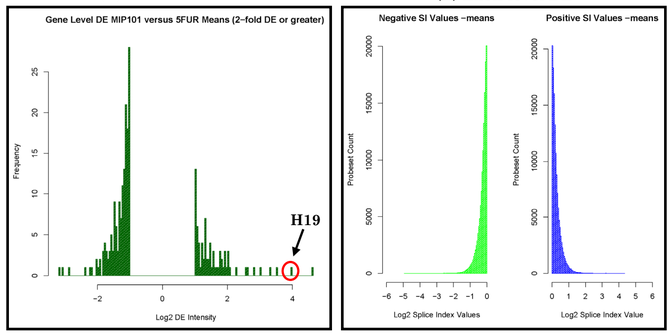
Mean expression values for the control gene, RPL11 (mean of MIP101 triplicates)



Differential expression of genes and exons

(A) Differential expression (DE) at the gene level was calculated as the mean log2 difference of gene expression values between sensitive and resistance samples (sensitive minus resistant). The following 'gene-level' figure shows only DE values of 2-fold or greater. The gene, H19 was 15.98 +/- 1.36 fold over-expressed in sensitive compared to resistant cells. **(B)** A 'splicing index' value was calculated for every exon to assess differential expression at the exon level. A total of ~1,500 exons had a 2-fold or greater change in expression between sensitive and resistant cells after normalizing for changes in expression at the gene-level.

(A) Gene-level DE **(B) Exon-level DE**



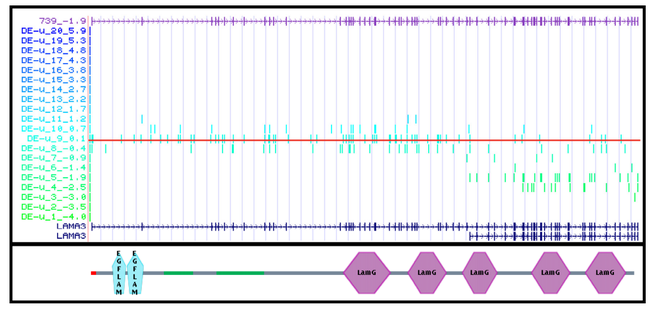
Summary of candidate differentially expressed isoforms

A total of 21 genes were found to be differentially expressed and 35 genes had evidence for differential expression of isoforms (4-fold difference or greater). Of these 35 candidate alternative transcription events, 25 are predicted to affect the ORF, 9 are confined to the 5′ UTR and 1 is confined to the 3′ UTR. A description of five of these genes follows.

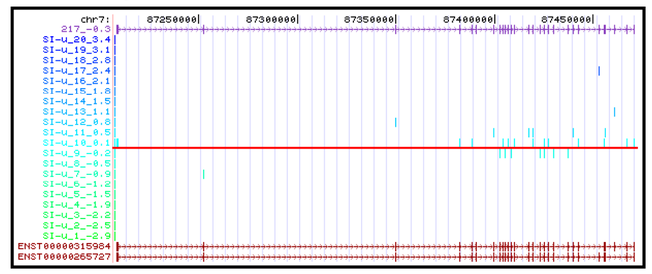
Gene Name	Gene Description	Description of Event
ADAM22	A metallopeptidase. Involved in cell adhesion.	Differential expression of two known isoforms which differ by skipping of a single exon (exon 24 of 32).
GNG2	G-protein gamma. Membrane component. Involved in signal transduction and cell proliferation.	Differential expression of two known isoforms, one with two additional 5' exons and an extra internal exon relative to the shorter isoform.
H19	Imprinted maternally expressed untranslated mRNA. Largely unknown function.	Differential expression (15.98 +/- 1.36 fold change) is observed for all 6 exons of this gene. There is also evidence for retention of the first intron. Expression of this gene is high in sensitive cells and almost completely lost in resistant cells
HHIP	'Hedgehog interacting protein'. Regulatory component of the hedgehog pathway.	Differential expression of two known isoforms, a long isoform of 13 exons and a short isoform of only the first 4 exons. Expression of the long isoform is lost in the resistant state. The short isoform lacks two EGF motifs and one transmembrane domain compared to the long isoform.
LAMA3	Member of the laminin family. Component of the ECM. Involved in cell adhesion, signal transduction and differentiation.	Differential expression of two known isoforms, a long isoform of 76 exons and a short isoform of only the last 38 exons. The short isoform lacks several of the protein motifs found in the long isoform (including an EGF-like domain).

4. Visualization of examples

Differential expression of LAMA3 isoforms



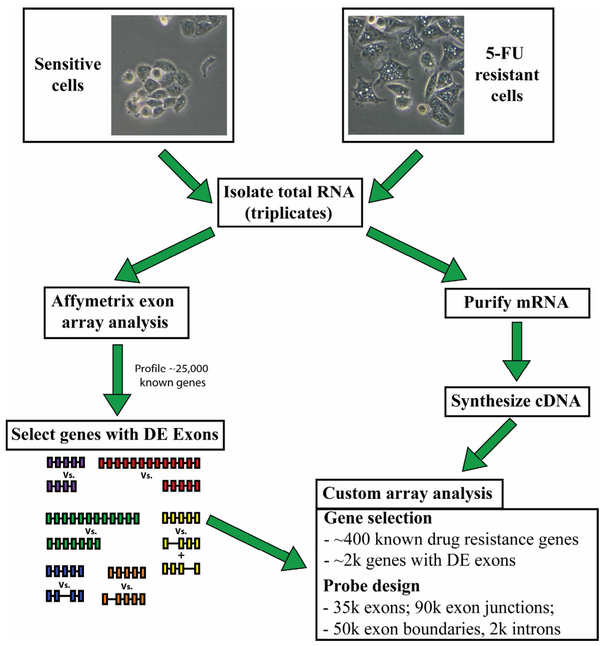
Differential expression of ADAM22 isoforms



5. Conclusions and future work

The approach described in this work identified a list of candidate differentially expressed isoforms associated with the transition from 5-FU sensitivity to resistance. qRT-PCR will be conducted on a subset of candidates to confirm the accuracy of exon level expression profiling; cloning and sequencing will be used to determine the precise structure of predicted DE isoforms; and functional assays will be conducted to study the potential role of selected genes in chemotherapy resistance. Experiments are currently being conducted to assess the sensitivity and specificity of Affymetrix exon arrays by comparing to hybridizations of the same samples on a custom array (experimental design is depicted below).

Cross-platform validation



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