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Excessive CpG island hypermethylation in cancer cell lines versus primary human malignancies

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Cancer cell lines are widely used in many types of cancer research, including studies aimed at understanding DNA hypermethylation of gene promoters in cancer. Hypermethylation of promoters is capable of repressing the expression of tumor suppressor genes and may play a role in the development and/or progression of cancer. Although both primary malignancies and cancer cell lines exhibit this epigenetic phenomenon, there has been no direct comparison between them. In order to address this question, we have utilized restriction landmark genomic scanning to measure the hypermethylation phenotypes of cancer cell lines and compared these data with the same analysis performed on primary malignancies. In all cases, cancer cell lines exhibit significantly higher levels of CpG island hypermethylation than the primary malignancies they represent. Colon cancer cell lines are most similar to their respective tumors, with only a 5-fold increase in hypermethylation, while head and neck squamous cell carcinoma cell lines show a 93-fold increase in hypermethylation. Furthermore, >57% of the loci methylated in cell lines are never methylated in 114 primary malignancies studied. Seventy percent of loci hypermethylated in cell lines are hypermethylated in lines from more than one type of cancer. These data indicate that most CpG island hypermethylation observed in cancer cell lines is due to an intrinsic property of cell lines as opposed to the malignant tissue from which they originated.

INTRODUCTION

CpG islands are GC-rich sequences usually located in promoters of genes, and normally unmethylated, with the exceptions of the inactive X chromosome, imprinted genes, and genes that are developmentally regulated, directly or indirectly, by methylation. Hypermethylation of these CpG islands contributes to the spectrum of genomic abnormalities found in human cancers and shows heterogeneity both within and among different tumor types (1–3). Through the recruitment of chromatin remodeling complexes (4), CpG island hypermethylation is capable of inactivating the expression of tumor suppressor genes and therefore contributing to carcinogenesis (1,5).

Cancer cell lines are widely used and important tools in cancer research, including DNA methylation studies and pharmacological studies aimed at manipulating DNA methylation (6,7). Mammalian cells grown in culture exhibit differences in DNA methylation compared with normal uncultured cells. Primary cultures approaching senescence demonstrate decreasing DNMT1 activity and decreasing 5-methylcytosine (5mC) content, while immortalized cultures do not (8,9). Established cancer cell lines, however, show significantly higher levels of 5mC than corresponding tumor tissues (10). In addition to global differences in 5mC content, CpG island hypermethylation occurs in both immortalized (11) and cancer cell lines, with as much as 61% of randomly cloned CpG islands exhibiting hypermethylation in one report (12).

Cancer cell lines clearly exhibit aberrant CpG island methylation (12)—as do primary malignancies—yet they differ from their respective malignant tissues in 5mC content (10). Therefore, the degree and quality of their similarity is in question. Nevertheless, there has been no comprehensive study comparing CpG island hypermethylation in cancer cell lines with the primary malignancies they represent. Restriction

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landmark genomic scanning (RLGS) has previously been used to demonstrate increasing hypermethylation in mouse cell lines with respect to normal brain tissue, with increasing passage in culture (13). Our recent application of RLGS (14–16) to assess the methylation state of 1184 CpG islands in 114 human malignancies of eight different types (2,17) affords a unique opportunity to compare the hypermethylation phenotypes of primary malignancies with their respective cell lines. Here we assess the methylation status of these same 1184 CpG islands in 24 cancer cell lines—three for each of the eight cancer types. Our data indicate significant qualitative and quantitative differences between established cancer cell lines and their corresponding malignant tissues.

RESULTS

Quantitative comparison

In order to determine whether CpG island hypermethylation in cell lines is similar to that seen in primary malignancies, RLGS profiles were prepared from established cell lines for the following eight malignancies: breast (BRE), colon (CLN), lung (LNG) and head and neck squamous cell carcinomas (HN), glioblastoma (GLI), acute myeloid leukemia (LEU), medulloblastoma (PNET) and testicular germ cell tumors (TST). RLGS profiles were analyzed by assessing the presence or absence of 1184 RLGS fragments previously determined to be present and non-polymorphic in peripheral blood lymphocyte (PBL) DNA (2). Absence of an RLGS fragment is indicative of DNA methylation of the corresponding CpG island (2). Portions of RLGS profile analyses are illustrated in Figure 1, indicating three prominent methylation events in the primary tumor and 15 in the corresponding cell line. Within each cell line type, the loss pattern is not uniform—specific fragments show significantly elevated loss [$P < 0.0001$ for each type; statistical methods are detailed in a previous report (2)]. The degree of hypermethylation in each cell line is shown in Table 1. RLGS fragment loss in these cell lines ranges from 60–95 fragments in TST cell lines, to 317–569 in LEU cell lines. As shown in Table 2, most methylation events occurred in only one of the three cell lines for each type. Methylation in all three cell lines of a given type was seen in as many as 30% of the loci (CLN) and as few as 4% of the loci (PNET). Comparison of the mean levels of CpG island methylation in our primary tumor data sets (2,17) with those found in the corresponding established cancer cell lines demonstrates that CpG island hypermethylation is greatly elevated in cell lines (Fig. 2) and varies significantly across

