Mutation of the *p53* gene in human astrocytic tumours correlates with increased resistance to DNA-damaging agents but not to anti-microtubule anti-cancer agents

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Summary Astrocytic tumours often become resistant to a variety of chemotherapeutic agents in advanced stages and frequently possess mutations in the *p53* tumour-suppressor gene. Previous studies using established cell lines to investigate the relationship between mutated *p53* genes and altered resistance to anti-cancer agents brought inconsistent results. In this report, we examined the status of the *p53* gene in 56 astrocytic tumour specimens by single-strand conformation polymorphism and their in vitro chemosensitivity to 30 different kinds of anti-cancer agents. The chemosensitivity was determined by drug-induced cell death using flow cytometry. We found that the mutated *p53* gene correlated with increased resistance to DNA-damaging agents but the sensitivity to anti-microtubule agents was independent of the mutation, suggesting a clinical significance of the status of *p53* gene in astrocytic tumours and a rational application of anti-microtubule agents to the patients with *p53*-mutated astrocytic tumours.

Keywords: astrocytic tumour; *p53*; chemosensitivity; microtubule; DNA damage

The *p53* gene is a tumour-suppressor gene that is frequently mutated in various human cancers, including astrocytic tumours which make up more than 60% of primary brain tumours. The 5-year survival rate of the most malignant type, glioblastoma multiforme, is less than 5% (Walker et al, 1980), and this devastating outcome is ascribed to marked resistance to chemotherapy and radiotherapy. Wild-type *p53* gene product functions as a checkpoint control protein and is responsible for G1 arrest that is observed at the time of DNA damage (Lane, 1992). The inhibition of the cell cycle after DNA damage allows more time for DNA repair, however, if optimal repairs are not accomplished *p53* can trigger a putative apoptotic pathway and eliminate damaged cells. Recent studies have demonstrated that DNA-damaging stimuli elevated the intracellular *p53* protein level (Kasten et al, 1991) and that the induction of apoptosis by chemotherapeutic agents was affected by the status of the *p53* gene (Lowe et al, 1993; Fujiwara et al, 1994). Consequently, tumour cells lacking functional *p53* protein become resistant to a variety of chemotherapeutic agents. Several lines of study have supported this notion (Ass et al, 1996; Perego et al, 1996). In contrast, increased sensitivity to paclitaxel or cisplatin was observed in primary or non-transformed cells without functional *p53* protein (Hawkins et al, 1996; Wahl et al, 1996). This discrepancy may be attributable to alterations in genes other than the *p53* gene and/or cell type specificity. In addition, the action mechanism of anti-cancer agents is also a crucial factor influencing chemosensitivity.

In this study, we investigated a possible relationship between the status of the *p53* gene in clinical specimens from astrocytic tumours and their chemosensitivity, with reference to the mechanism of pharmacological action. Such an approach using a number of clinical samples can randomize the effect of cell type differences derived from undetermined genetic alterations, and allows us to explore the relationship in human tumours.

MATERIALS AND METHODS

Tumour samples

All the specimens, aseptically obtained from 56 patients, were reviewed by several neuropathologists and they were diagnosed as astrocytic tumours according to the WHO classification. They included seven cases of grade II, ten cases of grade III (anaplastic astrocytoma) and 39 cases of grade IV (glioblastoma multiforme). The specimens were processed both for in vitro chemosensitivity test and for DNA extraction.

Genomic DNA amplification and single-strand conformation polymorphism (SSCP) of *p53* gene

Genomic DNA was extracted from each sample and the primers used for polymerase chain reaction were the same as reported previously (Murakami et al, 1991) except exon 7, for which 5’-TGCCACAGGTCTCCCAAGG-3’, 5’-TATGGAAGAAAATCGGTAAAA-3’ were used for sense and antisense primers respectively. Amplification of DNA was performed as described previously (Murakami et al, 1991), and the products were subjected to electrophoresis in a 5% polyacrylamide gel.

