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Research Article

Expression Microarray Analysis and Oligo Array Comparative Genomic Hybridization of Acquired Gemcitabine Resistance in Mouse Colon Reveals Selection for Chromosomal Aberrations

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Abstract

Gemcitabine is a commonly used therapy for many solid tumors. Acquired resistance to this nucleoside analogue, however, diminishes the long-term effectiveness in a majority of patients. To better define the molecular background of gemcitabine resistance, a mouse colon tumor was selected during successive rounds of transplantation with continued treatment of gemcitabine. Expression microarray analysis was applied to determine which genes are consistently and highly overexpressed or underexpressed in the resistant versus the nonresistant tumor. For the statistical interpretation of the microarray data, a parametric model was implemented, which returns model-based differential gene expression (log-) ratios and their uncertainties. This defined a set of 13 genes, putatively responsible for the gemcitabine resistance in solid tumors. One of these, *RRM1,* was previously identified as an important marker for gemcitabine resistance in human cell lines. Five of the 13 genes, including *RRM1,* are located within a 3 Mb region at chromosome 7E1 of which four are highly overexpressed, suggesting a chromosomal amplification. Therefore, chromosomal copy number changes were measured, using oligo array comparative genomic hybridization. A narrow and high amplification area was identified on 7E1 that encompassed all five genes. In addition, reduced RNA expression of two other genes at 8E1 encoding *COX4I1* and *RPL13* could be explained by a decrease in chromosomal copy number on chromosome 8. In conclusion, the array comparative genomic hybridization biologically validates our statistical approach and shows that gemcitabine is capable to select for chromosomally aberrant tumor cells, where changed gene expression levels lead to drug resistance. (Cancer Res 2005; 65(22): 10208-13)

Introduction

Gemcitabine is a nucleoside analogue used for systemic treatment of patients with solid tumors like cancers of the breast, lung, pancreas, and bladder. The drug has a high initial activity against these tumors, but many tumors acquire resistance to the drug (1, 2). To further define the acquired resistance to gemcitabine, we used oligo expression arrays to identify genes that are differentially expressed in gemcitabine-resistant tumors compared with nonresistant mouse colon tumors. The difference in overall gene expression between the resistant and nonresistant tumors is intrinsically subtle. Only few genes in these microarray experiments qualified as "outliers" as a consequence of the gemcitabine resistance including a most probable gene, *RRM1* (2, 3). The identification of *RRM1* in the resistant tumor was straightforward by eye due to duplicate spots and repeatability in subsequent and dye swap experiments in combination with a high deviation from normal and high expression values (4). However, no other genes were immediately and obviously detected. Statistically, this analysis is less straightforward due to the high amount of measurements in relation with the small number of experiments, in this case 7,230 measurements for each of three experiments. Moreover, most genes are expressed in moderate intensity segments of the array, whereas the noise level increases with decreasing intensities. To assess this problem and give a qualifier to the significance of the gemcitabine resistance genes, we implemented a dedicated parametric model (5, 6). Permutation methods like significance analysis of microarrays (7) are not useful for such small sample cases. The three paired samples analyzed allow for only eight possible permutations using significance analysis of microarrays. As a consequence, the minimal P value before multiple testing correction is as large as 1 divided by 8, so that a P value of 0.05 can never be reached. By taking intensity values into account, the parametric model presented in this study circumvents this dilemma. Similar to significance analysis of microarrays, our parametric model ranks the genes by significance and is dedicated for all dual channel microarray experiments with small sample size. Unlike significance analysis of microarrays, it effectively uses the fact that all genes have undergone a similar hybridization experiment and, hence, all genes share common crucial variables. The application of this model and the biological results obtained are described in this article. This led to the identification of at least 13 genes that are expressed differentially in the resistant tumor with significance comparable with *RRM1*. Four of the 13 genes identified by this method were biologically validated by the measurement of chromosomal copy number changes using oligo array comparative genomic hybridization (CGH). The results of this genomic approach reveal that the nucleoside analogue, gemcitabine, can induce drug resistance by selection for chromosomal aberrations.

