Concordant Copy Number and Transcriptional Activity of Genes Mapping to Derivative Chromosomes 8 During Cellular Immortalization In Vitro

Lesa Begley,[1] David Keeney,[1] Ben Beheshti,[2] Jeremy A. Squire,[2] Rajiv Kant,[1] Hassan Chaib,[1] James W. MacDonald,[3] Johng Rhim,[4] and Jill A. Macoska[1,3]*

[1]Department of Urology, University of Michigan, Ann Arbor, Michigan
[2]Ontario Cancer Institute, University of Toronto, Toronto, Ontario, Canada
[3]Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan
[4]Center for Prostate Disease Research, USUHS, Rockville, Maryland

Deletion, rearrangement, or amplification of sequences mapping to chromosome 8 are frequently observed in human prostate and other tumors. However, it is not clear whether these events alter the transcriptional activity of the affected genes. To examine this question, we have utilized oligonucleotide microarray technology and compared the transcriptional patterns of normal human prostate tissues and five immortalized cell lines carrying either two normal chromosomes 8 or one normal and one derivative chromosome 8. Comparison of the transcriptional profiles of the tissues and cell lines identified 125 differentially expressed transcripts specific to chromosome 8, with 46 transcripts mapping to 8p and 79 transcripts mapping to 8q. The majority of genes mapping to 8p (44/46, 96%) were transcriptionally down-regulated in cells hemizygous for 8p, whereas the majority of genes mapping to 8q (58/79, 73%) were up-regulated in cells carrying three copies of 8q. Moreover, hemizygous alleles on 8p exhibited sub-haploinsufficient transcript levels for several genes that could be induced to haploinsufficient levels under hypomethylating conditions, suggesting that epigenetic regulation is a common mechanism for gene silencing in cells deleted for one copy of 8p. The results of these studies clearly demonstrate that alterations of gene copy number and transcriptional activity are directly correlated in cell lines harboring derivative chromosomes 8, and that these events are commonly observed during cellular immortalization in vitro.

INTRODUCTION

A PubMed search conducted in March 2005 identified 170 citations linking chromosome 8 and human prostate cancer, the most citations for any chromosome. Many of these studies have clearly demonstrated that deletion, rearrangement, or amplification of sequences mapping to chromosome 8 are frequently observed in human prostate and other tumors (Bergerheim et al., 1991; Wolman et al., 1992; Bova et al., 1993; Macoska et al., 1993, 1994; MacGrogan et al., 1994; Sakr et al., 1994; Trapman et al., 1994; Bova et al., 1996). Several studies have identified discrete or large deletions of the short arm of chromosome 8, suggesting that one or more tumor-suppressor genes critical for prostate tumorigenesis map to 8p. Even so, only a few genes have been identified thus far as candidate tumor-suppressor genes mapping to 8p, including *NKX3.1* at 8p21, *LZTS1* at 8p22, and *MSR1* at 8p22 (He et al., 1997; Ishii et al., 1999; Xu et al., 2002). Other studies have demonstrated amplification for all or part of the long arm of chromosome 8 in prostate tumors, suggesting that one or more oncogenes important for prostate tumorigenesis map to 8q (Macoska et al., 1994; Takahashi et al., 1994). In particular, amplification of the 8q24.21 region harboring the *MYC* oncogene has been implicated as an independent prognostic factor predictive for prostate tumor recurrence, metastasis, and poor patient outcome (Jenkins et al., 1997; Sato et al., 1999).

Although the studies cited above successfully defined regions of genomic loss or gain involving chromosome 8, they did not globally examine the potential effects of these large dosage changes on

*Correspondence to: Jill A. Macoska, Ph.D., Department of Urology, University of Michigan, 6217 CCGC, 1500 East Medical Center Drive, Ann Arbor, MI 48109-0944. E-mail: jcoska@umich.edu

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the transcriptional levels of genes mapping to these sequences. This deficiency can be addressed by the use of high-throughput technologies, which now permit the dual analysis of genomic and transcriptional alterations in human tumors. Using these techniques, we and our colleagues have shown that reduction to hemizygosity and transcriptional down-regulation for genes mapping to 8p is largely coincident in human prostate tumors and that genes mapping within the 8p11–p12 amplicon are largely transcriptionally up-regulated in three human breast cancer cell lines (Chaib et al., 2003; Ray et al., 2004).

We now report the utilization of high-throughput techniques for precisely "mapping" the transcriptional levels of genes on 8p and 8q for normal and derivative chromosomes 8 in normal human prostate tissues and immortalized human prostate epithelial cell lines. These studies show that alterations of gene copy number and transcriptional activity are directly correlated in cell lines harboring derivative chromosomes 8. Moreover, several genes mapping to hemizygous alleles on 8p exhibited sub-haploinsufficient transcript levels that could be induced to haploinsufficient levels under hypomethylating conditions, suggesting that epigenetic regulation is a common mechanism for gene silencing in cells deleted for one copy of 8p. Taken together, these observations suggest that alterations in copy number and transcriptional activity of genes mapping to derivative chromosomes 8 are associated with human prostate epithelial cellular immortalization in vitro.

MATERIALS AND METHODS

Establishment of Cell Cultures

Normal prostate tissue samples were obtained aseptically from patients undergoing radical prostatectomy after cancer diagnosis. All tissue acquisition and processing protocols conformed to those reviewed and approved by the University of Michigan Institutional Review Board. The tissue was minced into pieces < 1 mm^2; one piece was fixed in 5% formalin for further histopathological evaluation, whereas the rest was plated into 60-mm plates coated with Vitrogen 100 (Cohesion Laboratories, Palo Alto, CA) in keratinocyte growth medium supplemented with bovine pituitary extract, epidermal growth factor (EGF), and antibiotics (Invitrogen, Carlsbad, CA). Cultures that exhibited both fibroblastic and epithelial cell populations were subjected to brief trypsinization for removal of fibroblast cells. Cells were immortalized through transduction with the recombinant LXSNE6E7 retrovirus harboring the human papilloma virus E6 and E7 genes as described previously (Bright et al., 1997). Transduced cells were selected by use of 400 μg/ml geneticin. After an initial round of cell death and crisis, cells resistant to geneticin grew out and were considered immortal after 10 passages. Immortalized cell cultures were diluted 1:24 and individual subclones established. Selected subclones were plated into sterile chamber slides, grown to ~80% confluency, fixed in 50% methanol/50% acetic acid, and stained using a pan-cytokeratin monoclonal antibody mixture containing clones C-11, PRK-26, CY-90, KS-1A3, M20, and A53-B/A2 for detection of epithelial cells, or a monoclonal antivimentin antibody (Sigma-Aldrich, St. Louis, MO) for detection of stromal fibroblastic cells. A representative subclone homogenously staining with the pancytokeratin antibodies but not the antivimentin antibody was chosen from each culture for further analysis. Cells at the following passage numbers (where p indicates passage) were used for all experiments: N15C6 early, $<$ p25; N15C6 late, $>$ p40; N17A3, p25–p30; N33B2, p25–p30; 1532T, p40–45; 1542T, p40–45.