Table 1. RLGS analysis of fragment loss in 24 established cancer cell lines

Cancer cell lines[a]		Methylated loci	% Methylated loci[b]
NCCIT	TST	60	5
DAOY	PNET	63	5
SCC-4	HN	73	6
NTERA-1	TST	85	7
SCC-9	HN	89	8
TERA1	TST	95	8
T47D	BRE	111	9
D283 MED	PNET	111	9
D341 MED	PNET	112	9
SCC-25	HN	116	10
HS578T	BRE	123	10
H522	LNG	135	11
MDA-435	BRE	151	13
H125	LNG	162	14
DLD-1B	CLN	179	15
H1299	LNG	198	17
LN235	GLI	200	17
DLD-2	CLN	206	17
COLON A	CLN	213	18
T98	GLI	255	22
U251	GLI	294	25
TI-1	LEU	317	27
Kasumi-1	LEU	338	29
HL-60	LEU	569	48

[a]The tumor type represented by the cell lines is listed to the right.
[b]Out of a total of 1184 analyzed RLGS fragments per RLGS profile.

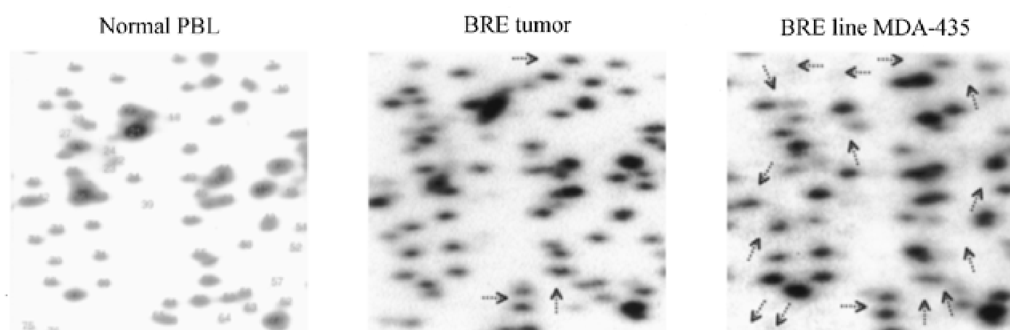

Figure 1. RLGS analysis was performed as described previously (16). Portions of RLGS profiles for normal peripheral blood lymphocytes (PBL), a primary breast carcinoma (BRE), and a breast cancer cell line (MDA-435) are shown. 1184 non-polymorphic fragments from the PBL profile (2) were assessed. Arrows in BRE tumor and BRE cell line profiles indicate methylation.

Table 2. Summary of locus methylation in cell lines

Cell line type	Total loci methylated in at least one line	Methylated in 1 of 3 lines (%)	Methylated in 2 of 3 lines (%)	Methylated in 3 of 3 lines (%)
BRE	277	67	27	6
CLN	323	45	25	30
GLI	477	58	28	14
HN	189	64	24	12
LEU	749	51	35	14
LNG	320	59	28	13
PNET	220	74	22	4
TST	163	63	26	11

types (Kruskal–Wallis test, $P < 0.004$). Interestingly, however, the relative degree of hypermethylation in cell lines is positively correlated with their respective malignancies (Fig. 2). TST, HN, PNET and BRE tumors exhibit the lowest mean levels of hypermethylation, as do their respective cell lines, while CLN, GLI, LNG and LEU show the highest. As many as 88% (LEU) but minimally 41% (BRE) of the CpG islands hypermethylated in primary malignancies are also hypermethylated in their respective cell lines (Fig. 2).