Materials and Methods

Tissue collection. Sources and characteristics of the mouse colon 26 tumor model were described previously (1). Briefly, one solid mouse tumor, colon 26A, was routinely maintained by successive transplantation. A subset of mice with this tumor was treated at the maximum tolerable dosage of

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Table 1. Data set up according to GEO database, including accession numbers, validation, and control samples

Slide no. and GEO accession no.	Dye	Sample	Sampling date
IV	Cy5	Solid tumor A5	08-04-1987
GSM43959	Cy3	Solid tumor G6	03-09-1995
III	Cy5	Solid tumor A4	07-22-1994
GSM43960	Cy3	Solid tumor G3	04-27-1995
II	Cy3	Solid tumor A2	09-03-1996
GSM43962	Cy5	Solid tumor G1	07-06-1995
I	Cy5	Solid tumor G1	07-06-1995
GSM43963	Cy3	Solid tumor G1	07-06-1995

NOTE: RNA was isolated from samples taken of independent passages of colon tumors 26A and 26G and hybridized to the array without matching the time of harvest of the different passages.

gemcitabine (120 mg/kg every 3rd day ×4) during five successive transplants. The tumor resistant to the highest dose was transplanted and these mice were treated 17 times at 120 mg/kg. The most resistant tumor from this group seemed to be completely resistant in the next passages and was designated as colon 26G.

Expression microarrays. A total of four microarray expression experiments were done (Table 1). Arrays were done essentially according to Bergman et al. (4). For the expression arrays, the mouse oligo library version 1.0 (Compugen/Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) was spotted in duplicate using the SA 72 (Perkin-Elmer, Zaventem, Belgium) with Telechem SMP3 pins (TeleChem International, Inc., Sunnyvale, CA), containing 7,524 oligonucleotides of 65 bp in length representing 7,230 separate genes. RNA isolations and labeling, as well as scanning and image analysis, were done as previously described (4). All data were of high quality: Background signals were very low compared with the foreground signals. Hence, neither spot filtering nor background subtractions were done. Oligo nucleotide sequences (accessible at http://www.ensembl.org/) were verified for the 13 identified genes by Blast onto *Mus musculus* build 33.1. All 13 sequences matched their designated gene ID. However, *RPL13* only had homology with the distal 32 bases of the oligonucleotide sequence. No other significant similarities were found with the first 28 bases; thus, no artifact can be expected from this maldesigned oligonucleotide sequence. Furthermore, the sequence for the spotted oligonucleotide representing *HBB-B1* (AF071431) was AAACCCCCTTTCCT**GATTT**TGCCTGTGAA-ACAATGGTTAATTGTTCCCAAGAGAGCATCTGTCAGT, whereas the actual chromosomal sequence at 7E1 for *HBB-B1* has 2 bp differences in the regions printed in bold, which should have been G**C**T**C**T according to Ensembl. No significant homologies with other mouse genes were observed.

Statistics. For the analysis of this small data set (Table 1), a parametric model was used. The parametric analysis was done using a hierarchical Bayesian model (Supplementary Data) implemented and validated in WinBugs (Medical Research Council Biostatistics Unit, Cambridge, United Kingdom). We did regression-type, within-array normalization, which basically rescales the Cy3 versus Cy5 channels around a 45-degree line using SPSS (SPSS, Inc., Chicago). Visual inspection of the MA plots (mean log intensity versus log ratio) showed no need for nonlinear normalization of these arrays. The model returns estimates of differential gene expression (log) ratios and their uncertainties. One additional self-self hybridization was done, but was not used in the calculations because it did not improve the accuracy of the log ratio estimates.

