Analysis of *PTEN* Mutations and Deletions in B-Cell Non-Hodgkin's Lymphomas

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The *PTEN* gene is involved in 10q23 deletions in several types of cancer, including glioma, melanoma, endometrial and prostate carcinomas. The *PTEN* gene product is a dual-specificity phosphatase with putative tumor suppressor function. Deletions and rearrangements of 10q22–25 have been reported in ~5%–10% of non-Hodgkin's lymphomas (NHLs), raising the possibility of *PTEN* involvement in these tumors. In order to address this question, we analyzed a panel of NHLs (n = 74) representative of the main histologic subtypes for mutations and homozygous deletions of *PTEN*. We report somatic coding/splice site mutations in 20% (2 of 10) of Burkitt's lymphoma cell lines and in 3% (2 of 64) of primary NHL cases analyzed. No homozygous deletions were found in these tumors. Interestingly, this study showed that cytogenetically characterized NHL cases (n = 6) with 10q22–q25 abnormalities displayed neither biallelic deletions nor mutations of *PTEN*. These results suggest that a tumor suppressor gene distinct from *PTEN* may be involved in 10q deletions in this subgroup of NHL cases. *Genes Chromosomes Cancer 24:322–327, 1999.*

INTRODUCTION

Non-Hodgkin's lymphoma (NHL), the fifth most common human malignancy, is most often of B-cell origin and is characterized by marked biological and clinical heterogeneity. The pathogenesis of this tumor is associated with chromosomal translocations that lead to the deregulated expression of various proto-oncogenes found at the breakpoint junction area by their juxtaposition to regulatory elements of genes, most often the immunoglobulin genes (Gaidano and Dalla-Favera, 1997).

Relatively less is known about the involvement of tumor suppressor genes (TSGs) in NHL pathogenesis. Genetic alterations of *TP53* have been reported in 20% of diffuse large-cell lymphoma and in 30% of Burkitt's lymphoma cases, respectively (Gaidano et al., 1991; LoCoco et al., 1993; Ichikawa et al., 1997). Alterations have also been described in $p16^{INK4A}$ principally occurring in mantle cell lymphomas, an uncommon lymphoma subtype (Cohen et al., 1997). Karyotypic analysis has revealed recurrent deletions at 10q22–25, suggesting that this chromosomal locus may harbor a tumor suppressor gene relevant to B-cell lymphomagenesis (Offit et al., 1991; Speaks et al., 1992).

Recently, a putative tumor suppressor gene, *PTEN* (for phosphatase and tensin homologue, located on chromosome 10) was identified based on its involvement in 10q23 deletions in a variety of solid tumors (Li et al., 1997; Steck et al., 1997). The gene was found to be mutated or homozygously deleted in glial, prostate, endometrial, kidney, testis, and breast cancers as well as in leukemia and melanoma (Guldberg et al., 1997; Rhei et al., 1997; Tashiro et al., 1997; Teng et al., 1997; Wang et al., 1997). In addition, germline mutations of *PTEN* have been found in a group of inherited predisposition cancer syndromes, including Cowden disease and Bannayan-Zonona syndrome (Liaw et al., 1997; Marsh et al., 1997). *PTEN* encodes for a ubiquitously expressed cytoplasmic tyrosine phosphatase, which contains a region of homology to tensin and auxilin, which are known as cytoskeletal proteins (Li et al., 1997).

To determine if the target of 10q anomalies in NHL (Offit et al, 1991; Speaks et al, 1992) involve the *PTEN* gene, we analyzed a panel of NHL biopsies and cell lines for mutations and deletions within the coding and exon-intron boundary domains of this putative TSG.

M.P.B. was supported by the Mater College Hospital, Dublin, and the Vocational Education Committee, Co. Donegal, Ireland, and is a registered Ph.D. student from the University College Dublin, Ireland.

Supported by: National Institutes of Health; Grant number: CA-44029.