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Chemosensitivity test of brain tumours with flow cytometry

Surgically resected tumour cells were minced and suspended in RPMI-1640 medium with 10% fetal calf serum. The viability in each preparation was examined using a dye exclusion test and was constantly over 95%. An aliquot of the cell suspension was incubated individually with 30 different agents classified as six types: 4-hydroperoxycyclophosphamide (CPM) (10 μg ml⁻¹), 4-hydroperoxycyclophosphamide (IFOs) (10 μg ml⁻¹), melphalan (MPL) (0.5 μg ml⁻¹), carboquone (CQ) (0.1 μg ml⁻¹), nimustine (ACNU) (2 μg ml⁻¹) and ranimustine (MCNU) (2 μg ml⁻¹) as alkylating agents; methotrexate (MTX) (3 μg ml⁻¹), 5-fluorouracil (5-FU) (10 μg ml⁻¹), thiostine (4-MPR) (3 μg ml⁻¹) and cytosome arabinoside (CA) (4 μg ml⁻¹) as anti-metabolites; mitomycin C (MMC) (0.2 μg ml⁻¹), bleomycin (BLM) (1 μg ml⁻¹), peplomycin (PEP) (0.5 μg ml⁻¹), chlomomycin A3 (TM) (0.01 μg ml⁻¹) and neocarzinostatin (NCS) (0.15 μg ml⁻¹) as antibiotics; actinomycin D (ACD) (0.01 μg ml⁻¹), aclacrubin (ACR) (0.6 μg ml⁻¹), doxorubicin (DOX) (0.3 μg ml⁻¹), daunomycin (DM) (0.6 μg ml⁻¹), pirarubicin (THP) (0.3 μg ml⁻¹), epirubicin (4'-EPI) (0.4 μg ml⁻¹), mitoxantrone (MIT) (0.06 μg ml⁻¹), etoposide (VP-16) (3 μg ml⁻¹) and camptothecin (CPT-11) (2 μg ml⁻¹) as topoisomerase inhibitors; cisplatin (CDDP) (0.5 μg ml⁻¹) and carboplatin (JM-8) (4 μg ml⁻¹) as platinum agents; vincristine (VCR) (0.1 μg ml⁻¹), vinblastine (VLB) (0.1 μg ml⁻¹), vindesine (VDS) (0.1 μg ml⁻¹) and paclitaxel (TAX) (0.6 μg ml⁻¹) as anti-microtubule agents. Each concentration tested corresponded to one-tenth of the peak plasma concentration of clinically recommended doses. Cells were incubated with each agent at 37°C in 5% carbon dioxide for 8 h, and then cultured in fresh RPMI-1640 medium for 72 h. They were mixed in phosphate buffered-saline pH 7.2, 0.1% Triton X-100, 0.1 mg ml⁻¹ RNAase, 0.01% sodium azide for 15 min, and were incubated with 100 μg ml⁻¹ propidium iodide (Sigma, St Louis, MO, USA) for 5 min. Isolated nuclei were analysed with a flow cytometer (FACScan: Becton Dickinson, Mountain View, CA, USA) (Nicoletti et al, 1991; Iwadate et al, 1997). Our previous study showed that the results obtained with the concentration used in this assay could represent the data from various concentrations of the agents (Iwadate et al, 1997).

Statistical analysis

Statistical analysis was performed with Fisher's exact probability test, Mann–Whitney test or Mantel–Haenszel χ² test.

RESULTS

The status of the p53 gene of astrocytic tumours was examined by SSCP on amplified genome DNA products. Among 56 samples tested, we detected altered electrophoretic mobility in 23 samples (41%), which included six cases of dual mutations (Table 1). The frequency of the mutated p53 gene in grade II astrocytic tumours was relatively great compared with that of grade III or IV cases without statistical significance (P = 0.09, Fisher's exact probability test). The overall mutation rate in astrocytic tumours matched with previous reports (Lang et al, 1994), but the reason for the high mutation rate in grade II cases is currently unknown.

We also examined the chemosensitivity of the specimens to 30 different anti-cancer agents. The agents used here are clinically

| Number of patients | Status of the p53 gene |
|--------------------|------------------------|
| II | III | IV |
| Wild-type | 2 | 8 | 23 |
| Mutated | 5 (71%) | 2 (20%) | 16 (41%) |
| Exons 5 and 6 | 1 | 1 | 6 |
| Exon 7 | 5 | 2 | 11 |
| Exons 8 and 9 | 2 | 0 | 1 |

in use and classified into six types based on the mechanism of action (see Materials and methods). The chemosensitivity was assayed by flow cytometrical analysis after staining with propidium iodide (Figure 1). The technique enables us to detect nuclear degradation that appears as a hypodiploid peak and represents DNA fragmentations induced by apoptotic process (Nicoletti et al, 1991). We judged the agent as effective in cases in which more than a 70% reduction of the integrated diploid peak compared with that of untreated control cells was observed (Iwadate et al, 1997). The increase in the hypodiploid area corresponded to the decrease in the number of live cells. Our previous dose–response experiment showed that the result obtained by the present method using a single dose point could represent the data accumulated using different concentrations of the agents (Iwadate et al, 1997).