The Bayesian hierarchical model implemented is made of several levels that mimic some of the crucial steps in the microarray experiment, such as hybridization and slide preparation. Errors are propagated through the model, resulting in realistic log ratio estimates. One of the consequences is

Table 2. Results from top 13 genes

Gene name	Gene ID	Gene description	Gene ontology	Chromosome location	AER2	Array Ratio G/A
OLFR683	AF121981	Odorant receptor 683. S51	Olfaction	7E1	5.71	53.59
TRIM21	NM_009277	Tripartite motif protein 21	Regulation of transcription. DNA-dependent	7E1	3.88	15.73
POLM	NM_017401	DNA polymerase μ	DNA replication	11A1	6.01	12.06
RRM1	NM_009103	Ribonucleotide reductase M1	DNA replication	7E1	9.38	3.82
DNTT	NM_009345	Deoxynucleotidyl transferase terminal	DNA replication	19	4.27	4.00
COX4I1	NM_009941	Cytochrome *c* oxidase subunit IV isoform 1	Energy pathways	8E1	3.46	0.38
RPL13	NM_016738	Ribosomal protein L13	Ribosome biogenesis	8E1	3.79	0.39
CRIP1	NM_007763	Cysteine-rich protein 1	Cell proliferation	12F1	3.65	0.34
KRT2-8	M21836	Keratin complex 2 basic gene 8	Cytoskeleton organization and biogenesis	15F2	7.91	0.13
HBB-B1	AF071431	Hemoglobin β adult major chain	Oxygen transport	7E1	5.68	0.13
HBA-A1	NM_008218	Hemoglobin α adult chain 1	Oxygen transport	11A4	7.19	0.11
II	X00496	Ia-associated invariant chain	Antigen processing	18 E1	4.41	0.11
HBB-BH1	NM_008219	Hemoglobin Z β-like embryonic chain	Oxygen transport	7E1	3.04	25.40

NOTE: Gene name, gene description, and gene ontology given by the oligo manufacturer; gene ID, National Center for Biotechnology Information gene identification accession numbers given by the oligonucleotide manufacturer; chromosomal location according to National Center for Biotechnology Information (m33); array ratio G/A and M: log 2 ratio G/A, model-based expression ratios; A, average log 2 intensity (Fig. 1); p2, posterior probability of the gene to be differentially expressed with >2-fold; p3, posterior probability of the gene to be differentially expressed with >3-fold; LC-PCR ratio G/A, expression, PCR-verified expression ratios with SD (in parentheses; see also Materials and Methods); array CGH copy number, change in copy number of tumor 26G relative to 26A (Fig. 2); N/A, not done.

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that the more inaccurately measured spots are down-weighted in the analysis (see Supplementary data). The parametric model provides an accurate compromise between the k-fold rule and t test. The k-fold rule is a biological rather than statistical criterion that preferentially selects for most differentially expressed genes. The standard t test is limited inherently considering the small number of arrays and focuses on statistical significance. This compromise is the posterior probability of a gene being k-fold expressed. Such a probability depends on both the intensity of the mean differential gene expression and the amount of agreement between measurements of the genes on the three arrays of the solid tumors. Alongside, the 2-fold adjusted expression ratios (AER2) across the three arrays are computed, which is the amount by which the log 2 ratio exceeds 1 [= log 2(2)] divided by the SD.

