Novel Tumor Suppressor Loci on 6q22-23 in Primary Central Nervous System Lymphomas

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Abstract

Deletions on the long arm of chromosome 6 (6q) are one of the most common chromosome alterations in systemic high-grade non-Hodgkin's lymphomas. However, the locations of allelic deletions and their roles have not yet been reported in primary central nervous system lymphomas (PCNSLs), most of which are classed as non-Hodgkin's lymphoma. We thus performed fine loss of heterozygosity (LOH) mapping of 6q in 29 samples of surgically resected PCNSLs using 39 microsatellite markers to identify commonly deleted regions. LOH was found at 1 or more loci at 6q22-23 in 19 samples (66%); furthermore, 18 of these samples shared a deletion in the same small (~140 kb) region flanked by D6S1030 and D6S1690, a region in which the human PTP-x gene (PTPRK) is reported to be located. Reverse transcriptase-PCR analysis of the mRNA from 4 cases with 6q deletions confirmed loss of this gene, and loss of PTPRK expression was observed in 76% (22 of 29) of tumors with immunohistochemistry. In addition, LOH on 6q22-q23 significantly correlated to shorter patient survival (12.8 ± 4.3 versus 23.4 ± 3.5 months; P < 0.0001). Our results suggest that a 140-kb deletion located at 6q22-23 may contain the putative tumor suppressor, PTPRK, that appears to be relevant to the pathogenesis and prognosis of PCNSLs.

Introduction

The incidence of PCNSLs in nonimmunodeficient patients has been markedly increasing over the past decades, and currently they are estimated to account for >6% of all primary brain tumors. Morphologically, 98% of them are B-cell, diffuse, large lymphomas that are categorized as high-grade non-Hodgkin's type (1). Regardless of the similarities in the phenotypes of these CNS and non-CNS lymphomas, there are no lymph nodes or lymphatics within the CNS, and therefore, the pathogenesis and histogenetic origin of PCNSL in immunocompetent patients is still poorly understood. The peculiar clinicopathological setting of PCNSLs suggests the presence of distinct molecular events from non-CNS lymphomas underlying their pathogenesis, whereas the cytogenetic and molecular profile of PCNSL has not been well characterized and seems inconsistent: in both CNS and non-CNS lymphomas, the p14ARF and p16INK4a genes are reportedly frequently inactivated by homozygous deletion (40–60%); and less commonly by 5’-CpG hypermethylation (15–30%; Refs. 2–4). In contrast, p53 mutations are frequent genetic events in systemic lymphomas but appear to be extremely rare in PCNSLs (3, 4). Promoter hypermethylation of both RB1 and p27Kip1 have been observed in a small fraction of PCNSL, whereas genetic alterations such as p21WAF1, MDM2, CDK4, CCND1, MYC, and REL have not been detected (2, 3).

LOH analysis can be used to assist in the identification of TSGs by narrowing the size of the search interval, thus providing investigators with a better-defined region in which to focus their efforts. Allelic losses on the long arm of chromosome 6 (6q) are among the most frequent chromosome aberrations in systemic malignant non-Hodgkin's lymphomas and in acute lymphoblastic leukemias, and two regions, 6q21-23 and 6q25-27, have been isolated that possibly contain different genes involved in lymphoma development (5, 6). However, a number of genes have been localized to these regions, among which are the B-cell surface marker CD24 (7), the cyclin C gene (8), and PTPRK (9, 10), but their precise roles have not been ascertained. Interestingly, the presence of 6q deletions correlates with poor patient prognosis in systemic lymphomas, suggesting that the identification of the target TSGs on 6q may provide a more specific marker of potential clinical significance (11). Although fine-scale mapping with LOH analysis has not yet been reported, comparative genomic hybridization studies on PCNSL have demonstrated that deletion on 6q was the most common chromosomal change detected (1), making it likely that 6q deletions are part of an important pathway in the development of PCNSL.

We analyzed 29 PCNSLs for LOH in 39 microsatellite loci spanning the entire 6q to refine the location of TSGs on 6q and to correlate allelic losses with the clinical course of this particular subset of brain tumors.

Materials and Methods

Tumor Samples and DNA Extraction. Twenty-nine primary malignant non-Hodgkin's lymphomas of the CNS were obtained from immunocompetent patients surgically treated between 1984 and 2001 in the Department of Neurosurgery, Nara Medical University (Nara, Japan). Additional tumor samples were also obtained at the time of relapse from four of these same patients (cases 17, 19, 25, and 28) with a full review of all available clinical data. DNA was extracted from paraffin sections to be used for microsatellite analysis. Control genomic DNA was derived from tissues separate from and not involved by the tumor.