Spectral Karyotyping Analysis

Spectral karyotyping (SKY) analysis was carried out on previously G-banded slides. Images were captured, and the microscope coordinates were noted. Residual oil was removed with xylene, followed by destaining with methanol. The slides were then rehydrated in a descending ethanol series and fixed with 1% formaldehyde in a 50 mM $MgCl_2$/phosphate buffer solution for 10 min. Slides were dehydrated and denatured for 30–45 sec at 75°C in 70% formamide/2× SSC (saline sodium citrate), followed by a final dehydration. The SKY paints (Applied Spectral Imaging, Carlsbad CA) were denatured for 7 min at 75°C, reannealed at 37°C for 1 hr, and then placed on the slide and covered with a glass coverslip. The coverslip was sealed with rubber cement, and hybridization was carried out in a humidified chamber for 24 hr at 37°C. Posthybridization washes were carried out according to established techniques and according to the manufacturer's instructions (Shrock et al., 1996). Images of metaphase chromosomes were captured for each preparation by use of an SD 200 spectral bio-imaging system (ASI Ltd., Migdal Haemek, Israel) attached to a Zeiss microscope (Axioplan 2) and stored on a SKY image-capture workstation. The passage number (p) and number of metaphase images captured and analyzed [in

brackets] were as follows for each cell line: N15C6 p20 [10]; N15C6 p45 [10]; N17A3 p26 [10]; N33B2 p21 [9]; 1532T p44 [10]; and 1542T p44 [10]. The images were analyzed with SKYView software (Applied Spectral Imaging). G-banding and SKY analyses were performed sequentially on each of the cell lines with the same 10 metaphase images captured for G-banding also analyzed by SKY.

SNP Analysis

Cells were grown to 90% confluency. Genomic DNA was harvested with use of the Wizard Genomic Purification Kit (Promega Corp., Madison, WI) according to the manufacturer's protocol. Allelic discrimination PCR was run by use of Assays on Demand FAM and VIC-labeled probes (Applied Biosystems, Foster City, CA) according to the manufacturer's protocols, with 13.5 ng of genomic DNA as the template. The specific SNP assays used were: *RANBP16*, C__1959574_20; *CSMD1*, C__1423109_10; *TACC1*, C_11861239_10; *NEFL*, C___322260_10; *RBPMS*, C_15880850_10, and *MSR1*, C_12104846_10. All reactions were performed in triplicate, and no-template controls were included. Alleles for any locus (arbitrarily termed "A" or "B") were identified by the SNP assays from averaged intensity values from triplicate determinations after subtraction of no-template control values. The ratio of B/A was interpreted to indicate two alleles at a given locus if $0.5 < B/A < 1.5$ (indicative of both alleles, AB). One allele was assessed if $B/A > 1.5$ (indicative of the B allele) or if $B/A < 0.5$ (indicative of the A allele).

Affymetrix U133A GeneChip Data Acquisition

RNA was purified from histologically verified (>70% epithelial) normal human prostate tissues from three separate specimens or from trypsinized cultured cells by homogenization in Trizol (Invitrogen, Carlsbad, CA) and additional processing with the RNeasy (Qiagen, Valencia, CA) cleanup procedure. Ten micrograms of RNA obtained from tissues or cell lines was used to obtain labeled cRNA. Following an in vitro transcription reaction, the labeled cRNA was fragmented and then hybridized to Affymetrix U133A GeneChips (Affymetrix, Santa Clara, CA) for 16 hr at 45°C. The array was then washed and stained with streptavidin-phycoerythrin and scanned. Expression intensity values for each gene were estimated by a method called robust multiarray average (RMA), which is implemented in the Affymetrix library of the Bioconductor package for the statistical language R. This method uses a robust modeling strategy to estimate the average intensity of each gene on each chip. Detailed information can be found at http://www.bioconductor.org. GeneChip expression values were analyzed by use of a *t*-test-based analysis adjusted for false discovery rate (FDR) with the Significance Analysis of Microarrays (SAM) program (Tusher et al., 2001). The resulting data were organized by use of a hierarchical clustering scheme for grouping genes with similar expression patterns (Eisen et al., 1998). The distance metric used in the hierarchical clustering was the Pearson correlation.

Quantitative Real-Time PCR

All quantitative real-time assays were conducted with an Applied Biosystems 7900HT instrument and reagents. One microgram of RNA was reverse-transcribed by use of Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The resulting cDNA was diluted 1:100. Real-time PCR was performed by use of Assays on Demand (Applied Bio-Systems, Foster City, CA) according to the manufacturer's instructions, except that Real Time Ready with Rox passive dye (QBioGene, Carlsbad, CA) was used in place of TaqMan Universal PCR Master Mix. The FAM-conjugated, gene-specific assays were as follows: *TNKS*, Hs00186671_ml; *MTUS1*, Hs00368183_m1; *NKX3.1*, Hs00171834_m1; *CLU*, Hs00156548_m1; *RBPMS*, Hs00199302_m1; *RCP*, 0368787_m1; *PPP2CB*, Hs00602137_ml; *TACC1*, Hs00180691_ml; *MYST3*, Hs00198899_ml; *IKBKB*, Hs00395088_ml; *RGS20*, Hs00186596_m1; *SNAI2*, Hs00161904_m1; *UBE2V2*, Hs00163342_m1; *MATN2*, Hs00242767_m1, and the control *RPLPO*, Hs99999902_m1.

Methylation Block

Cells were grown to ~50% confluency, then treated for 5 days with 5 μM 5Aza-2-dCTP in 50% acetic acid (Sigma-Aldrich, St. Louis, MO) or acetic acid alone (50 μl in 5 ml of medium; Mock treatment) in triplicate (Yamashita et al., 2002). RNA was prepared, and transcript levels for specific genes were assessed by use of quantitative real-time PCR. Statistical analysis was performed by ANOVA by use of averaged experimental (5Aza-dC) minus control (acetic acid) values.