Real-time LightCycler PCR. Seven genes identified by the microarray experiments were verified by real-time PCR with a LightCycler 1.0. (Roche Diagnostics, Almere, the Netherlands) according to the protocols described by Bergman et al. (4). Primers for all seven murine genes RNA were based on the sequence of the gene (http://www.ncbi.nlm.nih.gov/entrez/) designed with the program Primer3 (http://frodo.wi.mit.edu/); *OLFR683* forward primer: 5′-GATCAAAGCAGAGGGAGCTG, reverse primer: 5′-AAGGTTCCGTATTCCCTGCT; *TRIM21* forward: 5′-CCATGGTGGAGCCTATGAGT, reverse: 5′-GGTGAAGCTTCTCTCCATGC; *POLM* forward: 5′-CCCGAGTCAACTCAGCTTTC, reverse: 5′-CTGCACAACACCTCACTGCT; *COX4I1* forward: 5′-TTCTACTTCGGTGTGCCTTC, reverse: 5′-GCGAAGCTCTCGTTAAACTG; *RPL13* forward: 5′-TACTGAAGCCCCACTTCCAC, reverse: 5′-CGGACCTTGGTGTGGTATCT; *KRT2-8* forward: 5′-ATCGAGATCACCACCTACCG and reverse: 5′-TGAAGCCAGGGCTAGTGAGT. Primer sequences for *RRM1* and the reference gene *β-actin* were described by Bergman et al. (4). Expression levels were quantified on five different tumor passages relative to *β-actin* (4). Table 1 shows the median log 2 expression ratios of the gene in 26G relative to the expression in 26A with SD, thereby eliminating β-actin in the equation, such that it is directly comparable with the M:log 2 ratio G/A of the arrays.

Oligo comparative genomic hybridization microarray. DNA from tumor and normal liver samples were isolated using the Wizard Genomic DNA purification kit according to the protocol of the manufacturer (Promega Benelux BV, Leiden, the Netherlands). Labeling and hybridization procedures for the oligo array CGH were done as previously described (8) with the following modifications: mouse oligo library version 2.0 (Compugen/Sigma-Aldrich Chemie B.V., Zwijndrecht, the Netherlands) containing 21,997 oligonucleotides (65 bp) representing 21,587 exon regions, mapped to the University of California Santa Cruz Mouse genome 2003 freeze, was spotted on the arrays. Prehybridization was omitted and Cot-1 concentrations during the hybridization were reduced to 100 μg. Hybridizations were done using a Hybstation12 (Perkin-Elmer). CGH arrays were scanned using a laser scanner (Agilent Technologies, Amstelveen, the Netherlands) and analyzed using Bluefuse Software v.2.0 (Bluegnome Ltd., Cambridge, United Kingdom). Images show fused values; values with confidence higher then 0.35 with the overall Cy3 and Cy5 channels normalized to a log 2 ratio of 0. DNA was isolated from tumors 26A and 26G. The log 2 ratio for tumor 26G was slightly noisier than for tumor 26A, which is generally a result of the DNA quality. Therefore, DNA from a second tumor 26G was isolated and hybridized. The results of this second hybridization were of the same quality as the first 26G hybridization and completely confirmed the results. Because bacterial artificial chromosome CGH arrays are often spotted in triplicate (9), we present the Bluefuse confidence-based average of values of the two spots on the separate arrays for 26G. Original data files for all three arrays were uploaded in MIAME format for expression arrays at GEO (http://www.ncbi.nlm.nih.gov/geo/; 26G accession nos. GSM44665 and GSM44666, and 26A accession no. GSM44664). For interpretation and visualization purposes, smoothing was done by version 2 of aCGH smooth (10), with λ set to 2.0.

Results

Parametric statistical analysis of expression microarray data generates high discriminative power of differentially expressed genes. Microarray data analysis using an improved parametric model identified a set of 13 of 7,230 genes differentially regulated in gemcitabine-resistant versus nonresistant mouse colon tumors (Table 2). When ranking the genes with respect to AER2, it was observed that this ratio dropped after the 13th gene (Supplementary Data). The results of all model-based estimates, log 2 ratio and log 2 sum, are graphically summarized in Fig. 1, which represent a common slide-based MA plot for all genes on the three arrays.

The expression of 7 of the 13 genes was verified by quantitative LightCycler PCR (LC-PCR). Log 2 ratio estimates were validated for the three respective tumor pairs that were hybridized to the microarrays (Table 1) as well as for two independent pairs of additional tumor passages. All genes on the array that were subjected to the LC-PCR analysis could be completely confirmed (Table 2), with only the amplitude of the up-regulation of *OLFR683* being less in the LC-PCR compared with the array measurements.