LOH Assay. Thirty-nine highly polymorphic markers were selected from the Genome Database and NCBI1 for the LOH assay based on heterozygosity frequency as well as by coverage and flanking of the region of interest. Chromosomal maps and distances of each marker were obtained from the Whitehead Institute web site and BLAST the Human Genome. The names of the polymorphic microsatellites and their linear order based on the consensus genetic and physical maps of 6q are shown in Fig. 1. For each marker, the sense primer was labeled by a fluorescent dye, and paired normal and tumor DNA samples from each patient were amplified for 30 cycles with an annealing temperature of 56–58°C. Aliquots of the PCR reactions were then mixed with a size standard and formamide, denatured, and subjected to capillary

1 Internet address: gdbwww.gdb.org.
3 Internet address: www-genome.wi.mit.edu.
electrophoresis on the Genetic Analyzer 310 (ABI, Foster City, CA). The automatically collected data were analyzed with Genescan software (ABI) as described in the manufacturer’s protocol. Only samples heterozygous for a given locus were regarded to be informative; loci homozygosity and/or microsatellite instability rendered any particular sample noninformative. Samples were considered to show LOH when a peak allele signal from tumor DNA was reduced by 50% compared with the normal tissue counterpart. The search for candidate genes and ESTs located within MCRD was assisted by use of GeneMap99.7

RT-PCR Analysis of PTPRK Transcripts. Total RNAs were isolated from four CNS tumors (cases 7, 16, 19 and 21) and from normal human prostate and kidney using RNA TRIzol reagent (Invitrogen Corp., Carlsbad, CA) following the manufacturer’s instructions. cDNA was synthesized from 2 μg of total RNA. Amplification of G3PDH served as a control for cDNA quality. The sequences of the primers were as follows: 5′-ACCTCGACTCTCCTGTG-3′ as sense primer; and 5′-CATATTCTCTTTGGAACCC-3′ as the antisense primer. The PCR cycle conditions were comprised of 1 cycle of 95°C for 5 min, followed by 32 cycles of 95°C for 30 s, 52°C for 30 s, and 72°C for 1 min, with the last cycle reaction continued at 72°C for 7 min. The PCR-generated products were analyzed by electrophoresis in 2% agarose gels and proved to be 137 bp for PTPRK and 452 bp for G3PDH.

**Immunohistochemical analysis of PTPRK Expression.** Expression of PTPRK was assessed immunohistochemically, using a polyclonal antihuman PTPRK antibody (SC1113; Santa Cruz Biochemicals, Santa Cruz, CA). The antibody was incubated overnight at 4°C with antibodies at a dilution of 1:200. Other detailed method for immunohistochemistry was described previously (3).

**Statistical Methods.** Fisher’s exact test was used to examine possible associations between LOH at 6q22-23 and PTPRK expression. Patient survival probability was calculated using the Kaplan-Meier method, and the significance of the difference between pairs of Kaplan-Meier curves was calculated using the log-rank procedure. Statistical significance was established as $P < 0.05$.

**Results**

Analysis of LOH. All 29 tumors proved to be informative (i.e., heterozygous) for at least one marker. Nineteen (66%) tumors exhibited LOH in at least one locus. The allelic deletion map for the selected cases that displayed LOH in at least one locus is detailed in Fig. 1. Three tumors (cases 3, 7, and 9) showed loss at every informative locus studied, suggesting a whole chromosome loss in each case. The remaining 16 tumors showed partial or interstitial deletions. The respective frequencies (LOH cases/informative cases) are listed in Table 1.

**Table 1. Analysis of LOH of chromosome 6q in 29 PCNSLs.** Only the cases that lost at least one marker are shown. Marker names, map position, and genetic distance are on the left, and case numbers are listed at the top. A thick vertical bar at the extreme right refers to the 140-kb minimum deletion region between D6S1030 and D6S1690. LOH/Inf (%)*, the number of cases with LOH/the number of informative cases (LOH frequency).

![Image](image-url)

![Diagram](diagram-url)
Fig. 1. The highest frequency of LOH (15 of 21 informative cases, 71%) was observed at D6S407, a marker mapped to 6q22.2-23.1; representative cases are depicted in Fig. 2. The MCRD was defined with proximal and distal boundaries marked by D6S1030 and D6S1690. Constitutional heterozygosity at D6S1030, ~118.6 kb proximal to D6S407, was retained in case 18, and four other cases (1, 8, 16, and 17) retained heterozygosity at D6S1690, which is ~20.6 kb distal to D6S407 (Fig. 1). These results indicate that a locus at D6S407 in the chromosomal subband 6q22.2-23.1 may harbor a TSG within a physical map distance of 139.2 kb defined by markers D6S1030 and D6S1690. Eighteen of 29 cases (62%) lost at least one marker from this small region of LOH. Cases 16 and 23 also show LOH at D6S275, with retention of heterozygosity in the flanking markers. This suggests a second region of allelic loss, but it was not considered significant because of the low percentage of samples showing LOH at this locus. In those available cases of recurrent disease, specifically cases 17, 19, 25, and 28, three samples (cases 17, 19, and 28) showed LOH of 6q at initial presentation, whereas the remaining sample (case 25) had a normal chromosome. All four tumors showed the same LOH pattern at relapse as at their initial presentation (Fig. 2).

