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DETECTION OF STRUCTURAL ABERRATIONS OF CHROMOSOME 17 IN MALIGNANT GLIOMAS BY FLUORESCENCE IN SITU HYBRIDIZATION (FISH)

TAESHIK KWAK, TAKAFUMI NISHIZAKI, HISATO NAKAYAMA, SYUNNSUKE KAKINO, HARUHIDE ITO and YUKA KIMURA

Isolated nuclei prepared from fresh-frozen tissue specimens were used to investigate structural aberrations of chromosome 17 in 18 malignant gliomas (7 anaplastic astrocytomas, 11 glioblastomas). Nuclear DNA was hybridized with four DNA probes specific for centromeric (D17ZI), telomeric (Tel 17p, Tel 17q) and 17p 13.1 sequences (p53) of chromosome 17, using fluorescence in situ hybridization (FISH). The rates of nuclei with one signal (OS) for Tel 17p and p53 were significantly higher than those for D17ZI and Tel 17q. This indicated that the majority of chromosome 17 aberrations was a deletion of the short arm including the p53 gene. When compared with the histological grading, the rates of OS for Tel 17p and p53 in anaplastic astrocytomas were higher than those of glioblastomas, suggesting that the deletion may be associated with the early events in tumorigenesis and that some glioblastomas without chromosome 17 aberrations may be independent from tumour progression via low-grade gliomas.

Tumorigenesis in gliomas, is associated with multiple genetic aberrations (1, 2). The allelic loss of the short arm of chromosome 17 (17p) is most frequently seen in gliomas by means of restriction fragment length polymorphism (RFLP) analysis (3). This chromosomal aberration might include deletion or rearrangement of chromosome 17p, mitotic recombination with balanced interchromosomal exchange and chromosome 17 loss with duplication (4, 5). However, such genetic information is derived from analysis of total DNA and it is limited to a description of the average characteristics of the tumor, and therefore provides little information about multiple clones within a tumor.

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From the Department of Neurosurgery (T. Kwak, T. Nishizaki, H. Nakayama, S. Kakino, H. Ito) and Pathology (Y. Kimura), Yamaguchi University School of Medicine, Kogushi Ube-shi, Yamaguchi, Japan.
Correspondence to: Dr Taeshik Kwak, Department of Neurosurgery, Yamaguchi University School of Medicine, 1144 Kogushi-Ube-shi, Yamaguchi 755, Japan.

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Fluorescence in situ hybridization (FISH) using probes to selected DNA sequences is a powerful technique for studying genetic aberrations within individual interphase tumor cells (6). This technique allows the detection of multiple clones with genetic changes. FISH is useful in studying chromosomal mechanisms leading to allelic loss. Structural and numerical aberrations in solid tumors have been detected by FISH (7–10). The chromosomal region 17p13.1 is the site of the p53 tumor suppressor gene. The gene is inactivated by a point mutation in one allele and a deletion or recombinational loss of the other, which is associated with the progression of glioma and other cancers (11, 12). Moreover, another tumor suppressor gene on chromosome 17p has been predicted (13).

The DNA probes used in the present study were specific for centromeric, telomeric and 17p13.1 sequences, so that structural aberration of chromosome 17 in malignant gliomas could be investigated.

Material and Methods

Cells. Eighteen tumors were obtained from patients by surgical excision and they consisted of 7 anaplastic astro-

cytomas (WHO grade III) and 11 glioblastoma multiforms (WHO grade IV). The fresh tissue was frozen at −70°C until use. The frozen specimens were defrosted and minced with scissors in phosphate-buffered saline (PBS), filtered through a nylon mesh. The following procedure was performed as described by Pinkel et al. (14) and Kuo et al. (15). The cells were centrifuged, resuspended in 75 mM KCl solution at 37°C for 10 min, fixed in three changes of methanol:acetic acid (3:1), dropped onto slides and air-dried. Cultured lymphocytes stimulated with phytohemagglutinin (PHA) and non-neoplastic brain tissue were used as controls.