Qualitative comparison

Analysis of all RLGS fragments hypermethylated in 114 malignancies and 24 cancer cell lines (Fig. 3A) shows that 57% are hypermethylated exclusively in cell lines. This demonstrates a high intrinsic level of hypermethylation in cell lines, which is not present in non-cultured primary neoplasias. Of the RLGS fragments that are hypermethylated in any of the 24 cell lines, 70% are hypermethylated in lines from multiple malignancies, with 2% lost in lines from all eight types and 32% lost in four or more types. These data suggest that a large portion of this CpG island hypermethylation is a consequence of properties common to cancer cell lines, but different from factors specific to the tissue of origin. The total number of RLGS fragments lost in each individual tumor type and respective cell line is shown in Figure 3B, with the majority of hypermethylation appearing in cell lines representing multiple tumor types. Less than 9% of the total cell line hypermethylation for each malignancy is unique to lines of that type (except for LEU lines, which show the highest hypermethylation rate), again demonstrating that most cancer cell line hypermethylation is not dependent upon the tissue of origin. This is further illustrated in Table 3, showing 23 cloned RLGS fragments with homology to known genes. In nearly all cases, the genes are methylated in more cell line types than tumor types. One such example is the *OTX1* gene (RLGS fragment 2D20) which is methylated in cell lines representing all eight tumor types, but primary neoplasias from only the colon and lung.

DISCUSSION

In this report, we have addressed the questions of quantitative and qualitative similarity in the hypermethylation phenotypes between primary malignancies and their corresponding

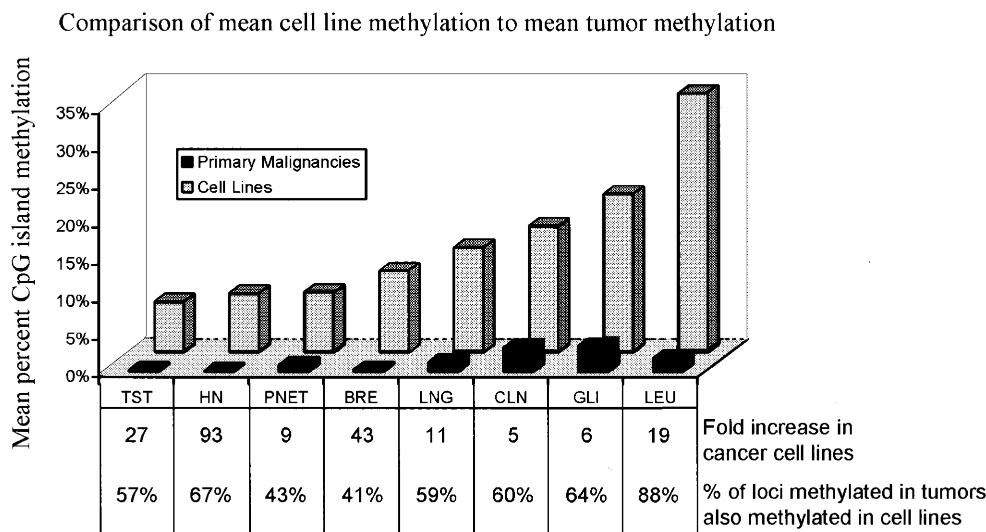

Figure 2. Mean CpG island hypermethylation compared among cell lines and respective malignant tissues (2), with hypermethylation fold increase in cell lines and percentage of hypermethylated loci in primary malignancies that are preserved in corresponding cell lines.