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Received 7 July 1998; Accepted 24 September 1998

TABLE 1. Frequency of *PTEN* Mutation/Deletion in NHL

NHL subtype[a]	Mutations	Homozygous deletions
SML	1/5	0/5
LPL	0/7	0/2
MCL	0/5	0/3
FCL	0/20	0/18
DLCL	1/27	0/10
BL	2/10	0/10
Total	4/74	0/48

[a]NHL subtype: SML, small lymphocytic lymphoma; LPL, lymphoplasmacytoid lymphoma; MCL, mantle cell lymphoma; FCL, follicular center-cell lymphoma; DLCL, diffuse large-cell lymphoma; BL, Burkitt's lymphoma (cell lines).

MATERIALS AND METHODS

Tumor Biopsies and Cell Lines

Sixty-four tumor biopsies from NHL patients were used for *PTEN* mutational analysis. The NHL panel was representative of the major B-cell NHL categories (Table 1). Ten Burkitt's lymphoma cell lines were also included. The karyotypic and immunophenotypic characterization of these cell lines has been reported previously (Lenoir et al., 1982; Parsa et al., 1994).

Mutation Analysis

Amplification of exons 1 to 9 of *PTEN* from normal/tumor DNA was achieved through the use of intron-based primers reported previously (Wang et al., 1997). PCR amplification was performed in a 25-μl reaction volume using 100 ng of genomic DNA, 1.5–2.0-mM $MgCl_2$, 100 μM each dNTP, 0.5-–M primer and 0.5-U Taq DNA polymerase (AmpliTaq, Perkin-Elmer, Foster City, CA). The cycling parameters were as follows: initial denaturation step at 94°C for 5 min, followed by 30 rounds of thermal cycling at 94°C for 30 sec, 56°C for 30 sec, 72°C for 30 sec, with a final extension period of 72°C for 5 min using a Perkin-Elmer Cetus 9600 GeneAmp PCR system; a different annealing temperature (58°C) was used for exon 4. PCR products were purified on columns (Wizard PCR Preps DNA purification system, Promega, Madison, WI) prior to sequencing by the dideoxy chain termination method using an automated sequencing system, ABI 373 (Applied Biosystems, Foster City, CA). Sequencing primers were the same as those used to amplify tumor DNA except for the following: exon 1 reverse 5'-ACGTTC-TAAGAGAGTGACAGA-3'; exon 5 forward 5'-ATTCTGAGGTTATCTTTTTTACC-3'; exon 8 forward 5'-GTAAATACATTCATACCAG -3'; exon 9 forward 5'-TCTCTAGGTGAAGCTGTACT-3'. All cases with mutations were analyzed twice to ensure reproducibility. To characterize the deletion mutations, PCR products were subcloned into the pGEM-T plasmid (Promega) and sequenced.

Homozygous Deletion Assessment

Biallelic deletions of *PTEN* were analyzed by Southern blot analysis using high-molecular-weight DNA available for 38 of the same 64 primary NHL cases used for sequence analysis. Briefly, 10 μg of tumor and normal tonsil DNA were digested with *Eco*RI and *Eco*RV restriction enzymes, run on 0.8% agarose gels, transferred to nitrocellulose membranes, and hybridized with probes specific for each *PTEN* coding exon. These probes were prepared from normal human DNA by PCR using the same primers used to amplify the tumor DNA for direct sequence analysis. All the PCR products were gel-purified followed by purification on columns (Wizard PCR purification system, Promega). Exon probes were first tested individually on *Eco*RI and *Eco*RV Southern blot filters prepared from normal tonsil DNA to determine the specific banding pattern given by each individual exon. Next, pools of the nine PCR-derived *PTEN* exon probes (Fig. 1) were used to screen NHL Southern blot filters. The signal intensity given by all probes was measured using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and normalized to the signal of a reference probe (exon 9 of the *BCL*-6 gene, which has no deletions in NHL) (Gaidano and Dalla-Favera, 1997). When using exon probes 5, 6, 7, 8, and 9, two or three bands were seen on Southern blot, which were found to represent *PTEN* homologous sequences. The identification of the *PTEN*-specific band in such cases was achieved by the hybridization of these exon probes to somatic cell hybrid Southern blot filters (full panel of 24 chromosomes), which were digested with *Eco*RI and *Eco*RV restriction enzymes (Mapping Panel #2, NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, Camden, NJ) (data not shown). By this approach we could show that each band seen on Southern blot (Fig. 1) corresponded to a single chromosomal locus (data not shown). The signals corresponding to the *PTEN* gene as well as the pseudogene (chromosome 9) (Teng et al., 1997) are both indicated in cases where an exon probe gives rise to two or more bands on Southern blot. The signal intensity of the *PTEN*-specific bands was measured and a >60% reduction in tumor versus