DNA probes. The D17ZI (Oncor, Gaithersburg, MD) was used as a biotin-labeled DNA probe for human alpha satellite DNA on chromosome 17, the p53 cosmid probe (Oncor) for 17p13.1 (immediately distal to the p53 gene), the Tel 17p cosmid (Oncor) for the most distal region of the chromosome 17p, the Tel 17q cosmid (Oncor) for the most distal region of the chromosome 17q. These cosmid probes were mixed with blocking DNA in 50% formamide, 2 × SSC.

In situ hybridization. Hybridization proceeded, as previously described, with modifications (14). The target DNA in the cells was denatured in 70% formamide, 2 × SSC (pH 7) at 70°C for 2 min then dehydrated in a 70%/85%/100% ethanol series. The hybridization mixture (10 μl total volume), consisting of 0.5 μg herring sperm DNA (sonicated to ~500 bp) and 10 ng alpha satellite DNA probe was denatured at 70°C for 5 min and then cooled on ice. The cosmid probe mix was incubated at 37°C for 5 min. The probe mixture was applied to a warmed slide and hybridized overnight under a coverslip at 37°C. The slides were washed three times in 50% formamide and 2 × SSC at 45°C for 5 min each and then in PN buffer (a mixture of 0.1 M NaH2PO4, 0.1 M Na2HPO4 and 0.1% Nonidet P-40, pH8) at 0°C for 3 min. The hybridized probes were detected by incubating the slides in FITC-conjugated avidin (5 μg/ml, Vector Laboratories, Burlingame, CA) followed by biotinylated goat anti-avidin antibody (5 μg/ml, Vector Laboratories) in PNM buffer (5% non-fat dried milk and 0.02% sodium azide in PN buffer) for 20 min each at room temperature. After further staining steps, the slides were washed three times in PN buffer for 10 min each. The DNA was counterstained with 2 μg/ml propidium iodide in an anti-fade solution.

Fluorescence microscopy. The number of signals per nucleus was counted in 100 nuclei per slide and the percentage of nuclei with one signal (OS) was determined. The evaluation criteria followed those detailed proviously (16). Nuclei with no signal were regarded as not reacting with probes and were excluded. When the rate of these nuclei exceeded 15%, the hybridization was repeated. Weak signals (minor hybridization site) were ignored and split signals were counted as one. The rate of OS among the four probes or between two WHO grades were analyzed by the Mann-Whitney test.

Table

The histological grade and rate of nuclei with one signal (OS)

Patient No.	Grade	Rate of OS(%) Tel 17p	p53	D17ZI	Tel 17q
Malignant gliomas					
1	III	33	38	11	17
2	III	49	53	50	42
3	III	50	50	62	60
4	III	46	48	23	28
5	III	46	43	10	18
6	III	46	45	18	22
7	III	20	20	21	8
8	IV	26	14	20	30
9	IV	42	45	12	16
10	IV	45	42	6	18
11	IV	23	26	23	14
12	IV	44	25	18	6
13	IV	29	40	5	18
14	IV	34	30	24	14
15	IV	30	38	42	14
16	IV	30	20	8	12
17	IV	33	30	12	15
18	IV	26	28	18	14
PHA-stimulated lymphocytes		6	20	20	8
Normal brain cells		18	20	30	16

Results

Photomicrographs obtained after the FISH procedure are shown in Fig. 1. The histological grade and rate of OS are demonstrated in the Table. The rate of OS in normal brain cells was 18.0 for Tel 17p, 20.0 for p53, 30.0 for D17ZI and 16.0 for Tel 17q. In all glioma specimens, the rate of OS for Tel 17p was 36.6 ± 2.4 (standard error of mean), 35.1 ± 3.0 for p53, 22.3 ± 4.3 for D17ZI and 21.2 ± 3.6 for Tel 17q. The rates of OS are plotted with

