Allelic Losses of Chromosome 10 in Glioma Tissues Detected by Quantitative Single-Strand Conformation Polymorphism Analysis

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Background: Detection of loss of heterozygosity (LOH) in clinical tissue samples is frequently difficult because samples are usually contaminated with noncancerous cells or because tumor cells in single tissues have genetic heterogeneity, and the precision of available techniques is insufficient for reliable analysis in such materials. We hypothesized that single-strand conformation polymorphism (SSCP) analysis can precisely quantify the gene dosage in mixed samples and is suitable for detection of LOH in clinical tissue samples.

Methods: We assessed the accuracy of a fluorescent SSCP method for the quantification of single-nucleotide polymorphism (SNP) alleles, using DNAs that were composed of cancerous DNA mixed with noncancerous DNA at various ratios. We applied this method to precisely characterize LOH in glioma tissue samples, using 96 SNPs that were evenly distributed throughout chromosome 10.

Results: LOH could be detected even in the cancerous DNA heavily contaminated (up to 80%) with noncancerous DNA. Using this method, we obtained LOH profiles of 56 gliomas with resolution at the SNP level (i.e., 1.5-Mbp interval). Anaplastic astrocytomas exhibited both 10p and 10q LOH, whereas diffuse astrocytomas frequently (63% of the cases) exhibited loss of 10p alone. We also found a possible new LOH region (around 10p13) in gliomas.

Conclusions: The present method is effective for precise mapping of LOH region in surgically obtained tumor tissues and could be applicable to the genetic diagnosis of cancers other than gliomas.

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Loss of heterozygosity (LOH)4 is a common genetic alteration in various kinds of cancer, and its detection is informative for diagnosis and prognosis (1). In brain tumors, combined 1p and 19q LOH is a statistically significant predictor of both chemosensitivity and longer survival in patients with oligodendroglial tumors (2, 3). In general, LOH is a genetic alteration that leads to the inactivation of tumor suppressor genes and is considered to be a critical event in cancer development (4). Thus, mapping of LOH regions is a practical approach to the identification of genes whose loss is related to tumorigenesis (5).

LOH on chromosome 10 is the most frequent genetic alteration in glioblastomas (GBMs), which are the most malignant grade of gliomas according to WHO criteria (6, 7). Investigation of segmental LOH on chromosome 10 in GBMs has revealed at least 3 common LOH regions: 10p14–15, 10q23–24, and 10q25–26 (8–14). It has therefore been suggested that multiple tumor suppressor genes exist on chromosome 10. The PTEN gene at 10q23 has been identified as a tumor suppressor gene in GBM (15, 16). The significance of LOH in the other chromosome 10 regions, however, is still controversial. Thus,
detailed mapping of LOH regions is important to clarify the mechanism of malignant glioma formation.

Although conventional LOH studies have used allelotype with microsatellite markers, these methods are not sufficient for the identification of specific genes because of the low density of the available markers. On the other hand, single-nucleotide polymorphisms (SNPs) are the most abundant form of sequence variations in the human genome (17), and worldwide efforts to collect SNPs have led to the accumulation of millions of these polymorphisms in public databases. Because of their overwhelming abundance in the genome, SNPs can serve as the basis for a high-density LOH analysis method. Many SNP-genotyping methods have been developed, and some of them can be applied to detect LOH (18, 19). A quantitative SNP genotyping method using an oligonucleotide array, called WGSAs, detects LOH with high resolution. The detection rate of LOH, however, is lower for DNA samples extracted from heterogeneous tumor tissue because of contamination by nonmalignant cells (20).

In gliomas, diffuse infiltration is frequently observed independent of histologic grade, and the tumor tissue is not demarcated from noncancerous tissue (21). Tolerance to contamination by noncancerous cells is therefore essential for LOH analysis of glioma samples. We previously developed a SNP-genotyping method, PLACE-SSCP, in which PCR products are postlabeled with fluorescent dyes and labeled products are analyzed in an automated capillary electrophoresis system under single-strand conformational polymorphism (SSCP) analysis conditions (22, 23). In this system, precise quantification of allele frequencies of SNPs is possible with use of pooled DNA samples (24).