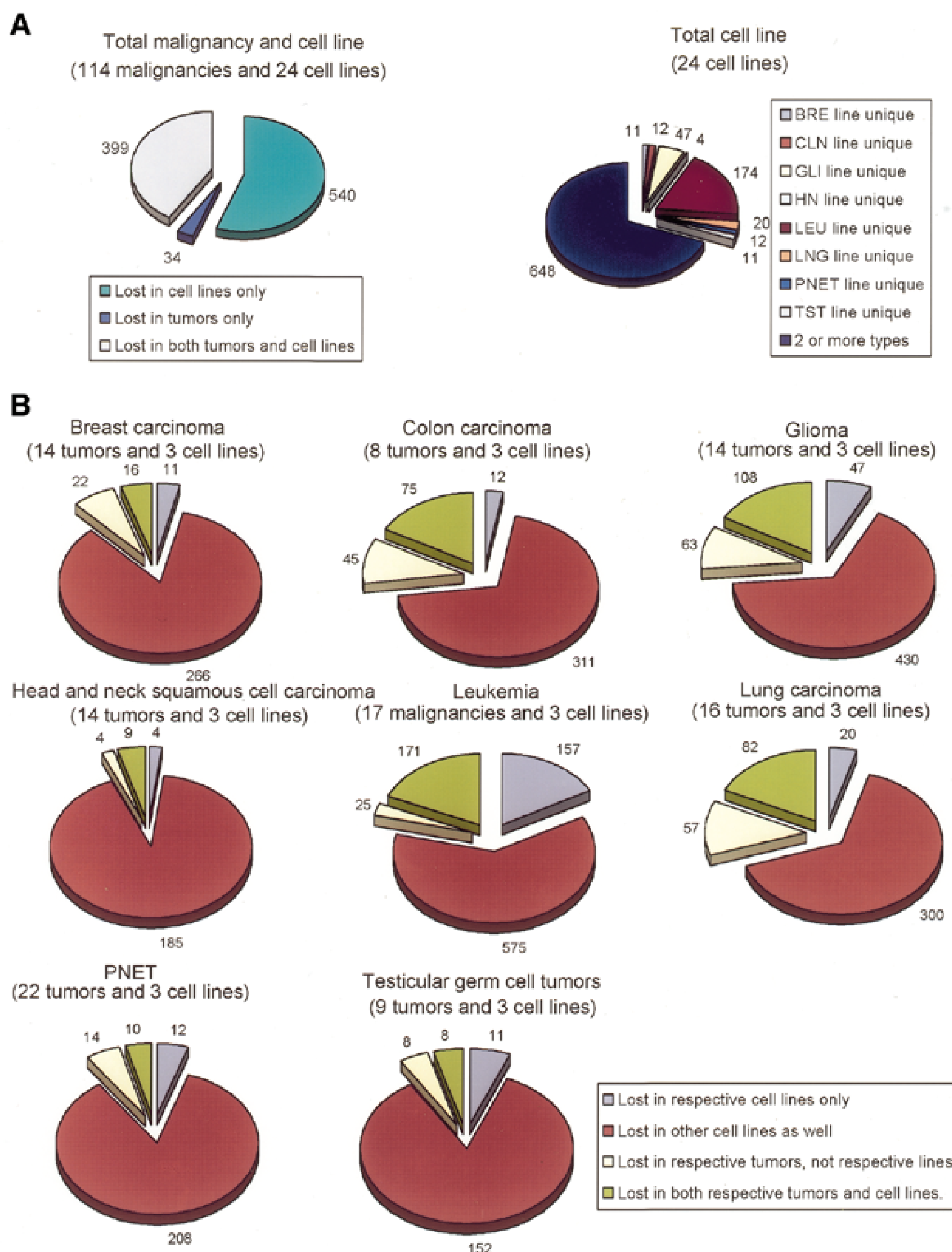

Figure 3. Distribution of RLGS fragment loss. Distribution of total (**A**) or individual malignancy (**B**) RLGS fragments lost in cell lines and/or malignancies.

established cancer cell lines. We have taken advantage of the unique ability of RLGS to provide a 'snapshot' of the CpG island hypermethylation phenotype in the genomes of primary malignancies and cell lines by assessing the methylation state of 1184 CpG islands in each sample. Through comparison of the data sets produced for 114 primary malignancies and 24 cell lines representing each of these malignancies, our analysis clearly demonstrates that cancer cell lines have much higher levels of CpG island hypermethylation than corresponding malignant tissues.

Interestingly, the degrees of the increased CpG island hypermethylation phenotypes in cell lines are not uniform across the types of cancers though they are positively correlated with the primary malignancies (Spearman's rank correlation 0.81, $P < 0.0001$). Cancer types that tend to exhibit relatively high levels of CpG island methylation also show relatively high levels in representative cell lines. The same is true of cancer types exhibiting relatively low levels of CpG island hypermethylation. This suggests that although the cell lines representing a particular cancer demonstrate much higher overall levels of hypermethylation, they have retained a measure of tissue-of-origin specificity limiting how much more hypermethylation they are capable of, or can withstand. TST cell lines, for example, seem to have a lower capacity for