Reduction of PTPRK mRNA and Protein Expression in PCNSLs. Genes located in the MCRD of interest were identified by searching through the GeneMap'99.7 We performed RT-PCR experiments on four representative PCNSLs and on normal prostate and kidney as positive controls. Although transcripts were retrieved in the positive controls, no PCR products corresponding to PTPRK were found in any of the tumor samples tested (Fig. 3A). PTPRK is of special interest because of its purported role relating to adhesion molecules. In the immunohistochemical assays, a total of 22 samples
(76%) examined showed reduced expression of PTPRK protein, and 7 tumors showed diffuse PTPRK immunoreactivity in cytoplasm. Among these cases, 19 tumors with LOH at 6q22–23 also lost PTPRK expression, whereas, in contrast, 3 tumors without detectable LOH at the same locus were immunopositive for PTPRK (Table 1). Fig. 3, B and C, shows representative immunopositive (case 11) and immunonegative (case 16) cases. We additionally found that the association between LOH at 6q22–23 and reduced expression of PTPRK was statistically significant (Table 1: \( P < 0.0001 \)).

**Correlation of LOH with Clinical Data.** The median survival time for all patients was 16 months (range, 8–30 months). We found that those patients whose tumors demonstrated the 6q deletion had a significantly shorter median survival when compared with those patients with tumors having a normal chromosome 6q (12.8 ± 4.3 versus 23.4 ± 3.5 months, respectively; \( P < 0.0001 \)). Fig. 3, D and E, show the Kaplan-Meier survival curves for all 29 patients with PCNSL. There was also a tendency toward earlier death in patients with tumors showing loss of PTPRK expression as compared with those maintaining PTPRK expression, although we could not find a statistically significant correlation (Fig. 3E; \( P = 0.053 \), log-rank test).

**Discussion**

Previous investigators have estimated that the most frequent chromosomal deletion previously reported in 20–40% of non-CNS lymphomas was in the 6q (5, 6). We performed a fine deletion mapping of 6q in PCNSLs to identify any MCRDs and were able to demonstrate that these lesions also showed frequent allelic losses on chromosome 6q22–23 and, additionally, that deletions within this region are a more frequent phenomenon (66%) than in lymphomas at other locations. Given the data, we feel our study indicates the likelihood of putative TSGs of particular significance to PCNSLs in this region of 6q. The difference in the frequency of 6q deletions may also be a function of the differences in pathogenesis-inducing genetic mechanisms between CNS and non-CNS lymphomas.

As a result of several LOH studies, deletions at 6q16–23 have been found in a variety of neoplasms, including prostate cancer (12), leukemia (6), and breast cancer (13). The MCRD reported by Barghoum et al. (14) to be on 6q22.1 in endocrine pancreatic tumors overlapped with the MCRD in PCNSL that we defined at 6q22–q23 in our study. This would suggest that both PCNSL and endocrine pancreatic tumors, as well as several other types of cancers, share common regions of 6q deletion and that the same gene or genes may be involved. The hypothesis that one or more TSGs may be located within 6q is supported by chromosome transfer experiments, which have shown that the introduction of all or part of chromosome 6 can suppress the cell growth and/or metastatic potential of melanoma cell lines (15). A similar effect was also reported in breast cancer (13) in which the region of tumor suppressor activity was within 6q23-25 (13). Fine deletion mapping on 6q by LOH analysis had not yet been performed in PCNSL when these earlier studies were undertaken. As a consequence, no recurrent breakpoint information was available to facilitate positional cloning studies in both PCNSLs and systemic lymphomas. Even so, similarities were repeatedly noted. Weber et al. (1), using comparative genomic hybridization methods, reported that the chromosome arm most frequently affected by losses of genomic material in large B-cell-type CNS lymphomas was 6q (47%), with a commonly deleted region mapping to 6q21-22. In systemic non-Hodgkin's lymphomas, several MCRDs along 6q16–23 were reported, including regions located at 6q23 (5), at 6q23-24 (16), at 6q14–21, and at 6q23–27 (17). It is thus likely that at least one TSG important in regulating the development of these high-grade lymphomas, including PCNSL, is located at 6q21–23, but because not all of the regions for common allelic loss overlap, the implication is that more than one TSG associated with lymphomagenesis resides in this region. Alternatively, of course, PCNSLs might be affected by totally different genetic events from non-CNS lymphomas.