In the present study, we explored the effectiveness of PLACE-SSCP for detecting allelic imbalance and developed a new sensitive approach, LOH estimation by quantitative SSCP analysis (LOQUS), to detect LOH at the SNP resolution level. We first evaluated the high sensitivity of this analysis, using a tumor cell line DNA artificially mixed with DNA from noncancerous cells. Subsequently, we analyzed LOH on chromosome 10 in glioma samples and investigated the correlation between the LOH profile and histologic grade. This method revealed a new LOH hot spot at 10p13, which is a putative area that may harbor tumor suppressor genes.

Materials and Methods

SAMPLES AND DNA PREPARATION

Glioma samples were obtained from patients during surgery at Kyushu University Hospital or other affiliated institutions. A part of the tumor tissue was saved for histopathologic examination; the rest was snap-frozen in liquid nitrogen and stored at −80 °C. Tumors were histologically diagnosed by a qualified neuropathologist and graded according to the WHO criteria (21). Tumor tissue samples were collected from 56 patients with gliomas, including 35 GBMs, 8 anaplastic astrocytomas (AAs), 8 diffuse astrocytomas (DAs), and 5 gliomas categorized as WHO grade I (3 pilocytic astrocytomas, 1 pilomyxoid astrocytoma, and 1 ganglioglioma). Among them, 13 GBMs were used in a previous study of LOH analysis with microsatellite markers (25). Tumor DNA was isolated from the frozen blocks by a standard phenol–chloroform extraction procedure. Corresponding wild-type DNA was isolated from a blood sample from the same patient by use of a QIAamp DNA Blood Kit (Qiagen). The present investigation was approved by the Ethics Committee of Kyushu University.

SINGLE-NUCLEOTIDE POLYMORPHISMS

We chose 384 SNPs on chromosome 10 from public databases: JSNP (http://snp.ims.u-tokyo.ac.jp/), TSC (http://snp.cshl.org/), and NCBI dbSNP (http://www.ncbi.nlm.nih.gov/SNP/). The frequencies of the minor alleles for the chosen SNPs were >20% in Japanese or Asians in at least one of the above public databases. Among them, 288 SNPs were mapped to the distal portion of chromosome 10q (10q25-telomeric end), and 96 SNPs were from other regions of chromosome 10. The genomic sequences including the chosen SNPs were downloaded from NCBI (http://www.ncbi.nlm.nih.gov/), and the repetitive sequences were masked by use of Repeatmasker (http://www.repeatmasker.org/). PCR primer pairs for all SNPs were designed for the nonredundant regions by use of Primer3 software (26) to obtain a product of 80 to 300 bp and a standardized primer melting temperature (Tm) of 55–65 °C. Oligonucleotide primer pairs (custom synthesized at SIGMA Genosys, Hokkaido, Japan) were made to carry either 5′-ATT or 5′-GTT for postlabeling purposes, as described previously (22).

PCR

PCR was performed in a total volume of 5 μL containing 25 ng of template DNA, 0.25 μM each of the primers, 0.2 mM each of the nucleotides, 0.125 U of AmpliTaq® DNA polymerase (Applied Biosystems), 27.5 ng of TaqStart™ antibody (Clontech Laboratories), 2 mM MgCl2, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 50 nL/μL dimethyl sulfoxide. The thermal cycle profile was 1 min at 94 °C for initial heating, followed by 40 cycles of 30 s at 94 °C, 30 s at 60 °C, and 1 min at 72 °C.

PLACE-SSCP AND DATA ANALYSIS

Post-PCR labeling was performed, followed by removal of the residual fluorescent nucleotides by gel filtration, as described previously (23). Electrophoresis was performed under SSCP conditions in a 36-cm capillary in the ABI Prism® 3100 genetic analyzer (Applied Biosystems). The samples were dissolved in 0.5 mmol/L EDTA, loaded by electrokinetic injection at 2 kV for 10 s, and separated at 15 kV for 30 min, as described previously (27). Output data
were converted to ASCII format and then imported to Quantitative Interpretation of SSCP in Capillary Array (QUISCA) software for analysis.