Table 3. Cloned RLGS fragments methylated in various cell line and tumor types

RLGS fragment	Reference[a]	Gene		Tumor types methylated[b]	Cell line types methylated[c]
1G20	–	*SOX2*	SRY-Box 2	LEU	CLN, LEU
2B53	–	*NCL*	Nucleolin	HN	CLN, GLI
2D14	(17,19)	*CD8B*	T-cell glycoprotein CD8 beta	HN, LEU	BRE, CLN, GLI, LEU, LNG, PNET, TST
2D20	(2)	*OTX1*	Orthodenticle (*Drosophila*) homolog 1	CLN, LNG	BRE, CLN, GLI, HN, LEU, LNG, PNET, TST
2E24	(19)	*MCT3*	Monocarboxylate transporter 3	GLI, LEU, LNG, PNET	BRE, CLN, GLI, HN, LEU, LNG, PNET, TST
2E25	–	*FKBP8*	FK506-binding protein 8	BRE	TST
2F50	(2,19)	*POU3F1*	POU domain class 3, transcription factor 1	GLI, LEU	BRE, GLI, LEU
3B36	(19)	*CYP1B1*	Cytochrome P450, subfamily I, polypeptide 1	CLN, GLI, LEU, LNG, PNET	BRE, CLN, GLI, HN, LEU, LNG, TST
3B55	–	*TBR1*	T-Box, brain, 1	BRE, CLN, LNG, PNET	CLN, GLI, LEU, TST
3C1	(2,17,19)	*GNAL*	G-alpha olfactory	CLN, GLI, LEU, LNG	BRE, CLN, GLI, LEU, LNG, TST
3D21	–	*LOC51193*	Zinc finger protein ANC_2H01	LEU	None
3D24	–	*USP6*	Ubiquitin-specific protease 6	GLI, LEU, LNG	BRE, LNG, PNET, TST
3E55	(17,19)	*IPF1*	Insulin promoter factor 1	LNG	CLN, GLI, HN, LEU, LNG, PNET, TST
3E59	–	*BNPI*	Brain-specific Na-dependent inorganic phosphate cotransporter	LNG	CLN, LEU
3F64	–	*GAK*	Cyclin G associated kinase	LNG	GLI, HN, LEU, LNG
3F72	(19)	*GPR88*	G-protein coupled receptor 88	LEU, LNG	CLN, LEU
3G46	–	*DPAGT2*	GlcNac-1-P transferase	None	LEU
4B56	–	*XT3*	Orphan transporter XT3	BRE, LEU	BRE, GLI, LEU
4F15	(17)	*BMP3B*	Bone morphogenetic protein-3b	CLN, LNG	GLI, LEU, LNG
5D9	–	*NGEF*	Neuronal guanine nucleotide exchange factor	HN, LNG, TST	BRE, CLN, GLI, LEU, LNG, PNET, TST
5E25	–	*FMN2*	Formin 2-like	CLN, LNG	CLN, HN, LEU, PNET
5E34	(2,15,19)	*WIT1*	Wilms' tumor associated protein	GLI, LEU	BRE, CLN, LEU, LNG, PNET, TST
5E4	–	*MYO9B*	Myosin IX B	BRE, GLI, LEU, LNG	BRE, CLN, GLI, HN, LEU, LNG, TST

[a]References where the RLGS fragment was first reported to be methylated.
[b]Breast carcinoma (BRE), $n = 14$; colon carcinoma (CLN), $n = 8$; glioblastoma (GLI), $n = 14$; head and neck squamous cell carcinoma (HN), $n = 14$; acute myeloid leukemia (LEU), $n = 17$; lung carcinoma (LNG), $n = 16$; medulloblastoma (PNET), $n = 22$; testicular germ cell tumors (TST), $n = 9$.
[c]Three cell lines from each of the eight tumor types.

hypermethylation than LEU cell lines, which is reflective of the primary malignancies. It is unclear whether the limiting factor explaining why TST lines do not hypermethylate their genomes as much as LEU lines is a reflection of their ability to hypermethylate, or their lack of tolerance to hypermethylation.

Analysis of which loci become hypermethylated in primary malignancies and their respective cell lines is striking. More than 57% (540 loci) of the loci that become hypermethylated do so in cell lines but in none of the 114 primary malignancies. Even taking into account the higher overall level of hypermethylation in cell lines, it is remarkable that such a large portion of hypermethylation events is exclusive to cell lines. Furthermore, the majority of cell line hypermethylation is common to multiple lines independent of the tissue of origin. Seventy percent (648 events) of the hypermethylation events which occur in any cell line occur in cell lines representing more than one type of cancer. Thus, it appears that the majority of the hypermethylation events seen in cancer cell lines are related to an intrinsic property of mammalian cells grown in culture rather than dependent upon the cell of origin. A portion of this may reflect the repression of both non-essential genes as well as genes involved in terminal differentiation (12), thus conferring an *in vitro* growth advantage. It is likely that repression of many of these genes could not be tolerated *in vivo*.