Three of 13 genes with differential expression in resistant colon tumors are involved in DNA replication. To identify a common functional denominator or pathway, we collected data

Table 2. Results from top 13 genes (Cont'd)

Gene name	M: Log 2 ratio G/A	A	p2	p3	LC-PCR log 2 ratio G/A	Array CGH copy number G/A
OLFR683	5.74	5.26	1.000	1.000	0.72 (0.96)	Amplified
TRIM21	3.98	5.13	1.000	1.000	5.43 (1.13)	Amplified
POLM	3.59	6.20	1.000	1.000	6.79 (1.21)	Same
RRM1	1.93	7.50	1.000	0.983	4.69 (0.74)	Amplified
DNTT	2.00	6.29	1.000	0.833	N/A	Decrease
COX4I1	−1.41	6.83	0.983	0.208	−0.80 (0.34)	Decrease
RPL13	−1.37	7.42	1.000	0.092	−1.10 (0.31)	Decrease
CRIP1	−1.54	6.51	0.983	0.433	N/A	Same
KRT2-8	−3.00	6.73	1.000	1.000	−3.06 (1.23)	Same
HBB-B1	−2.99	6.59	1.000	1.000	N/A	Amplified
HBA-A1	−3.14	6.48	1.000	1.000	N/A	Same
II	−3.22	6.11	1.000	1.000	N/A	Same
HBB-BH1	4.67	4.51	0.983	0.304	N/A	Amplified

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for all 13 genes, such as gene description, gene ontology, oligo sequence, and chromosomal location (Table 2).

One of the highly up-regulated genes identified by our statistical approach, *RRM1,* was verified by both real-time PCR (Table 2) and Western blot analysis (4). For *RRM1, DNTT, POLM*, and *KRT2-8*, our statistical approach not only showed high log 2 ratios and high posterior probabilities of >2-fold differential expression, but also high intensity levels. These intensity levels indicate high RNA expression levels especially because the hybridization efficiency of the oligo probes are largely normalized in their design. According to their gene ontology, 3 of 13 genes are involved in DNA replication, which includes *RRM1* in addition to *POLM* and *DNTT* (Table 2).

Five of 13 genes identified by expression array analysis are located in a 3Mb region on chromosome 7E1. Five of the 13 genes were found to reside in a 3 Mb region of chromosome 7E1. Moreover, two genes that are both down-regulated are located on a 2.5 Mb region of chromosome 8. The close proximity of the genes and their respective up- or down-regulation suggest chromosomal copy number changes as a consequence of the gemcitabine treatment. To test this hypothesis, we did oligo array CGH. Array CGH detected high-level amplifications at 7E1 spanning a 3.1 Mb chromosomal region (Fig. 2). This high-level amplification encompasses all five genes (Fig. 2*C*). No additional genes in this region were detected with our expression array analysis. The reduced RNA expression of the two genes at 8E1, *COX4I1,* and *RPL13* coincide with a decrease in copy number of the entire chromosome 8 relative to the sensitive tumor 26A. In addition to the anticipated chromosomal changes, a decrease in copy number of chromosome 19 is observed in tumor 26G relative to 26A.

Discussion

Gemcitabine is capable to select for chromosomally aberrant tumor cells, with changed gene expression leading to drug resistance. Thirteen of ~7,000 genes were identified by expression microarray analyses that are highly regulated in gemcitabine-resistant versus nonresistant tumors using a Bayesian parametric model. Seven of the 13 genes were verified by LC-PCR, confirming our expression array platform and data analysis. The identification of 8 of these 13 genes could straightforwardly be verified and explained by literature, gene ontology, or chromosomal copy number changes as measured by oligo array CGH. The gene *RRM1* was previously identified to be involved in drug resistance by several independent research groups in both cell lines and solid human tumors (2, 3, 11). Gemcitabine is a fluorinated deoxycytidine analogue and thereby interferes with DNA replication (1). Our expression array analysis identified *DNTT* and *POLM* as highly up-regulated, highly expressed, with high posterior probabilities and the same gene ontology as *RRM1:*DNA replication. This suggests that they could act in the same pathway to convey resistance to the tumors. Four additional genes, *HBB-BH1, HBB-B1, OLFR683*, and *TRIM21,* are located at the same chromosomal location, 7E1. *HBB-BH1, OLFR683*, and *TRIM21* are also up-regulated along with *RRM1.* By oligo array CGH, a high and specific amplification of 7E1, encompassing all five genes, was identified. Gene function or ontology of *OLFR683* or the hemoglobin genes does not give a logical explanation for their identification by our expression array analysis. The up-regulation of *OLFR683, HBB-BH1,* and *TRIM21,* however, can be explained by chromosomal location rather than by gene function.