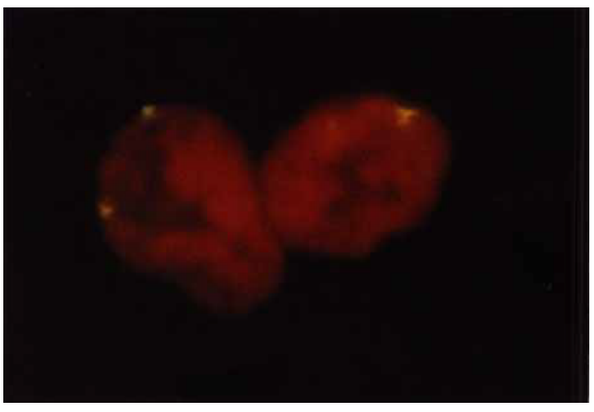

Fig. 1. Photomicrograph showing FISH using the p53 cosmid probe of isolated glioma cells (patient No. 10) (×400).

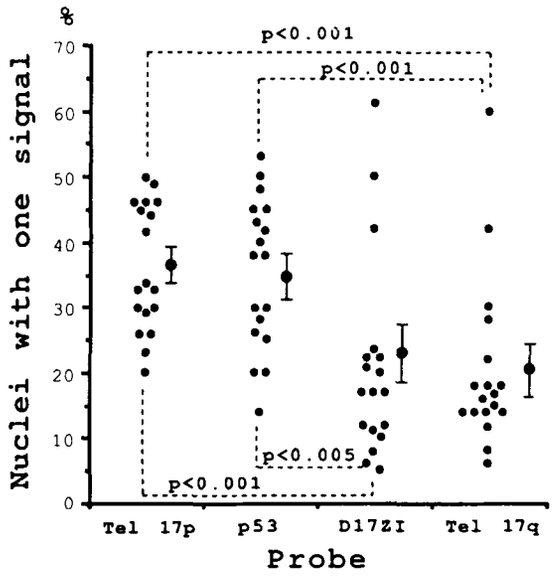

Fig. 2. Rate of nuclei with one signal (OS) with respect to the four probes specific for chromosome 17 in malignant gliomas. The rates of OS for Tel 17p and p53 were significantly higher than those for D17ZI and Tel 17q.

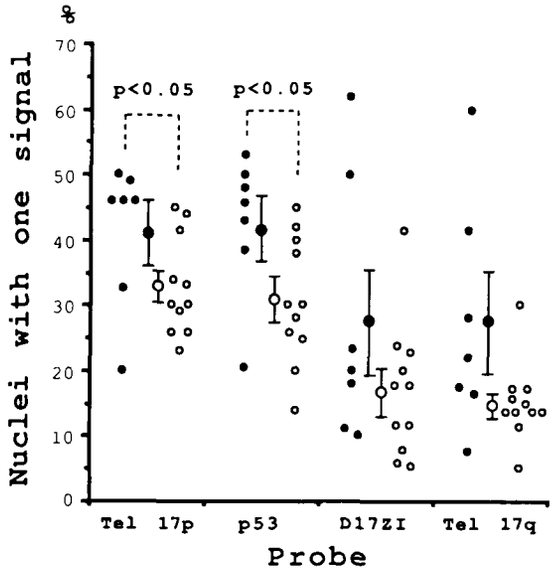

Fig. 3. Rate of nuclei with one signal (OS) with respect to the four probes specific for chromosome 17 in anaplastic astrocytomas (●) and glioblastomas (○). The rates of OS for Tel 17p and p53 in anaplastic astrocytomas were higher than those of glioblastomas ($p < 0.05$ and $p < 0.05$ respectively).

respect to the four probes in order to investigate the major patterns of chromosome 17 structural aberrations (Fig. 2). The rates of OS for Tel 17p and p53 were significantly higher than those for D17ZI ($p < 0.001$ and $p < 0.005$ respectively) and Tel 17q ($p < 0.001$ and $p < 0.001$ respectively). However, some specimens had a different pattern of chromosome 17 structural aberrations. For example, specimens from patients 2 and 3 had a high frequency of OS for all probes. That from patient 12 had high frequency of OS for only Tel 17p.