Despite the above observations, cancer cell lines do in fact show some evidence of hypermethylation specificity as most methylation events in primary malignancies also occur in their respective cell lines. Only two cancer types, PNET and BRE, have <50% of the loci (9/21 and 12/29, respectively) methylated in primary tumors that are also methylated in their respective cell lines. Thus, similar to the quantitative analysis described above, these qualitative data also indicate some retention of tissue-of-origin characteristics in their hypermethylation phenotypes. Given that CpG island hypermethylation is thought to contribute to repression of gene expression, this

retention of tissue-of-origin hypermethylation characteristics might be reflected in the set of genes expressed in these cell lines. This idea is supported by cDNA microarray data showing that cell lines from individual cancer types cluster together based on expression profiling (18).

In conclusion, the CpG island hypermethylation found in cell lines is significantly different from that seen in the primary malignancies they represent. It appears that the majority of hypermethylation events in cell lines can be thought of as 'background' events, which have little to do with the cancer, but much to do with the fact that these cells are growing in culture. Nevertheless, it is clear that these cell lines have retained some hypermethylation characteristics from their tumor of origin, particularly with regard to the degree of hypermethylation, and to a lesser extent, with regard to which loci become hypermethylated. These data have strong implications for certain types of DNA methylation research performed with cancer cell lines as the main experimental system. When cell lines are used to identify targets of promoter hypermethylation in a particular cancer type, these results indicate that 66% (LNG) to 92% (HN) of the loci identified are lost only in cell lines, not in cancer. Thus, we conclude that, given their high intrinsic level of CpG island hypermethylation, cancer cell lines are likely to be a poor resource for identifying novel targets of DNA hypermethylation involved in oncogenesis. However, since they often preserve hypermethylation from their corresponding tumors, they are indeed useful resources to study hypermethylation and its consequences at specific loci identified in tumors.

MATERIALS AND METHODS

Cell lines

The glioma cell line LN235 was kindly provided by Nicolas de Tribolet (Lausanne, Switzerland). The leukemia cell lines (TI1, HL60 and Kasumi1) were provided by one of the co-authors (M.A.C.). All other cell lines were originally obtained through ATCC (Rockland, MD). The breast carcinoma cell lines (T47D, HS578T and MDA4355) and the colon carcinoma cell lines (LN235, DLD2 and Colon A) were grown interchangeably in Dulbecco's modified Eagle's medium (DMEM) or mammary epithelial cell basal medium (Gibco BRL, Rockville, MD) with 5% fetal bovine serum. The glioma cell lines (T98, U251 and LN235) which were originally derived from human glioblastomas (WHO grade IV) were grown in minimal essential medium with 10% fetal calf serum (Gibco BRL). The head and neck squamous cell carcinoma cell lines (SCC4, SCC9 and SCC25) were grown in a mixture of 50% DMEM with 10% fetal bovine serum and 50% defined keratinocyte-SFM medium (Gibco BRL). The leukemia cell lines (TI1, HL60 and Kasumi1) were grown in RPMI-1640 medium with 20% fetal bovine serum (Gibco BRL), 1% antibiotic-antifungal agent (Gibco BRL) and 1% anti-PPLO agent (Gibco BRL). The lung carcinoma cell lines (H125, H522 and H1299) were grown in RPMI-1640 medium with 5% fetal calf serum. The medulloblastoma cell lines (Daoy, D283 Med and D341 Med) were grown in high glucose DMEM supplemented with 4 mM L-glutamine and 10% heat-inactivated human umbilical cord serum. The testicular non-seminoma cell line TERA1 was grown in McCoy's 5A medium modified (Gibco BRL) with 15% fetal bovine serum. The non-seminoma cell lines NCCIT and NTERA1 were grown in RPMI-1640 medium with 10% fetal bovine serum. All cell lines were grown at 37°C in a 5% CO_2 humidified atmosphere.

Standard RLGS procedure

RLGS on genomic DNA was performed as described by Okazaki *et al.* (14) and modified as described previously (16).

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