RESULTS

The Majority of Immortalized Prostate Epithelial Cells Exhibit 8p Loss

Five cell lines were successfully developed from normal prostate epithelium through transduction with the pLXSN16E6E7 recombinant retrovirus.

TABLE 1. Chromosome 8 Configuration in HPV-Immortalized Cell Lines

Cell line	Chromosome 8 configuration	Number of copies of 8p	Number of copies of 8q	Reference
N17A3	two normal	2	2	This study
1532T	one normal, one i(8)(q10)	1	3	Macoska et al., 2000
1542T	one i(8)(8q10), one der(8;20)(q10;p10), or one der(8;21)(q10;p10)	1	3	Macoska et al., 2000
N33B2	one normal, one i(8)(q10)	1	3	This study
	p20: one normal, one der(8)t(8;19)(q10;p10), two der(8)t(8;20)(p10;q10)	2	2	
N15C6	**p45**: one normal, one der(8;19)(q10;q10)	1	2	Macoska et al., 2004

Development and characterization of the 1532T, 1542T, and N15C6 cell lines were reported previously (Bright et al., 1997; Macoska et al., 2000, 2004; Schwab et al., 2000). The N17A3 and N33B2 cell lines are described here for the first time. The karyotypes for the 1532T, 1542T, and N15C6 cell lines have been reported elsewhere (Macoska et al., 2000, 2004). The karyotypes for the remaining two cell lines are as follows: N17A3 (at passage 26, 10 cells analyzed), 34–48,X,Y, −11 (8/9 cells), −19 (7/9 cells), +20 (8/9 cells), −22 (8/9 cells), der(15)t(11;15)(q14;q24) (5/9 cells); and N33B2 (at passage 21, 9 cells analyzed), 40–44,X,Y, −19 (4/7 cells), −22 (5/7 cells), der(1)t(1;13)(p36;q32) (6/7 cells), i(8q) (5/7 cells), der(13)t(11;13)(p10;q10) (2/7 cells), der(15)t(15;19)(q10;p10) (3/7 cells).

Two cell lines—N15C6 and 1542T—exhibited structural alterations of chromosome 8 that included translocation of chromosome 8 sequences to 19p (N15C6), 20q (1542T), or 21q (1542T). Three cell lines—1532T, 1542T, and N33B2—exhibited i(8q) chromosomes. Four of the six cell lines were hemizygous for the short arm of chromosome 8 (8p). The configuration of chromosomes 8 in each of the five cell lines is detailed in Table 1 and shown in Figure 1. Only the N17A3 cell line demonstrated two normal chromosomes 8. SNP analysis confirmed hemizygosity for all six SNP loci examined on 8p in the N33B2, N15C6, 1532T, and 1542T cell lines and heterozygosity for the *MSR1*, *NEFL* and *TACC1* loci on 8p in the N17A3 cell line (Table 2).

Gene Transcript Levels Are Proportional to Gene Dosage on Chromosome 8

GeneChip expression values were compared between samples demonstrating two normal chromosomes 8 (the three normal prostate tissues and N17A3 cells) and those demonstrating derivative chromosomes 8 (N33B2, N15C6, 1532T, and 1542T cells) by use of a *t*-test-based analysis adjusted for FDR with the Significance Analysis of Microarrays program. The FDR is equivalent to the *q* value and is indicated as the *q* value in Figure 2. The FDR ranged from 0.03% to 5.0%. These analyses identified 125 differentially expressed transcripts specific to chromosome 8, with 46 localized to genes mapped to 8p and 79 localized to genes mapped to 8q. The majority of transcripts mapping to 8p (44/46, 96%) were significantly down-regulated in cells carrying derivative chromosomes 8, and was consistent with reduction to hemizygosity for 8p in the N15C6, N33B2, 1532T, and 1542T cell lines. This comparison also demonstrated that the majority of transcripts mapping to 8q (58/79, 73%) were significantly up-regulated in cells carrying derivative chromosomes 8 (Fig. 2) compared to N17A3 cells and normal tissues.

To examine further the relationship of 8q dosage to 8q gene transcript levels, we performed an additional SAM analysis that focused exclusively on the transcriptional levels of genes mapping to 8q. For these experiments, transcript levels for 8q genes were compared for N15C6 and N17A3 cells (both carrying 2 copies of 8q) with N33B2 and 1532T cells (both carrying 3 copies of 8q). This analysis identified 41 differentially expressed genes on 8q, with 34/41 (83%) up-regulated in N33B2 and 1532T cells compared to N17A3 and N15C6 cells. The results of these studies are consistent with a concordant increased transcriptional level and increased gene dosage for genes mapping to 8q in N33B2 and 1532T cells.

When organized by use of an unsupervised hierarchical clustering scheme with genes ordered based on chromosomal position (8pter–8qter), the gene expression data formed two large clusters,