DNA FOR MIXING EXPERIMENT
We purchased DNA samples extracted from a non-small lung cancer cell line (NCI-H2126) and its matched non-cancer cell line (NCI-BL2126) from the American Type Culture Collection. The DNA concentrations were determined by use of a PicoGreen ds-DNA quantification assay (Molecular Probes), and paired DNA of equal concentration was obtained by diluting with 0.1 M Tris-EDTA buffer. The tumor DNA was then diluted with NCI-BL2126 DNA of the same concentration in 10% increments.

FLUORESCENT IN SITU HYBRIDIZATION
Imprint preparations of tumor cells were prepared from unfixed snap-frozen samples, as described previously with some modifications (29). In brief, small pieces of cancer tissue were touched against silanized slides, and the slides were fixed 3 times in methanol–acetic acid (3:1 by volume) for 10 min each and subsequently air-dried. Cells on slides were denatured in 700 mL/L formamide in 2× standard saline citrate (SSC; pH 7) at 75 °C for 5 min. After dehybridization in an ethanol series (700 mL/L, 850 mL/L, and absolute ethanol), samples were treated with 0.1 g/L proteinase K (Merck) in phosphate-buffered saline (pH 7) at 37 °C for 3 min, followed by a second dehybridization in ethanol. Hybridization was performed with the LSI PTEN/CEP 10 Dual Color Probe, which is a mixture of a locus-specific probe (LSI) localized on band 10q23 and a subcentromeric probe (CEP) on band 10p11.1-q11.1 (Vysis). The probe mixture was denatured at 73 °C for 5 min and hybridized to the denatured cells on the slides. After cells were covered with an 18 × 18 mm coverslip and sealed with rubber cement, hybridization was allowed to proceed for 2 days. Slides were then washed twice at room temperature for 5 min in 2× SSC containing 3 mL/L NP-40, then at 73 °C for 2 min. After washing, nuclei were counterstained with 0.15 mg/L 4,5-diamino-2-phenyl-indole. Slides were scored for the number of fluorescent signals in each nucleus by use of a Zeiss Axioskop 2 plus fluorescence microscope equipped with a triple-pass filter (Aqua/Green/Orange; Vysis).

MICROSATELLITE ANALYSIS
Tumor and nontumor DNAs were evaluated by a PCR-based LOH assay using 8 microsatellite markers located at regions on chromosome 10 frequently deleted in gliomas (D10S591, D10S1649, D10S1652, D10S1765, D10S587, D10S216, D10S217, and D10S1655). PCR and fluorescence labeling were performed according to a previously described method (25). Capillary electrophoresis was performed with a 310 Prism Genetic Analyzer (Applied Biosystems). Raw electrophoresis data were analyzed with GeneScan Analysis software (Applied Biosystems). Allelic status was assessed based on the criteria established in a previous study (25). Microsatellite instability was determined by the appearance of extra bands on tumor DNA examination.

Results
SELECTION OF SNP MARKERS
We tested the effectiveness of PLACE-SSCP by assessing the LOH status of chromosome 10, which is the site of one of the most frequent genetic alterations associated with malignant gliomas (6, 7). We first chose 384 SNP markers
on chromosome 10 as described in the Materials and Methods, and PCR reactions on tumor and corresponding nontumor DNA were performed for each sequence-tagged site containing a SNP in the same batch. Subsequently, PCR products were divided and used for sequencing and PLACE-SSCP analyses for the same 8 individuals, and these data were interpreted independently. Among the 384 SNPs, a total of 70 (18.2%) were excluded from further analysis because the peak patterns of the SSCP analyses were not interpretable (22 SNPs), separation of allele peaks of heterozygous samples was insufficient for quantitative SSCP analysis (28 SNPs), or there was no heterozygote sequenced among the 8 individuals (20 SNPs). From the remaining 314 SNPs, we excluded SNPs with a minor allele frequency < 30% in the 8 individuals. The 96 SNPs that distributed evenly throughout chromosome 10 (marker intervals ~ 1.5 Mbp) were used for subsequent analyses.