The number of agents judged as effective varied among each sample tested, but the mean percentage of effective agents in the mutated and the wild-type groups of the p53 gene was 4.2 ± 1.1% (s.e.) and 17 ± 2.2% respectively (Table 2). Thus, the mutation in the p53 gene confers increased resistance to chemotherapeutic agents with statistical significance (P < 0.01, Mann–Whitney test).

We analysed the relationship between the susceptibility to each agent and the status of the p53 gene based on the mode of pharmacological action (Table 2). Although the number of effective agents varied among the specimens, the percentage of effective agents that belong to DNA-damaging types (alkylating agents, anti-metabolites, antibiotics and topoisomerase inhibitors) was higher in the wild-type than in the mutated cases (P < 0.01 in every type, Mantel–Haenszel χ² test), regardless of the nature of compounds. In the case of platinum agents, another DNA-damaging type, the statistical significance was not proved (0.05 < P < 0.06), Mantel–Haenszel χ² test; however, we observed the same tendency as in the high number of effective agents in the wild-type. Consequently, all the DNA-damaging agents tested, including platinum agents, were less effective in the p53-mutated cases (3.0 ± 1.0%) than in the wild-type p53 cases (18 ± 2.4%) (P < 0.01, Mann–Whitney test). In contrast, the percentage of effective agents that act on microtubules was not different between the cases (12 ± 2.7% for p53-mutated cases and 8.3 ± 3.1% for wild-type cases). Among the mutated cases, the percentage of effective drugs that belong to anti-microtubule agents was higher than that to other types (12 ± 2.7% vs 3.0 ± 1.0%, P < 0.01, Mann–Whitney test). Thus, these data collectively suggest that the mutation in the p53 gene confers resistance to DNA damage-based agents but not to anti-microtubule agents.
Figure 1  A representative flow cytometrical analysis of specimens with mutated p53 (A) or wild-type p53 gene. Sample A was resistant to all the agents except TAX, whereas sample B was sensitive to CPM, BLM, ADM but not to CA, CDDP and TAX. The abscissa represents fluorescence intensity and the ordinate represents cell number. Abbreviations of drug names are explained in Materials and methods.
Table 2  In vitro chemosensitivity of astrocytic tumour patients to various anti-cancer agents

| Agents                  | Mutated p53 (n = 33) (per cent of effective agents ± s.e.) | Wild-type p53 (n = 33) (percent of effective agents ± s.e.) |
|-------------------------|-------------------------------------------------------------|-------------------------------------------------------------|
|                         |                                                               |                                                             |
| Alkylating agents       |                                                             |                                                             |
| CPM                     | 0.00                                                        | 9.00                                                        |
| IFOS                    | 1.00                                                        | 9.00                                                        |
| MPL                     | 0.00                                                        | 2.00                                                        |
| CQ                      | 0.00                                                        | 1.00                                                        |
| ACNU                    | 0.00                                                        | 2.00                                                        |
| MCNU                    | 2.00                                                        | 4.00                                                        |
| Subtotal                | (2.2 ± 1.5%)                                                | (14 ± 4.5%)                                                 |
| Anti-metabolites         |                                                             |                                                             |
| MTX                     | 1.00                                                        | 3.00                                                        |
| 5-FU                    | 0.00                                                        | 2.00                                                        |
| 6-MPR                   | 0.00                                                        | 4.00                                                        |
| CA                      | 0.00                                                        | 13.00                                                       |
| Subtotal                | (1.1 ± 1.1%)                                                | (17 ± 7.7%)                                                 |
| Antibiotics             |                                                             |                                                             |
| MMC                     | 0.00                                                        | 4.00                                                        |
| BLM                     | 0.00                                                        | 3.00                                                        |
| PEP                     | 0.00                                                        | 6.00                                                        |
| TM                      | 0.00                                                        | 3.00                                                        |
| NCS                     | 0.00                                                        | 1.00                                                        |
| Subtotal                | (0 ± 0%)                                                    | (10 ± 2.5%)                                                 |
| Topoisomerase inhibitors|                                                             |                                                             |
| ACD                     | 1.00                                                        | 4.00                                                        |
| ACR                     | 5.00                                                        | 15.00                                                       |
| DOX                     | 2.00                                                        | 10.00                                                       |
| DM                      | 0.00                                                        | 11.00                                                       |
| THP                     | 1.00                                                        | 10.00                                                       |
| 4'-EPI                  | 1.00                                                        | 10.00                                                       |
| MIT                     | 1.00                                                        | 5.00                                                        |
| VP-16                   | 2.00                                                        | 11.00                                                       |
| CPT-11                  | 0.00                                                        | 3.00                                                        |
| Subtotal                | (6.3 ± 2.2%)                                                | (27 ± 4.0%)                                                 |
| Platinum agents         |                                                             |                                                             |
| CDDP                    | 0.00                                                        | 7.00                                                        |
| JM-8                    | 1.00                                                        | 3.00                                                        |
| Subtotal                | (2.2 ± 2.2%)                                                | (15 ± 6.1%)                                                 |
| Anti-microtubule agents |                                                             |                                                             |
| VCR                     | 3.00                                                        | 5.00                                                        |
| VLB                     | 1.00                                                        | 0.00                                                        |
| VDS                     | 4.00                                                        | 3.00                                                        |
| TAX                     | 3.00                                                        | 3.00                                                        |
| Subtotal                | (12 ± 2.7%)                                                 | (8.3 ± 3.1%)                                                 |
| Total                   | (4.2 ± 1.1%)                                                | (17 ± 2.2%)                                                 |