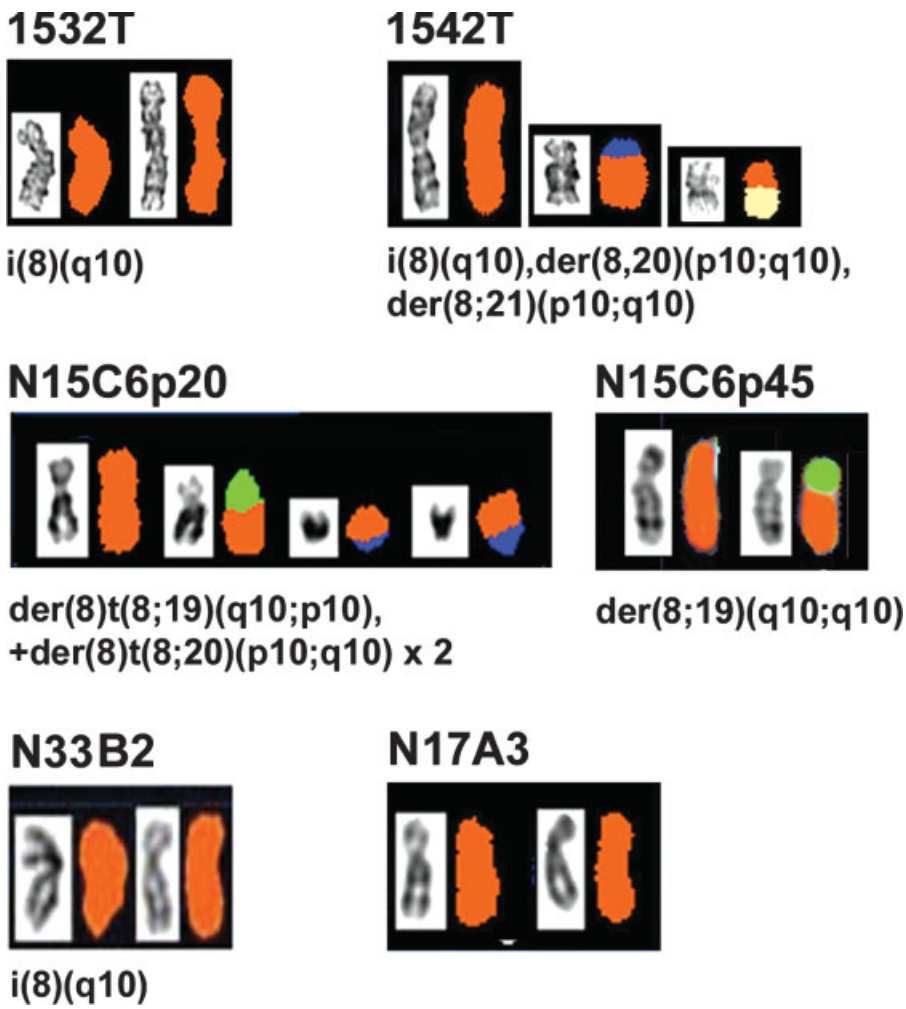


Figure 1. Spectral karyotype analysis of derivative chromosomes 8 in immortalized human prostate epithelial cells. Chromosomes are shown both before (G-banded, left) and after (pseudo-color application, right) hybridization with whole-chromosome paint probes. The compositions of derivative chromosomes 8 are indicated. Pseudo coloring indicates chromosome 8 in orange, chromosome 20 in blue; chromosome 21 in yellow, and chromosome 19 in green.

TABLE 2. SNP Allelotyping of Prostate Cell Lines

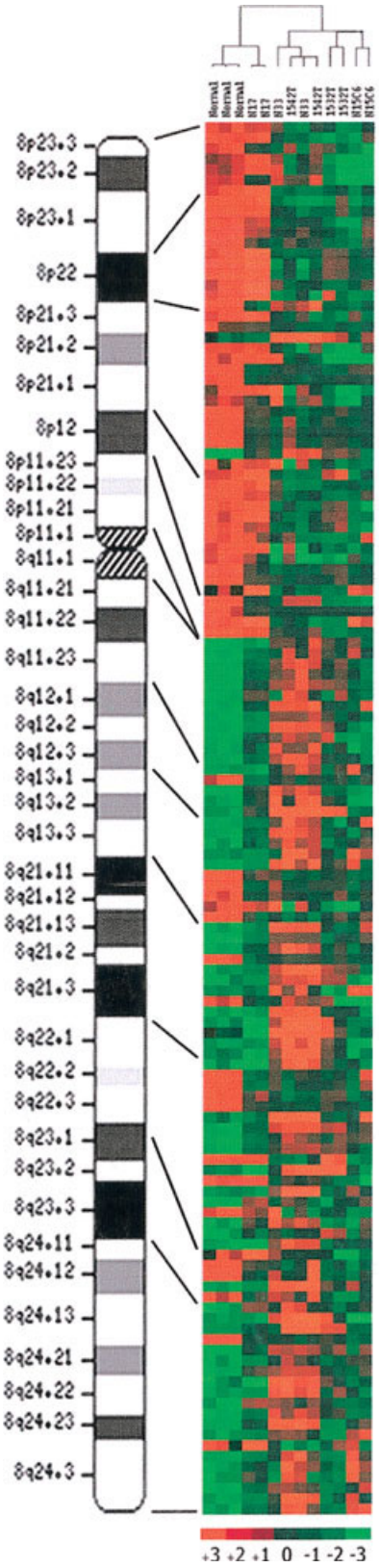
	Cell line				
Locus	N17A3	N33B2	N15C6	1532T	1542T
CSMD	A	A	B	B	B
MSR1	AB	B	B	A	B
NEFL	AB	A	B	B	B
RAN	B	A	A	A	A
RBPMS	A	B	A	A	A
TACC1	AB	A	B	A	A

Alleles at a locus have been designated as "A" or "B" according to these criteria: hemizygous for B when B/A > 1.5, hemizygous for A when B/A < 0.5, and heterozygous when 0.5 < B/A < 1.5.

one comprising the three normal tissues and the N17A3 cells, which carried two normal chromosomes 8, and the other comprising the N33B2, N15C6, 1532T, and 1542T cells, which carried derivative chromosomes 8 (Fig. 2). This result demonstrated that the observed trends in gene expression identified by the transcriptional profiles were largely consistent with the physical structure (normal or derivative) of the chromosomes 8 of the evaluated tissue or cell line. It should be noted that a large block of genes localized pericentromerically on 8q11 were transcriptionally up-regulated in N33B2, 1532T, and 1542T, the three cell lines that were hemizygous for 8p and carrying i(8q) chromosomes (Fig. 2).

Transcript Levels Are Frequently Reduced to Sub-Haploinsufficient Levels in Cells Hemizygous for 8p

The transcriptional levels of 14 genes—10 mapped to 8p (*TNKS*, *MTUS1*, *NKX3.1*, *CLU*, *RBPMS*, *RCP*, *PP2CB*, *TACC1*, *MYST3*, *IKBKB*) and 4 mapped to 8q (*RGS20*, *SNAI2*, *UBE2V2*, *MATN2*)—identified as differentially expressed in the GeneChip experiments were assessed by quantitative real-time PCR. As seen in Table 3, the expression trends for genes evaluated by both GeneChip and real-time PCR methodologies were similar. All 10 genes mapping to 8p were trans-