To determine whether and which candidate TSG or TSGs may reside on chromosome band 6q22–23 in PCNSL, we combined information from BLAST the Human Genome, GeneMap99, NCBI Entrez Genome, and our own physical map. Markers D6S1030, D6S407, and D6S1690 are localized on the same fragment (NT_025741.9), indicating that they are closely related. The predicted distance between D6S1030 and D6S1690 does not exceed 140 kb. Also, markers D6S1436, D6S1705, and D6S1572 are localized on the same contiguous segment, suggesting the distance between all of these markers to be less than ~4 cm. Sequence-based mapping of the PTPRK gene, obtained from BLAST the Human Genome and NCBI Entrez Genome, revealed that the 29 exons of this gene are contained between the markers D6S1436 and D6S1690, making PTPRK the lead contender for a TSG in this MCRD.

Yang et al. (10) showed that transforming growth factor-β1 inhibits human keratinocyte proliferation in vitro, possibly through induction of PTPRK gene expression. They additionally suggested that PTPRK might be involved in the regulation of cell contact and adhesion via dephosphorylating β-catenin and γ-catenin/plakoglobin or cadherins, thereby contributing to the formation and maintenance of intact adherens junctions. An additional involvement of the PTPRK gene in cell proliferation, tumor invasiveness, and metastatic spread has also been indicated. PTPRK is expressed ubiquitously in normal tissues, at low levels in renal tissues, and at high levels in prostate tissues (18). However, from four PCNSL patients in our investigation for whom RNA samples were available, we showed that the PTPRK gene is not expressed in tissues demonstrating a chromosome 6q22–23 deletion. Furthermore, loss of PTPRK protein expression was observed in 76% of PCNSL specimens, which reveals a strong association between LOH at 6q22–23 and loss of PTPRK expression (\( P < 0.0001 \)) and argues that altered expression of the PTPRK gene is characteristic of PCNSL with 6q22–23 LOH and that there is a potential tumor suppressor role for this gene in this neoplasm.

The structural aberrations on 6q have occasionally been correlated with clinical features of non-Hodgkin’s lymphoma such as tumor progression, transformation, and survival in systemic lymphomas (11). Despite the similarities in the phenotypes of CNS and non-CNS lymphomas, the prognosis for patients with the disease has remained poor, although some patients with non-CNS lymphoma can survive longer than those with PCNSL after treatment with high-dose chemotherapy. Our results were in line with those of systemic lymphomas. Loss of chromosomal material on 6q was significantly correlated with shorter survival compared with patients with retention of 6q (12.8 ± 4.3 versus 23.4 ± 3.5 months; \( P < 0.0001 \)), and the incidence of 6q LOH in PCNSL is higher than in non-CNS lymphomas (66 versus 20–40%; Refs. 5, 6). Tumor LOH was also analyzed at the time of relapse in four patients. All these patients showed the same 6q structure as their first presentation, suggesting, albeit with a small patient sample size, that 6q deletions may be an initial event in PCNSL pathogenesis and may occur less frequently during the progression of this tumor. However, the sample size of relapsed patients

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| Table 1 Correlation between PTPRK expression and LOH at 6q22–23 in 29 PCNSLs | }
is too small in this study for statistical analysis of the data. Longer follow-up periods will be required for relapse of more patients. The relapse data, however, does not contradict our impression that 6q deletions have a stronger influence on clinical behavior of PCNSL than on that of systemic lymphomas. In addition, we noticed a tendency toward earlier death in patients with tumors having reduced PTPRK expression, implying a potential prognostic value in tissue PTPRK status and supporting the possible tumor suppressor capacity of the gene.

To recapitulate, we demonstrate that a minimum deletion interval (~140 kb) on chromosome band 6q22-23 is likely to contain a lymphoma-related TSG and, additionally, that PTPRK is a strong candidate. We hope, in light of this and previous studies, that additional work on the genes located at 6q22-23, with more samples of various types of human malignancies, would be done to elucidate their roles in human tumorigenesis. It would be intriguing to investigate, on a thorough and extensive scale, whether silencing of the PTPRK gene may affect the uncontrolled cellular proliferation and invasiveness that takes place during PCNSL tumorigenesis; various genetic and epigenetic mechanisms that could silence this gene need to be scrutinized, including deletions, rearrangements, point mutations, aberrant mRNA splicing, posttranscriptional processing, and methylatation status.

References


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