Abbreviations of the agents names are explained in Materials and methods. *Mantel–Haenszel χ² test.

**DISCUSSION**

In this study we have shown that the chemosensitivity of astrocytic tumours to DNA-damaging agents but not to anti-microtubule agents is influenced by the status of the p53 gene. The role of p53 protein in the sensitivity to chemotherapeutic agents that include anti-microtubule agents is controversial (Lowe et al, 1993; Wosikowski et al, 1995; Della et al, 1996; Hawkins et al, 1996; Perego et al, 1996; Wu and El-Deiry, 1996). Overexpression of the wild-type p53 gene increased the sensitivity in some experiments (Fujiwara et al, 1994) but inactivation of the wild-type p53 gene did not always decrease the sensitivity (Hawkins et al, 1996; Wu and El-Deiry, 1996). Several reasons for the inconsistent results have been put forward: (a) species and/or cell type specificity used in various experimental system (Della et al, 1996; Wu and El-Deiry, 1996); (b) additional genetic changes acquired during the process of established cell lines; and (c) genetic instability incurred by loss of function of the p53 gene (Livingstone et al, 1992). Examination of a number of clinical specimens as presented in this report, even although heterogeneity of clinical cases is unavoidable, can circumvent the above arguments and evaluate the clinical importance of the mutated p53 gene in a certain malignancy.

There are two types of anti-microtubule agents regarding their action mechanisms, i.e., depolymerization and stabilization of...
microtubules. Vinca alkaloids such as vinblastine and vincristine depolymerize microtubules and induce the arrest of the cell cycle at the mitotic stage. In contrast, paclitaxel binds directly to polymerized tubulins and impels cell cycle blockage during mitosis by promoting the microtubule assembly and inhibiting the disassembly (Horwitz, 1992). As both types primarily induce dysfunction of microtubules rather than direct DNA damage and prevent the completion of mitosis, consequent cell death would be less dependent on functional p53. In fact, increased p53 by genotoxic agents preferentially induces G1 arrest rather than G2/M arrest (Jacks and Weinberg, 1996). Accordingly, p53 may be less involved in apoptotic pathways during G2/M phase triggered by chemotherapeutic agents (Wahl et al, 1996).

Our present results show that the role of p53 in chemosensitivity depends on the type of agents, and suggest clinical benefits of the regimens including anti-microtubule agents to the patients with p53-mutated tumours who are not susceptible to DNA damage-based therapies. Current clinical trials of paclitaxel in brain tumours suggest that paclitaxel can cross the blood–brain barrier (Chamberlain and Kornamik, 1995; Glantz et al, 1996a) and that the status of the p53 gene may influence the responsiveness (Glantz et al, 1996b). Randomized clinical trials regarding the mutation and the chemotherapeutic based on its sensitivity test will be an important step in future.

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