Fig. 3. LOQUS assay of glioma tissue samples. (A), LOQUS assay of a GBM (GB31) tissue, plot of $V_T$, showing 10q LOH. Microsatellite analysis data confirmed 10q LOH, as shown by □, □, and □ for allelic imbalance, noninformative, and retained heterozygosity, respectively, on the right. (B), LOQUS assay of an AA (AA27) tissue, revealing LOH at 10q21–26 although $V_T$ values were high (0.7–0.8). (C), LOQUS assay of a GBM (GB35) tissue sample. Two distinct LOH regions are revealed: one, on 10p15–10q21, showing high $V_T$ (around 0.5–0.6), and the other, on 10q21–26, showing low $V_T$ (around 0.1–0.2). The vertical dotted line in each panel indicates the threshold for LOH detection (0.83).
The reproducibility of peak-height ratios of alleles in nontumor cells from persons heterozygous for SNPs is crucial for the sensitivity of LOH detection by PLACE-SSCP. The accuracy of the estimation of relative allele abundance in the samples, which in turn depends on the reproducibility of peak-height ratios of alleles in either nontumor cells from individuals heterozygous for the SNP being evaluated or in tumor samples. We defined \( R_{Nj} \) as the height ratio of the peak for the first allele peak to the peak for the second allele peak in nontumor DNA from individuals heterozygous for each SNP. For each SNP, PCR reactions from the same DNA were performed in 2 wells of the same microtiter plate, and 2 \( R_{Nj} \) values (\( R_{N1j} \) and \( R_{N2j} \)) were obtained for all SNPs in nontumor cells showing heterozygosity. We analyzed the 96 SNPs, using nontumor DNA from 8 heterozygous individuals, and determined the variability of the peak-height ratio obtained for the same nontumor DNA (\( V_N \)) by \( R_{N1j}/R_{N2j} \) for all SNPs showing heterozygosity (326 determinations). The mean (SD) \( V_N \) was 1.000 (0.057), with a CV of 5.7%, and the data were fitted to a gaussian distribution by use of the Shapiro–Wilk \( W \) test with JMP software (Ver. 5.0.1; SAS Institute; http://www.sas.com/; Fig. 1) (30). The test revealed a gaussian distribution (\( W = 0.9626 \)) with high significance (\( P < 0.0001 \)). We define \( V_T \) as an indicator of the fraction of the DNA with allelic imbalance in the tumor sample, where \( R_T \) is the peak height ratio of the tumor sample (see Fig. 2 of the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol52/issue3/). We thus conclude that \( V_T < 0.83 \) (3 SD from the mean) can be regarded as an LOH at a confidence level of \( > 99.7 \% \) (according to the probability density of the gaussian distribution). We adopted this criterion as an indication of LOH in the LOQUS assay described below.

Mixed DNA Experiment

Glioma tissue samples frequently show heterogeneity or infiltration by noncancerous cells. To further estimate the sensitivity of LOQUS to detect the allelic status in such situations, we performed reconstitution experiments in which a pair of DNA samples from tumor and healthy tissues of the same individual were mixed at various ratios and subjected to LOQUS analyses. The DNA samples for this experiment were obtained from a lung cancer cell line (NCI-H2126) and its matched noncancer cell line DNA (NCI-BL2126), in which LOH was detected in all informative microsatellite markers examined on chromosome 10 (31). Among the 96 SNPs, SSCP analysis of NCI-BL2126 indicated heterozygosity for 22. The LOQUS assay performed with these SNPs indicated that \( V_T \) increased proportionally in response to an increase in nontumor DNA (Fig. 2). At a mixing ratio of 20 (i.e., 20\% tumor DNA and 80\% nontumor DNA), LOH was detectable in 95\% of the examined SNPs (21 of 22), and above
this ratio (>20% tumor DNA), LOH was detected with every SNP. Even at a 10% mixing ratio, LOH was still detectable for 10 of 22 SNPs (46%). These results indicate that the LOQUS assay can tolerate a mixed sample contaminated with up to 80% noncancer DNA.