GENBANK	T-STAT.	FOLD CHANGE	Q-VALUE	LOCATION	TREND
AF070613	-4.4342	0.4406	0.0309	9458854	DOWN
AL080178	-6.0889	0.5475	0.0309	11179409	DOWN
AA872727	-3.3196	0.4759	0.3713	11697663	DOWN
NM_001908	-3.9450	0.5385	0.1126	11739235	DOWN
NM_001908	-3.9333	0.4998	0.1126	11739235	DOWN
BE875786	-2.7255	0.4965	1.2049	11739235	DOWN
AL096842	-8.0875	0.5475	0.0309	17545583	DOWN
AL096842	-7.5271	0.4087	0.0309	17545583	DOWN
AL096842	-6.3723	0.2019	0.0309	17545583	DOWN
AI924817	-4.8110	0.4509	0.0309	17824778	DOWN
AI205598	-4.4944	0.5389	0.0309	17824778	DOWN
NM_006197	-3.7747	0.4786	0.1749	17824778	DOWN
AI379338	-11.9106	0.3392	0.0309	17958220	DOWN
AI934569	-7.3500	0.1850	0.0309	17958220	DOWN
U87674	-7.1944	0.1804	0.0309	17958220	DOWN
NM_000662	-8.6179	0.5020	0.0309	18111894	DOWN
AW117368	-7.2923	0.5177	0.0309	18432348	DOWN
NM_018371	-2.3454	0.1135	2.5813	19305951	DOWN
NM_022749	-6.5923	0.5596	0.0309	22002659	DOWN
NM_004430	-2.1451	0.3370	4.5722	22601118	DOWN
NM_002318	2.9462	3.9888	0.5294	23210640	UP
AB002390	-5.4221	0.5407	0.0309	23346095	DOWN
NM_018579	-4.2278	0.5536	0.0568	23442424	DOWN
AF247704	-3.2664	0.0455	0.3713	23592172	DOWN
NM_006158	-3.2064	0.2799	0.4374	24866239	DOWN
AL132665	-2.3251	0.4993	2.5813	26296439	DOWN
AF233336	-3.9352	0.3667	0.1126	27404577	DOWN
M25915	-3.6124	0.0575	0.2362	27510367	DOWN
AI982754	-3.4538	0.2272	0.2676	27510367	DOWN
M25915	-3.2683	0.0498	0.3713	27510367	DOWN
NM_018492	3.2307	2.4276	0.2362	27723058	UP
NM_024567	-6.8729	0.5264	0.0309	28804143	DOWN
NM_015254	-6.3544	0.3534	0.0309	28980714	DOWN
NM_016127	-5.7693	0.2948	0.0309	30040172	DOWN
D84109	-6.5051	0.2334	0.0309	30361584	DOWN
NM_006867	-6.0639	0.2863	0.0309	30361584	DOWN
D84109	-4.6485	0.2741	0.0309	30361584	DOWN
AL442077	-4.3745	0.4963	0.0309	37713268	DOWN
AA176833	-4.8794	0.5559	0.0309	37738832	DOWN
AL136616	-3.6356	0.5473	0.1991	37738832	DOWN
BF511315	-3.6032	0.2804	0.2362	37773931	DOWN
AI655003	-3.4031	0.4929	0.3235	37773931	DOWN
NM_025151	-3.2468	0.1992	0.4374	37837342	DOWN
NM_006283	-3.1404	0.4777	0.5294	38763937	DOWN
NM_020130	-3.2279	0.2483	0.4374	40130143	DOWN
NM_006766	-5.7062	0.5831	0.0309	41907425	DOWN
AU153366	-6.2303	0.3425	0.0309	42247985	DOWN
U47077.5	6.5300	2.8604	0.0309	48848221	UP
U34994.3	4.7604	2.6153	0.0309	48848221	UP
X74794	4.4223	2.2824	0.0309	49036046	UP
AI859865	4.0575	2.6991	0.0309	49036046	UP
AI859865	3.9601	2.5953	0.0309	49036046	UP
X74794	3.0142	1.8508	0.4374	49036046	UP
U62136.2	4.2036	2.0307	0.0309	49083547	UP
AI572079	5.4230	3.6689	0.0309	49992795	UP
AF074979	4.7380	3.1350	0.0309	54956117	UP
BG288007	2.7045	1.7178	0.9647	55121490	UP
NM_014175	4.3277	2.4672	0.0309	55210333	UP
NM_024831	5.2902	1.8585	0.0309	56848254	UP
NM_006211	-2.6010	0.3120	1.5781	57516069	DOWN
AF306765	3.0797	2.1609	0.3713	62578373	UP
AF289489	2.7169	2.0926	0.9647	62578373	UP
NM_003878	3.6433	3.9839	0.0850	64090194	UP
NM_018120	4.1311	1.8104	0.0309	66677628	UP
NM_014637	4.0439	1.7695	0.0309	66719527	UP
BF214329	3.9403	2.4425	0.0309	66719527	UP
BC001811	4.7770	1.9038	0.0309	67503816	UP
NM_006837	4.8676	1.7068	0.0309	68117869	UP
AW043713	-3.9067	0.5317	0.1462	70541426	DOWN
AW043713	-3.9083	0.2821	0.5294	70541426	DOWN
AW043713	-2.8614	0.4283	0.7890	70541426	DOWN
AI040324	-3.5758	0.5264	0.2362	71186820	DOWN
AU145806	-3.1616	0.5096	0.4374	71186820	DOWN
NM_005648	5.2334	1.8692	0.0309	75021187	UP
NM_001151	4.8329	2.4200	0.0309	75021187	UP
NM_017866	4.0030	1.8435	0.0309	75050997	UP
NM_024721	-2.8135	0.3581	0.9647	77778834	DOWN
NM_014018	4.3177	1.7432	0.0309	80993650	UP
NM_001444	3.9453	3.4549	0.0309	82355339	UP
U75642	2.5709	1.8160	1.5781	86276810	UP
BF131791	-2.6579	0.5660	1.5781	87424109	DOWN
NM_016033	5.0996	2.5894	0.0309	87555453	UP
AK001017	5.0175	1.9302	0.0309	91014888	UP
NM_002485.2	2.8428	1.7261	0.6524	91014888	UP
AI796269	3.5700	1.8040	0.1126	91082755	UP
NM_015942	3.9032	1.8111	0.0309	97320908	UP
NM_014754	6.4814	2.4729	0.0309	97343342	UP
J04621	-2.8451	0.2473	0.9647	97575071	DOWN
J04621	-2.8225	0.3588	0.9647	97575071	DOWN
NM_016134	-4.2432	0.5209	0.0568	97726674	DOWN
NM_006102	-3.4338	0.4575	0.3235	97726674	DOWN
AI972475	4.2478	1.8714	0.0309	98725582	UP
NM_018407	4.6021	2.3940	0.0309	98856984	UP
AW149681	4.5560	2.5469	0.0309	98856984	UP
T15777	4.1703	1.8991	0.0309	98856984	UP
NM_002380.2	-4.4990	0.1573	0.0309	98969380	DOWN
D31765	5.1706	2.2593	0.0309	99199243	UP
AI811298	-4.8151	0.4119	0.0309	100025861	DOWN
AF311312	4.3347	2.1245	0.0309	101230831	UP
AF251061	-3.6012	0.5154	0.2362	102768135	DOWN
NM_003506	3.7150	1.7787	0.0787	104380275	UP
NM_030780	6.5722	3.3653	0.0309	104480042	UP
NM_018002	-3.8503	0.5817	0.1462	107739503	DOWN
U83508	-3.2886	0.4733	0.3713	108330885	DOWN
NM_001146	-2.7182	0.4561	1.2049	108330885	DOWN
NM_020189	5.3908	2.1716	0.0309	110415811	UP
NM_014112	-2.6691	0.3957	1.2049	116489899	DOWN
NM_000127	3.2964	2.6165	0.1991	118887686	UP
NM_024094	3.0916	1.9993	0.3713	120915396	UP
NM_022783	-3.1718	0.2251	0.4374	120955145	DOWN
NM_014078	3.9543	2.3093	0.0309	121477264	UP
NM_024295	4.6138	2.0412	0.0309	124094768	UP
NM_014109	3.1928	1.9717	0.2676	124401921	UP
AF279365	5.5180	2.4761	0.0309	126080612	UP
AA639705	5.3777	3.0691	0.0309	126080612	UP
NM_002467	2.6548	2.0124	1.2049	128817685	UP
NM_016623	4.4012	2.1235	0.0309	130922899	UP
NM_018482	3.7004	2.2584	0.0787	131133534	UP
AI613483	3.7657	1.9994	0.0787	141610446	UP
NM_005672	-5.6623	0.1143	0.0309	143758914	DOWN
AF217197	4.2672	1.7842	0.0309	144970535	UP
NM_000445	3.7241	1.7633	0.0787	145061308	UP
Z54367	2.8341	2.4272	0.6524	145061308	UP
NM_001916	4.9740	1.9606	0.0309	145221986	UP
BG491842	4.8557	3.0858	0.0309	145456863	UP
AK021938	4.2867	2.5028	0.0309	145553032	UP