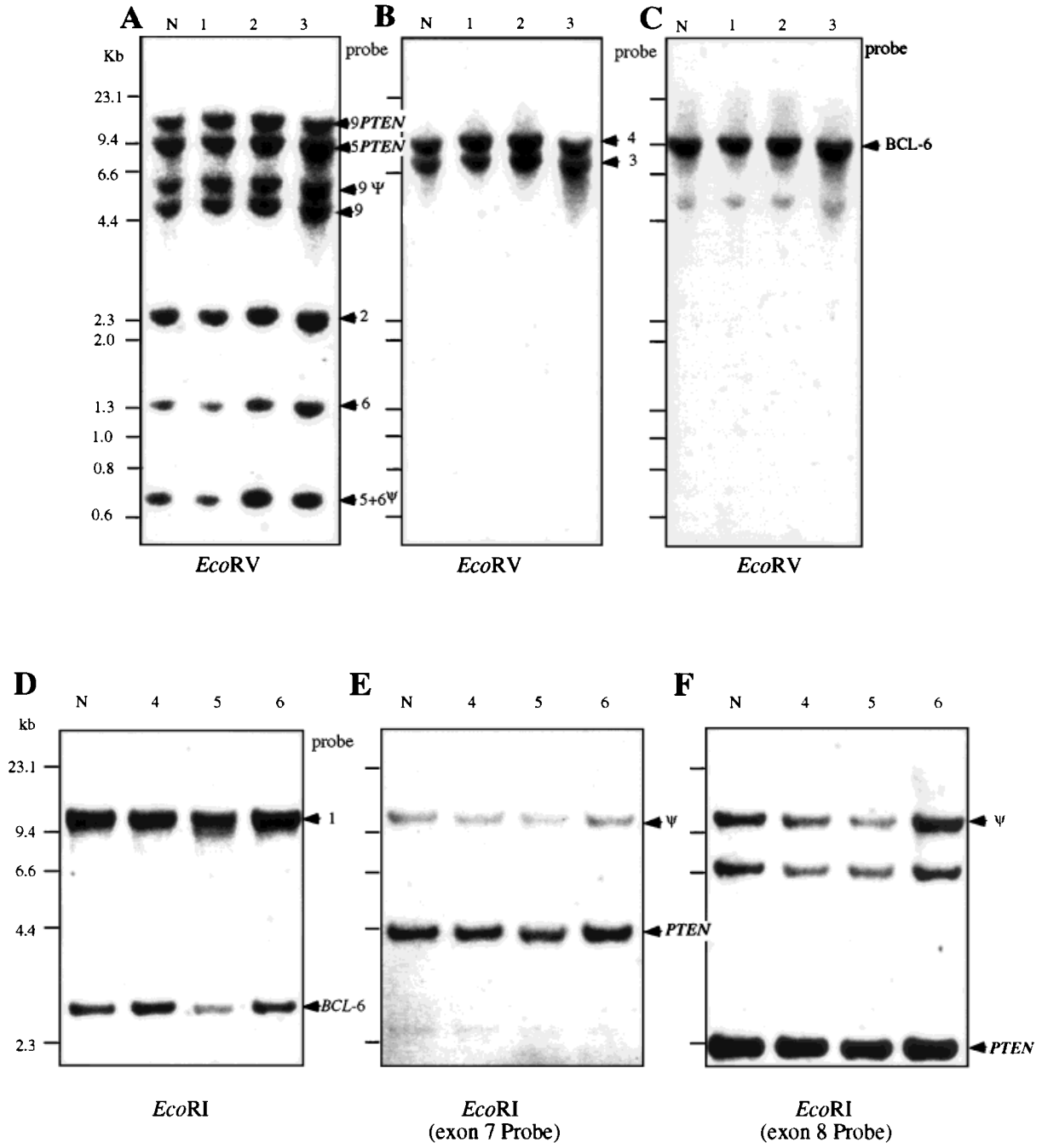

Figure 1. Homozygous deletion assessment of *PTEN* in representative NHL cases. **A–C:** Southern blots of DNAs digested with *Eco*RV. **D–F:** Southern blots using *Eco*RI. Sizes given by molecular weight marker are indicated in A and D. The bands produced by the exon probes are indicated by the arrows. A represents the hybridization of *PTEN* exons 2, 5, 6, and 9; B, that of exons 3 and 4; D, E, and F, that of exon probes 1, 7, and 8, respectively. The reference probe used was exon 9 of *BCL*-6, which is located at chromosomal locus 3q27 (see C and D). Exon probes producing two or more bands (exons 5, 6, 7, 8, and 9) represent cross-hybridization to the *PTEN* pseudogene (chromosome 9; represented by ψ), as well as to sequences located on additional chromosomes, which may represent *PTEN* homologues (data not shown). The first lane is normal tonsil DNA; lanes 1–3 are representative follicular center-cell lymphoma cases; lanes 4–6 are Burkitt's lymphoma cell lines.

TABLE 2. *PTEN* Mutations in NHL

Case	NHL subtype[a]	Exon/ intron	Codon[b]	Mutation	Predicted effect	Allelic status
BL30	BL	Exon 3	61	A182T	His > Leu	Homozygous
BL108	BL	Exon 8	335	C1003T	Arg > Stop	Heterozygous
857	SML	Intron 3	Splice site	210-3delCTTT	Splicing variant	Heterozygous
1687	DLCL	Exon 8	307	G921A	Glu > Glu	Heterozygous
1494	FCL	Intron 8	NA	1026 + 43 G > A	–	Heterozygous
1335	MCL	Intron 1	NA	79 + 11 C > T	–	Heterozygous