When compared with the histological grade, the rates of OS in anaplastic astrocytomas were 41.4 ± 4.5, 42.4 ± 4.5, 27.9 ± 8.2 and 27.9 ± 7.2, whereas in glioblastomas they were 32.9 ± 2.5, 30.7 ± 3.4, 17.1 ± 3.7 and 15.5 ± 1.8, for Tel 17p, p53, K17ZI and Tel 17q respectively. The rates of OS for D17ZI and Tel 17q in anaplastic astrocytomas were not significantly different from that in glioblastomas, but the rates of OS for Tel 17p and p53 in anaplastic astrocytomas were greater than that in glioblastomas (Fig. 3, $p < 0.05$ and $p < 0.05$ respectively).

Discussion

By microscopic analysis of FISH, pairing of two signals may be identified as the single spot; this resulted from so-called 'somatic pairing' phenomenon (17). This phenomenon cannot be ignored in evaluating the loss of target sites using FISH. Moreover, loss of target DNA during hybridization procedure or inaccessibility to probes will decrease signal number. In our study, 6–30% of normal brain cells displayed monosomy for the different DNA probes, which was probably caused by these factors. Therefore, we compared the rates of nuclei with one signal for four regions on chromosome 17 in order to evaluate the true loss of target sites.

El-Azouzi et al. (18) reported that the allelic loss of chromosome 17p was due to a deletion of the region in 2 of 5 gliomas. James et al. (19) reported that an allelic loss of chromosome 17p in gliomas most frequently involves mitotic recombination. In the present study, the statistical frequency of one signal indicates that a simple deletion of chromosome 17p, including the p53 region, is one mechanism of the allelic loss in gliomas. This mechanism of chromosome 17p loss has been implied in other solid tumors (20). In one sample (patient No. 12), the increased signal for Tel 17p compared with 17p13 indicates that the breakpoint is distal to p53 (13). A second tumor suppressor gene or other mechanisms than loss of tumor suppressor gene are possible. In addition, in 2 samples (patients Nos 2 and 3), more than 50% nuclei were monosomic for all four probes, suggesting the non-disjunctional loss of a total chromosome.

A cytogenetic study using RFLP analysis has shown that the allelic loss of chromosome 17p is associated with gliomas of all grades (11, 13). Our results also indicated a deletion of chromosome 17p in both anaplastic astrocytomas and glioblastomas. However, anaplastic astrocytomas had a significantly increased frequency of chromosome 17p deletion compared with glioblastomas. Fults et al. (3) reported frequent allelic loss of chromo-

some 17p in anaplastic astrocytoma compared with glioblastoma (6 of 10 versus 7 of 22) and our data agree with their results. Von Deimling et al. (21) reported that there were at least two glioblastoma types with different genetic pathways: one arises through progression from low-grade gliomas with loss of chromosome 17p, whereas the other arises de novo without loss of chromosome 17p. Our results indicate that deletion of chromosome 17p was more associated with anaplastic astrocytomas than with glioblastomas, suggesting that the deletion may be associated with early events in tumorigenesis and that some glioblastomas without chromosome 17 aberrations may be independent from tumor progression through low-grade gliomas.

We tried to investigate structural aberrations of chromosome 17 in human gliomas using statistical comparison. If instead we would use multicolour FISH, we might be able to hybridize with two or more probes simultaneously and perhaps be able to outline the individual rearrangements in each cell. Moreover, if the signal intensity of hybridized probes is quantified using flow-cytometry, more accurate evaluation of the signal number may be possible, without being affected by 'somatic pairing' (22). We conclude that FISH is a powerful technique for detecting deletions of specific genes in solid tumor.

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