Figure 2. Hierarchical clustering of chromosome 8 molecular signatures for prostate tissues and cell lines. Normal human prostate tissues and the N17A3 immortalized human prostate epithelial cell line carry two normal chromosomes 8, whereas the N33B2, 1542T, N15C6, and 1532T immortalized human prostate epithelial cell lines carry derivative chromosomes 8. Normal human prostate tissues and the N17A3 cell cluster together on the left; the N33B2, 1542T, N15C6, and 1532T cell lines cluster together on the right of the diagram. Differentially expressed genes are designated by GenBank accession numbers and are arranged according to chromosomal position, from 8pter (top) to 8qter (bottom). Relative gene expression intensities are represented by color, with the highest expression indicated by light red and lowest expression by light green (see color bar). The data columns to the right of the heat map indicate the GenBank accession number, *t* statistic, -fold change, q value (false discovery rate), physical location on chromosome 8 in base pairs relative to 8pter (at 0), and expression trend for each gene. The physical location of the indicated genes is shown with reference to the chromosome 8 ideogram on the left.

TABLE 3. Gene Expression Levels Relevant to Allelic Copy Number and Methylation Status

Gene[a]	Cell line[b]	GeneChip expression Intensity	GeneChip relative difference from N17A3	Real-time PCR relative difference from N17A3	Descriptive expression level[c]	Magnitude of expression of 5-Aza-dCTP/mock treatment[d]
TNKS 8p23.1	N17A3	8.5349			Normal diploid	1.27 (0.16)
	N15C6	7.5549	0.51	0.22	Haploinsufficient	0.84 (0.42)
	N33B2	7.5700	0.51	0.09	Haploinsufficient	1.03 (0.57)
	1532T	7.4964	0.50	0.50	Haploinsufficient	0.76 (0.08)
	1542T	7.6001	0.52	0.45	Haploinsufficient	0.99 (1.48)
MTUS1/ATIP/MTSG1	N17A3	9.0427			Normal diploid	1.09 (0.10)
8p22	N15C6	8.1129	0.52	0.53	Haploinsufficient	0.84 (0.02)
	N33B2	7.0428	0.25	0.51	Sub-haploinsufficient	0.90 (0.07)
	1532T	7.2312	0.28	0.62	Sub-haploinsufficient	0.81 (0.03)
	1542T	7.1544	0.27	0.52	Sub-haploinsufficient	0.94 (0.04)
NKX3.1 8p21.2	N17A3	8.7627			Normal diploid	0.96 (0.10)
	N15C6	7.6702	0.47	0.42	Haploinsufficient	0.93 (0.01)
	N33B2	7.4763	0.41	0.59	Haploinsufficient	1.03 (0.01)
	1532T	7.5506	0.43	0.62	Haploinsufficient	0.93 (0.00)
	1542T	7.5211	0.42	0.46	Haploinsufficient	0.99 (0.16)
CLU 8p21.1	N17A3	9.7010			Normal diploid	1.18 (0.02)
	N15C6	7.8431	0.28	0.23	Sub-haploinsufficient	1.38 (0.01)
	N33B2	7.8727	0.28	0.65	Sub-haploinsufficient	1.20 (0.04)
	1532T	7.6853	0.25	0.42	Sub-haploinsufficient	1.15 (0.14)
	1542T	8.0263	0.29	0.33	Sub-haploinsufficient	0.97 (0.09)
RBPMS 8p12	N17A3	7.8116			Normal diploid	1.17 (0.96)
	N15C6	6.5243	0.41	0.31	Haploinsufficient	1.44 (0.31)
	N33B2	6.6881	0.46	0.80	Haploinsufficient	1.14 (0.49)
	1532T	7.0163	0.58	0.31	Haploinsufficient	1.13 (0.07)
	1542T	6.7768	0.49	0.39	Haploinsufficient	1.35 (0.07)
RCP 8p12	N17A3	8.9545			Normal diploid	0.77 (0.09)
	N15C6	8.0395	0.53	0.58	Haploinsufficient	0.99 (0.10)
	N33B2	6.7461	0.22	0.31	Sub-haploinsufficient	1.00 (0.08)
	1532T	6.7558	0.22	0.26	Sub-haploinsufficient	0.93 (0.09)
	1542T	7.0416	0.27	0.06	Sub-haploinsufficient	0.83 (0.03)
PPP2CB 8p12	N17A3	11.4297			Normal diploid	0.84 (0.17)
	N15C6	11.1333	0.81	0.33	Haploinsufficient	1.03 (0.48)
	N33B2	10.8176	0.65	0.40	Haploinsufficient	0.66 (0.01)
	1532T	10.6311	0.57	0.40	Haploinsufficient	1.25 (0.92)
	1542T	10.9211	0.70	0.19	Haploinsufficient	1.60 (0.22)
TACC1 8p11.23	N17A3	8.7967			Normal diploid	1.80 (0.11)
	N15C6	7.8278	0.51	0.50	Haploinsufficient	1.08 (0.14)
	N33B2	8.2347	0.68	0.02	Haploinsufficient	1.61 (0.96)
	1532T	7.8839	0.53	0.80	Haploinsufficient	1.50 (1.07)
	1542T	8.4415	0.78	0.84	Elevated	0.67 (0.20)
MYST3 8p11.21	N17A3	10.3441			Normal diploid	0.82 (0.09)
	N15C6	9.9824	0.78	0.51	Haploinsufficient	1.23 (0.50)
	N33B2	9.5280	0.57	0.27	Haploinsufficient	0.90 (0.07)
	1532T	9.3938	0.52	0.26	Haploinsufficient	1.18 (0.06)
	1542T	9.4300	0.53	0.54	Haploinsufficient	1.89 (0.36)
IKBKB 8p11.21	N17A3	7.3251			Normal diploid	0.99 (0.08)
	N15C6	6.6220	0.61	0.43	Haploinsufficient	1.09 (0.02)
	N33B2	6.6027	0.61	0.41	Haploinsufficient	1.03 (0.05)
	1532T	6.4703	0.55	0.33	Haploinsufficient	1.13 (0.03)
	1542T	6.6620	0.63	0.56	Haploinsufficient	1.08 (0.05)
RGS20 8q11.23	N17A3	6.4388				1.08 (0.08)
	N15C6	7.3215	1.84	1.47		1.20 (0.08)
	N33B2	8.7131	4.84	1.90		1.05 (0.03)
	1532T	8.0973	3.16	1.85		1.16 (0.02)
	1542T	8.7364	4.92	15.38		1.22 (0.06)
SNAI2 8q11.21	N17A3	9.3423				1.10 (0.31)
	N15C6	11.2486	3.75	1.12		2.68 (0.14)
	N33B2	11.8038	5.51	9.88		1.84 (0.15)