[a]NHL subtype: BL, Burkitt's lymphoma (cell line); SML, small lymphocytic lymphoma; DLCL, diffuse large-cell lymphoma; FCL, follicular center-cell lymphoma; MCL, mantle cell lymphoma.
[b]NA, not applicable.

TABLE 3. Mutation and Deletion Analysis of *PTEN* in NHL Cases With 10q Abnormalities

Case number	NHL subtype[a]	Cytogenetics	Mutation	Homozygous deletion
1381	FCL	del(10)(q22q24)	Negative	Negative
1382	DLCL	t(4;10)(q31;q24)	Negative	Not done
1383	FCL	del(10)(q22q24)	Negative	Negative
1384	LPL	t(10;14)(q24;q32)	Negative	Negative
1385	DLCL	t(9;10)(q34;q24)	Negative	Not done
1386	LPL	add(10)(q24)	Negative	Not done

[a]NHL subtype: FCL, follicular center-cell lymphoma; LPL, lymphoplasmacytoid lymphoma; DLCL, diffuse large-cell lymphoma.

normal DNA was interpreted as evidence of biallelic deletion (normal contaminating DNA is present in NHL biopsies).

RESULTS

PTEN Mutation Analysis

The entire coding domain of *PTEN*, including splice site junction regions, was examined for sequence variations in a total of 64 NHL cases and in 10 cell lines. The NHL panel used to investigate *PTEN* mutational inactivation is given in Table 1 and is representative of the major B-cell NHL histological subtypes. Systematic scanning of all nine exons of *PTEN* led to the identification of coding/splice site mutations in 2 of 10 (20%) Burkitt's lymphoma cell lines, and in 2 of 64 (3%) primary NHL cases (Table 2). No mutations or biallelic deletions were observed in the NHL cases karyotypically defined as having 10q abnormalities (Table 3).