**LOQUS ASSAY IN Glioma Tissue Samples**

We performed the LOQUS assay with glioma tissue samples. The result of examination of a GBM sample (GB31), in which LOH of the entire 10q was detected, is shown in Fig. 3A. This LOH was confirmed by both microsatellite markers and fluorescence in situ hybridization analyses (see Fig. 1 in the online Data Supplement). We could also detect LOH on 10q in samples for which the $V_T$ values were in the range 0.7–0.8 (Fig. 3B). The results obtained for an AA sample (AA24), in which a microdeletion measuring <250 kbp was detected by narrowing the region of analysis by use of densely located SNPs, are shown in Fig. 4.

**LOH Profiles of Chromosome 10 in Gliomas**

We analyzed 56 glioma samples, and the mean number of informative loci was 40.91 (42% of examined SNPs). LOH profiles of examined samples are shown in Fig. 3 of the online Data Supplement. The majority of GBMs (68%) had LOH at all informative loci, and were therefore interpreted to be a monosomy of chromosome 10. In contrast, AAs and low-grade gliomas showed no such LOH pattern, except for 1 AA sample. LOH on 10q was observed in all of the AA cases, and their LOH regions included multiple loci. On the other hand, all LOH on 10q observed in DAs and grade I gliomas involved loss of a single region. LOH on 10p was observed in 63% of DAs and AAs, whereas none of the grade I gliomas had LOH in this chromosome region. As for DAs, LOH on 10p included multiple loci, in contrast to LOH on 10q.

**Identification of an LOH Hot Spot at 10p13**

Among the 56 gliomas, the LOH ratio (ratio of samples with LOH to all informative samples) of 96 SNPs varied from 40% (rs724444 at chromosome position 77 558 207 bp) to 77.8% (rs726451 at chromosome position 122 403 971 bp). On the basis of the LOH ratio plot shown in Fig. 5, we identified 3 LOH hot spots: 10p13–15, the PTEN region at 10q23, and 10q25–26. The latter 2 regions were consistent with the previously reported commonly deleted lesions in malignant gliomas (8, 11, 12). The first deletion hot spot overlapped the commonly deleted region (10p14–15) reported in other studies (9, 10, 14), but somewhat extended to the centromeric side. We therefore focused on the 10p LOH samples. To identify the minimal 10p LOH region, we compared 40 samples with at least one LOH locus on 10p: 30 GBMs, 5 AAs, and 5 DAs (Fig. 6). We found that 2 samples (GB9 and DA23) had an interstitial LOH localized at the centromeric portion of 10p13, and all informative samples showed LOH of rs1376690 at chromosome position 15 720 079 bp.

We are currently performing investigations to identify genes possibly involved in the development of gliomas.

**Discussion**

Traditionally, LOH has been estimated by detecting allelic imbalance by use of microsatellite markers. The use of SNP markers for LOH detection has the advantage of much higher resolution because SNPs are available at an overwhelmingly higher density. The results are also more reliable because SNPs are more stable markers than microsatellites in vivo and microsatellite instability during tumor evolution or formation is avoided. Slippage during PCR and the appearance of stutter peaks are frequently encountered problems of microsatellite analysis, which does not exist for SNP markers.

We demonstrated high reproducibility (CV = 5.7%) of the signal intensity ratio of SNP alleles in the analysis of DNA from samples heterozygous for the SNPs studied in the LOQUS analysis. This reproducibility is comparable to that of the bacterial artificial chromosome–based comparative genomic hybridization array (32, 33) and better
than the reproducibility of other methods such as multiplex ligation-dependent probe amplification analysis (34, 35), multiplex amplification and probe hybridization analysis (36), and SYBR Green I–based real-time PCR analysis (30).

Recently, oligonucleotide microarray analysis was applied to detect LOH at the SNP level (19, 37). As an advantage of this method, both LOH and the copy number abnormality profile can be determined by single-platform analysis. Although oligonucleotide microarrays allow high-throughput analysis, their use requires a relatively high proportion of tumor DNA to detect LOH.