The particular region on 7E1 has been studied in great detail because it does not only contain an olfactor receptor cluster, but also the β-globin gene cluster (12). β-globin gene expression is highly specific and molecularly one of the best-characterized regions of the mouse and human genomes (13). The genes are arranged along the chromosome in order of their expression during development (14), such that the expression of embryonic and adult β-globin are mutually exclusive. HBB-BH1 is an embryonic β-globin and is highly up-regulated in the resistant tumor probably as a consequence of the high-level amplification. The expression of *HBB-B1,* which is located downstream of *HBB-BH1,* and one of the adult β-globins is down-regulated, despite its high-level amplification. In addition, one of the adult α-globin genes at chromosome 11 is also down-regulated in the resistant tumor. Thus, it is tempting to speculate that, as a consequence of the high up-regulation of *HBB-BH1,* both *HBB-B1* and *HBA-A1* RNA expression is down-regulated. Thus, *HBB-BH1* is then a consequence of the amplification along with *RRM1* and the down-regulation of *HBB-B1* and *HBA-A1* a consequence of a consequence.

Interestingly, the 7E1 region corresponds to the imprinted 11p15.5 region in humans (15). Loss or gain of imprinting is a

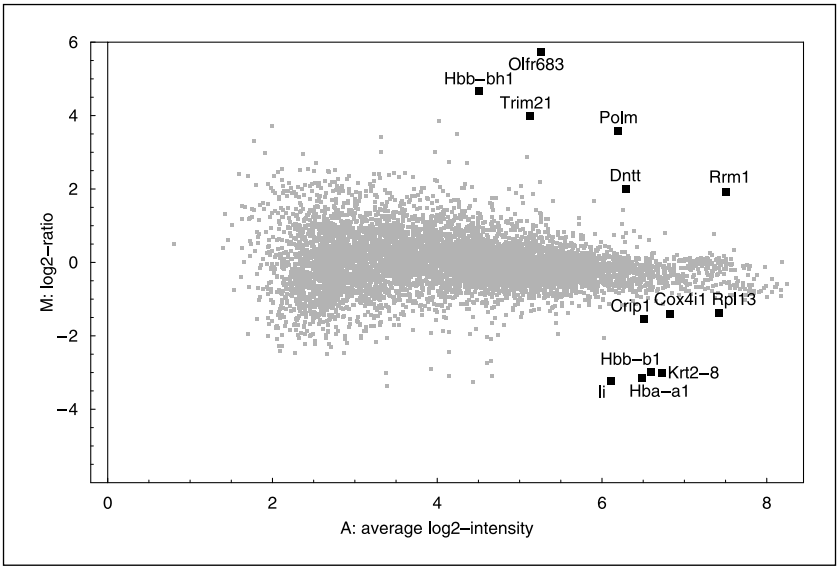

Figure 1. Model-based MA plot of tumor 26A versus tumor 26G. *X axis* (*A; average log 2 intensity*), average intensity values of both Cy3 and Cy5 channels together for each individual gene on the arrays. *Y axis* (*M; log 2 ratio*), ratio of 26G over 26A.