The mutations detected in the Burkitt's lymphoma cell lines were of somatic origin as they were not found in their normal DNA counterparts (Fig. 2, Table 2). Among these alterations, a missense mutation was detected in BL30, an A-to-T transversion resulting in a histidine-to-leucine change at codon 61. The other allele was deleted in this cell line. A second BL cell line, BL108, carried a nonsense mutation, a C-to-T transition at codon 335, and retained a normal second allele.

Among primary NHL cases, a small lymphocytic lymphoma case (case no. 857; Table 2) displayed a 4-bp deletion located 1 bp 5' to the acceptor splice site of exon 4. In addition, a diffuse large B-cell lymphoma (case no. 1687) carried a silent mutation, a G-to-A transition, at codon 307 within exon 8. Other sequence variants were found in two lymphomas displaying intronic base pair substitutions, in intron 1 (case no. 1335) and in intron 8 (case no. 1494). In all primary cases, a second wild-type allele was detectable, but we could not determine whether it was present in tumor cells or in contaminating normal cells. Finally, a recurrent missense mutation located 32 bp 3' to the splice donor site of exon 8 [TTG(T/G)TGACT] was detectable in multiple cases. This mutation was previously described in other individuals (Wang et al., 1997), suggesting that it may represent a germline population polymorphism.

Deletion Assessment of *PTEN*

Homozygous deletions of *PTEN* were studied by Southern blot analysis using probes specific for each of the nine exons of *PTEN*. Through the use of two restriction enzymes, it was possible to identify an exon-specific banding pattern (Fig. 1), thus allowing for the detection of deletions of individual exons. No biallelic deletion of *PTEN* was detectable among 38 primary NHL cases. Monoallelic loss could not be reliably assessed due to the presence of normal DNA contaminating the samples.

DISCUSSION

Mutations and deletions of the *PTEN* gene on 10q23 are emerging as a common genetic alteration in a variety of sporadic human cancer types as well as in some hereditary tumor predisposition syn-