When contamination by noncancer DNA reaches 30%–50% of the total, there is a significant loss of detection of LOH by the microarray analysis (20). In contrast, the reproducibility of the peak-height ratio for SNP alleles obtained with the LOQUS method is high, as shown in Fig. 1, and this method can tolerate a mixed sample with up to 80% contamination by DNA from noncancerous cells and still detect an allelic imbalance. For specimen with a low percentage of tumor cells, further experiments, e.g., microdissection to enrich tumor cells, should provide more convincing results on the determination of LOH status.

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The Ward method (40) was used for hierarchical clustering of samples by use of JMP software. The samples with LOH at all informative loci, which were interpreted to be a monosomy of chromosome 10, or retention of heterozygosity at all informative loci, were excluded. SNP markers are shown on the left. Case numbers are indicated at the top. ■, LOH; □, retention of heterozygosity; □□, not informative (homozygous individual or not determined).
Contamination by nontumor cells in tumor tissue samples is inevitable because contribution of ancillary cells such as fibroblasts and endothelial cells is essential for tumor maintenance, and they frequently infiltrate the tissue (38). Although methods such as culturing or laser capture microdissection have been used to enrich tumor cell content, obtaining pure tumor cell populations remains difficult. Furthermore, based on the clonal multistep tumor evolution theory, tumor cells do not have a uniform genetic change in clinical tumor tissues (39). The LOQUAS assay also can detect the heterogeneity of tumor cells, as shown in Fig. 3C, which revealed 2 distinct LOH regions showing high and low $V_T$. These results demonstrate the robustness of our method in the examination of clinically obtained tumor tissue samples, which are often mixed with an excess of healthy cells or consist of a heterogeneous population of 2 or more malignant cell types.

Other advantages of our method, as well as other targeted methods such as multiplex ligation–dependent probe amplification or multiplex amplification and probe hybridization assay, over microarray-based genome-wide methods are flexibility of experimental design and the cost of analysis. LOQUAS analysis requires only widely available instrumentation, i.e., a conventional PCR instrument and capillary sequencer. The throughput of this system is low compared with other methods, e.g., microarray-based techniques. Use of the ABI Prism® 3100 genetic analyzer, which is the most appropriate instrument for PLACE-SSCP (27), enables analysis of 96 loci in 1 day, including PCR steps. However, PLACE-SSCP has the advantage of flexibility, e.g., additional high-density investigations could be done simply by selecting additional SNPs from public databases and designing the appropriate PCR primers. The recent enhancement of public databases allows selection of SNPs with high heterozygosity, which is essential for efficient analysis of allelic imbalance using the present system.

The LOH profiles in the present study indicated the following: (a) The majority of the GBMs had complete LOH of chromosome 10. (b) AAs frequently had partial LOH on both 10p and 10q. (c) DAs also frequently had 10p LOH, but seldom had 10q LOH. The progression of astrocytoma is associated with an increased loss of 10p and 10q sequences, probably reflecting the increased involvement of tumor suppressor genes (14). Our findings are consistent with these observations and, in addition, suggest that 10p LOH has a less malignant effect compared with 10q LOH in glioma formation or progression.

According to previous microsatellite analyses of astrocytic gliomas with different malignancy grades, 10p LOH is frequently observed in a subpopulation of tumor cells (9). Such subtle genetic abnormalities are more reliably detected by a highly sensitive method such as LOQUAS. Moreover, the higher frequency of 10p LOH in DAs detected in this study (63%) compared with previous reports (0%–35%) (9, 10, 14) might be attributable to the high sensitivity of the present method.

The reported regions on 10p commonly deleted in gliomas are concentrated at 10p14–15 (9, 10, 14). We identified an additional deletion hot spot at 10p13, however, suggesting that 10p13 is an additional putative area that might harbor previously undiscovered tumor suppressor genes. Further efforts are needed to identify genes at deletion hot spots on chromosome 10, including 10p13, to elucidate the significance of LOH in chromosome 10 regions in gliomas.

References