(Continued)

TABLE 3. Gene Expression Levels Relevant to Allelic Copy Number and Methylation Status (Continued)

Gene[a]	Cell line[b]	GeneChip expression Intensity	GeneChip relative difference from N17A3	Real-time PCR relative difference from N17A3	Descriptive expression level[c]	Magnitude of expression of 5-Aza-dCTP/mock treatment[d]
	1532T	11.3941	4.15	1.03		1.57 (0.12)
	1542T	11.9651	6.16	8.26		0.64 (0.04)
UBE2V2 8q11.21	N17A3	8.9640				1.16 (0.21)
	N15C6	10.0513	2.12	2.73		1.35 (0.12)
	N33B2	10.3167	2.55	2.09		1.31 (0.08)
	1532T	9.8244	1.82	1.78		1.74 (0.09)
	1542T	10.4446	2.79	1.00		1.84 (0.27)
MATN2 8q22	N17A3	9.4594				0.70 (0.04)
	N15C6	6.2641	0.11	0.82		0.77 (0.02)
	N33B2	6.4067	0.12	0.20		0.84 (0.04)
	1532T	8.5334	0.53	0.12		0.79 (0.02)
	1542T	6.7616	0.15	0.30		0.81 (0.02)

[a]Gene symbol and cytogenetic location are shown.
[b]N17A3 cells possess two copies of 8p, whereas N15C6, N33B2, 1532T, and 1542T cells possess one copy of 8p.
[c]For cells with one copy of 8p, haploinsufficient levels are defined as between 0.70 and 0.30 of normal, sub = haploinsufficient as < 0.30 of normal, and elevated as > 0.70 of normal.
[d]Gene expression is expressed as a ratio of that obtained for cells grown in 5 mM 5-Aza-2-dCTP and that for mock-treated cells (standard deviation is shown in parentheses).

criptionally down-regulated in the majority of cell lines hemizygous for 8p, compared to those heterozygous for 8p. However, the transcriptional profiles for each gene in each cell line hemizygous for 8p were somewhat heterogeneous and varied from haploinsufficient or sub-haploinsufficient levels to elevated levels. For example, after normalization to N17A3 transcript levels as a measure of normal heterozygous transcriptional activity, it was apparent that all cell lines hemizygous for 8p expressed NKX3.1 at haploinsufficient levels, that is, between 0.41- and 0.47-fold of that observed for N17A3 cells by GeneChip analysis. In contrast, all cells hemizygous for 8p expressed *CLU* at sub-haploinsufficient levels, between 0.25- and 0.29-fold of that observed for N17A3 cells. *TNKS*, *MTUS1*, *RBPMS*, *RCP*, and *IKBKB* were expressed at haploinsufficient or sub-haploinsufficient levels in cells hemizygous for 8p. Expression levels of *PP2CB*, *TACC1*, and *MYST3* were haploinsufficient for three of four cell lines hemizygous for 8p, but were elevated, that is, expressed at near-heterozygous levels, in one of the four cell lines (Table 3). In total, transcript levels were measured in 40 instances (4 cell lines hemizygous for 8p, 10 genes evaluated by quantitative real-time PCR) and were found to be haploinsufficient in 27/40 instances (68%), sub-haploinsufficient in 10/40 instances (25%), and elevated in 3/40 instances (8%) by GeneChip analysis. These observations demonstrate that transcript levels were reduced to haploinsufficient or sub-haploinsufficient levels in the majority (37/40, 92%) of the cases examined in this study.