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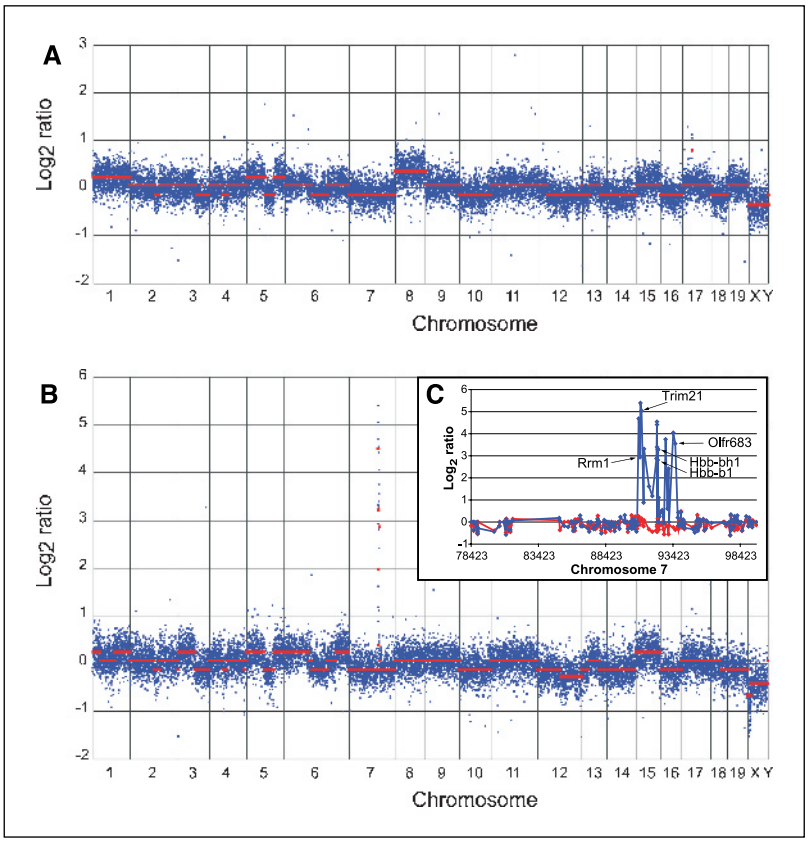

Figure 2. Genome-wide oligo array CGH tumor profiles, ratios ordered by chromosomal position. *A,* gemcitabine-sensitive tumor 26A with log 2 ratios (*blue*) and smoothed values (*red*). *B,* gemcitabine-resistant tumor 26G with log 2 ratios (*blue*) and smoothed values (*red*). *Inset* (*C*), chromosome 7E1 for tumor 26A (*red*) and 26G (*blue*). Genes located in this region from Fig. 1 and Table 2 are individually labeled.

feature of many tumors where changes in dosage compensation leads to changes in the expression levels of the genes involved with subsequent selective growth advantage of these tumor cells. To our knowledge, the relation between gemcitabine and 11p15 has only been described for non–small cell lung cancer (2). In the latter case, deletions are assumed to be involved. Our data show that a similar relation exists in colon tumor cells but involves amplification leading to overexpression of *RRM1, HBB-B1, OLFR683*, and *TRIM21.* Down-regulation of two other genes, *COX4I1* and *RPL13*, could also be explained by our array CGH analysis because down-regulation of these genes coincides with a deletion of chromosome 8. The genes identified by expression array analysis thus biologically validate that both our oligo array expression platform as well our parametric approach are robust.

Chromosomal instability is generally studied by DNA replication inhibitors such as Aphidicolin (16). It is well established that chromosomal instability is directly associated with DNA repair or delayed DNA replication (17). Here, we show in a solid tumor that, indeed, the nucleoside analogue gemcitabine is capable to convey drug resistance to tumors by chromosomal instability. The role of nucleotide availability in DNA replication and chromosomal stability has recently been shown by Debatisse et al. (18). Acquired drug resistance leading to chromosomal copy number was also observed in cell lines for other nucleoside analogues (19). Our studies, therefore, make it conceivable that this group of nucleoside analogues can convey chromosomal instability as a direct consequence of their interference in DNA replication.

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