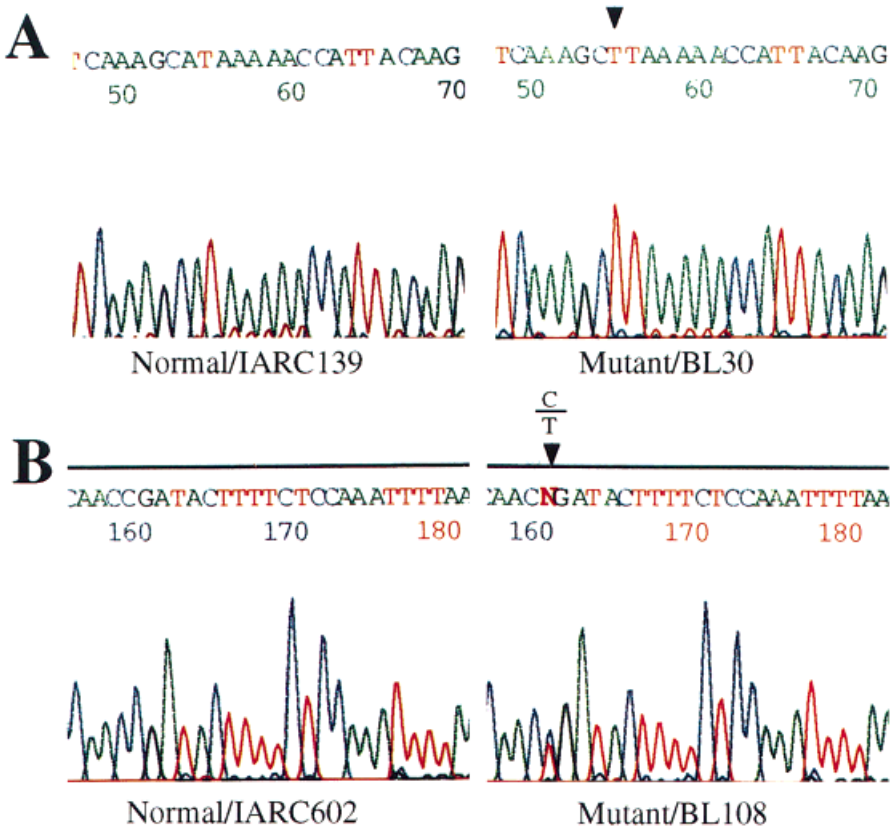

Figure 2. Detection of somatic mutations in Burkitt's lymphoma cell lines by direct sequence analysis. **A** shows the chromatograms of BL30 and its normal counterpart, IARC139: a missense mutation at codon 61 leads to an A-to-T transversion resulting in a histidine to leucine substitution (CAT to CTT). **B** gives the chromatogram of BL108 and corresponding normal DNA, IARC602. A nonsense mutation is detected at codon 335, as a result of a CGA-to-TGA change.

dromes. Based on the presence of chromosome 10q22–25 deletions and rearrangements in ~5%–10% of NHLs (Offit et al., 1991; Speaks et al., 1997), this study was aimed at assessing whether *PTEN* was involved in these cytogenetic abnormalities. Our results show that alterations of the coding region of *PTEN* are not common in NHL and have implications for the understanding of the significance of 10q deletions in these malignancies.

In this study, we detected mutations of the *PTEN* gene in 20% (2 of 10) of the BL cell lines and in 3% (2 of 64) of primary NHL cases. Both mutations detected in the BL cell lines may have functional significance. The missense mutation leading to a histidine-to-leucine alteration at codon 61 involves a residue conserved in the homologous domain, which in cytoskeletal tensin binds to actin filaments at focal adhesions (Li et al., 1997), suggesting that it may be part of an important regulatory domain in *PTEN*. Because the second *PTEN* allele was deleted in this cell line, it is conceivable that *PTEN* function may be lost in this case. The second BL cell line carried a nonsense mutation at codon 335, which has been recurrently observed in both glial and endometrial carcinomas (Risenger et al., 1997; Wang et al., 1997), suggesting that it may represent a frequent target for *PTEN* inactivation. Finally, a small deletion detected in a small lymphocytic lymphoma case near the acceptor splice site may disrupt the normal splicing of exon 4, leading to exon skipping and ultimately truncation of the *PTEN* protein product.

In one of the BL cell lines (BL108), the mutant *PTEN* allele coexisted with the wild-type allele; also in the small lymphocytic lymphoma the wild type allele was detectable, although its derivation from normal versus tumor cells could not be determined. These findings may represent the "first hit" (Knudson et al., 1971), while mutation/deletion of the second allele may not yet have occurred. Alternatively, the second allele may have been inactivated by mutation in the regulatory region of the gene or through methylation. Another possibility is that monoallelic inactivation of *PTEN* may be sufficient to produce a biological effect, as has been suggested for other TSGs (Donehower et al., 1995).

The availability of anti-*PTEN* antibodies as well as the analysis of mice with monoallelic *PTEN* loss should allow for these possibilities to be addressed.

The results presented in this study indicate that cytogenetically detectable alterations of 10q23 are significantly more frequent in NHL than the occurrence of *PTEN* mutations. Our findings have been corroborated by a recent report showing infrequent alterations of *PTEN* (2 of 14 cell lines and 2 of 170 primary tumors) among lymphoid malignancies (Gronbaek et al., 1998). The same observation is valid for thyroid, colon, ovarian, and pancreatic cancers, which all display 10q23 deletions, but infrequent *PTEN* mutations (Dahia et al., 1997; Tashiro et al., 1997; Teng et al., 1997). Indeed, we detected no mutations or biallelic deletions of *PTEN* in the lymphoma cases with cytogenetically detectable 10q anomalies included in this study. Thus, these results may suggest that haploinsufficiency of *PTEN* may produce a biological effect in these diverse tumor types or, alternatively, that another TSG may be located at 10q that is involved in these tumors.

ACKNOWLEDGMENTS

The authors thank Ulla Beauchamp for providing excellent technical assistance in DNA sequencing, and Anna Migliazza, Rob Hauptschein, Georgia Hatzivassiliou, and Huifeng Niu for critically reviewing this manuscript.

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