Epigenetic Mechanisms May Regulate Transcription of Hemizygous 8p Genes

The observed sub-haploinsufficient transcript levels for three genes—*MTUS1*, *CLU*, and *RCP*—mapping to 8p in cells hemizygous for the same sequences could be attributed to epigenetic mechanisms. To investigate this question, we grew cells heterozygous (N17A3) or hemizygous (N15C6, N33B2, 1532T, 1542T) for 8p in the presence of the methylation blocking agent 5Aza-2-dCTP or, as a control, in the vehicle solvent acetic acid. Of the 10 genes examined, exposure to the methylation blocking agent was associated with significantly increased transcript expression of *MTUS1*, *CLU*, and *RCP* in three or more cell lines hemizygous for 8p (Table 3). For genes mapping to 8q, none of the three genes that exhibited increased transcript levels compared to N17A3 cells by Gene Chip and quantitative RT-PCR analyses demonstrated significantly higher expression levels in more than two cell lines after treatment with the hypomethylating agent. In contrast, *MATN2*, which maps to 8q but exhibited decreased transcript levels compared to N17A3 cells by both gene expression profiling and quantitative RT-PCR studies, demonstrated significantly increased transcript levels in N15C6, N33B2, and 1542T cells after treatment with 5Aza-2-dCTP (Table 3). These data suggest that both allelic deletion and sequence

methylation may contribute to the transcriptional silencing of particular genes in immortalized and initially transformed human prostate cells.

DISCUSSION

Loss of 8p and gain of 8q are observed frequently in human prostate tumors. These observations are consistent with the hypothesis that tumor-suppressor genes mapping to 8p and oncogenes mapping to 8q play critical roles in prostate cellular immortalization and transformation. However, identifying specific genes that function as tumor-suppressor genes or oncogenes mapping to chromosome 8 has been difficult and labor intensive. We have attempted to address these issues by utilizing a high-throughput approach to identifying candidate tumor-suppressor genes and oncogenes associated with DNA sequences losses or gains, respectively, on chromosome 8.

Examination of the transcriptional profiles of prostate tissues and cell lines possessing two normal chromosomes 8 and of those possessing derivative chromosomes 8 revealed large-scale transcriptional up-regulation of genes mapping to 8q. Three of the four cell lines exhibiting derivative chromosomes 8—N33B2, 1532T, and 1542T—carry i(8q) chromosomes with consequent gain of 8q. Moreover, specific "blocks" of increased gene transcription were evident on the 8q arm. Notably, a large block of 20 genes localized pericentromerically on 8q was transcriptionally up-regulated in the four cell lines carrying derivative chromosomes 8 (Fig. 2). This suggests that long-range chromatin modifications may occur because of reduction to hemizygosity for 8p that result in increased transcriptional activity of pericentromeric genes. Other blocks of transcriptionally up-regulated genes on 8q, including one at 8q22 and most of the 8q24 region, are consistent with sites for putative oncogenes (Rubin, 2004; Savinainen, 2004; Wang, 2004).

Four of the five immortalized prostate epithelial cell lines utilized in the studies reported here were hemizygous for 8p and demonstrated transcriptional down-regulation for the majority (96%) of genes mapping to 8p. These four cell lines clustered with each other in the dendrogram shown in Figure 2, suggesting that they shared a common transcriptional molecular signature largely driven by hemizygosity for 8p. In the majority (68%) of cases examined, hemizygosity for 8p was associated with haploinsufficient transcript levels, that is, levels that were approximately half that observed for cells possessing two copies of 8p (Table 3). However, an additional 25% of cases examined demonstrated transcript levels that were further reduced to sub-haploinsufficient levels (Table 3). This finding suggested that some of the genes mapping to the sole copy of 8p in these cells might be transcriptionally inactivated through epigenetic mechanisms. Indeed, induction of a methylation block resulted in significantly increased expression of all transcripts detected at sub-haploinsufficient levels by GeneChip analysis. The results of these studies clearly demonstrated that methylation-mediated epigenetic regulation could be responsible for the observed sub-haploinsufficient 8p hemizygous allele transcript levels in immortalized and initially transformed prostate epithelial cells.

Surprisingly, transcripts originating from 8p hemizygous alleles were actually elevated above haploinsufficient levels in 3 of 40 (8%) of cases examined. This phenomenon was largely heterogeneous, and was observed for three genes—*PPP2CB*, *TACC1*, and *MYST3*—in one cell line each. *PPP2CB* was recently found to be down-regulated in a majority of prostate tumors examined but up-regulated in a minority of cases (Wissmann et al., 2003). *TACC1* has been described as transcriptionally down- and up-regulated in human breast tumors, the latter in conjunction with DNA amplification (Conte et al., 2002; Ray et al., 2004). Although transcript expression of *MYST3* has not been previously reported for prostate tissues, *MYST*3 is involved in t(8;16)(p11;p13) translocations and forms a fusion protein with *CREBBP* that is frequently associated with acute myeloid leukemia (Rozman et al., 2004). A survey of gene expression profiles of normal and malignant prostate tissues identified down-regulation for *MYST3* in three studies, up-regulation in one study, and lack of differential expression in four other studies (http://www.oncomine.org). These reports suggest that these three genes in particular may be subject to complex transcriptional and translational regulation in human prostate cells.

The observation of both haploinsufficient and sub-haploinsufficient transcript levels originating from hemizygous alleles of 8p-localized genes further suggests that deletion of one copy of 8p may result in either a one-hit or a two-hit mechanism for tumor initiation or progression. This hypothesis proposes that cells suffering deletion of one copy of 8p and expressing haploinsufficient levels of associated transcripts may be preferentially selected for clonal growth and immortalization, whereas similar cells that experience further transcript reductions to sub-haploinsufficient levels may be driven further down the road of malignant progres-

sion. This is a difficult hypothesis to test because of the typical presence of several genetic alterations in addition to 8p loss in cultured cells and tumors. However, evaluation of the simple cultured-cell models used in the current study showed that N33B2, 1532T, and 1542T cells exhibited subhaploinsufficient levels of 3 of the 10 genes examined on 8p, whereas N15C6 cells exhibited subhaploinsufficient levels of only 1 of the same 10 genes (Table 3). Moreover, previous studies have shown that 1532T and 1542T cells express a more advanced malignant phenotype than do N15C6 cells (Schwab et al., 2000; Macoska et al., 2000, 2004).

Taken together, these observations are consistent with a positive correlation between advanced expression of the malignant phenotype and sub-haploinsufficient levels of hemizygous 8p gene transcript levels. This is an interesting hypothesis that will require the study of more refined, gene-specific models for thorough testing and evaluation.

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