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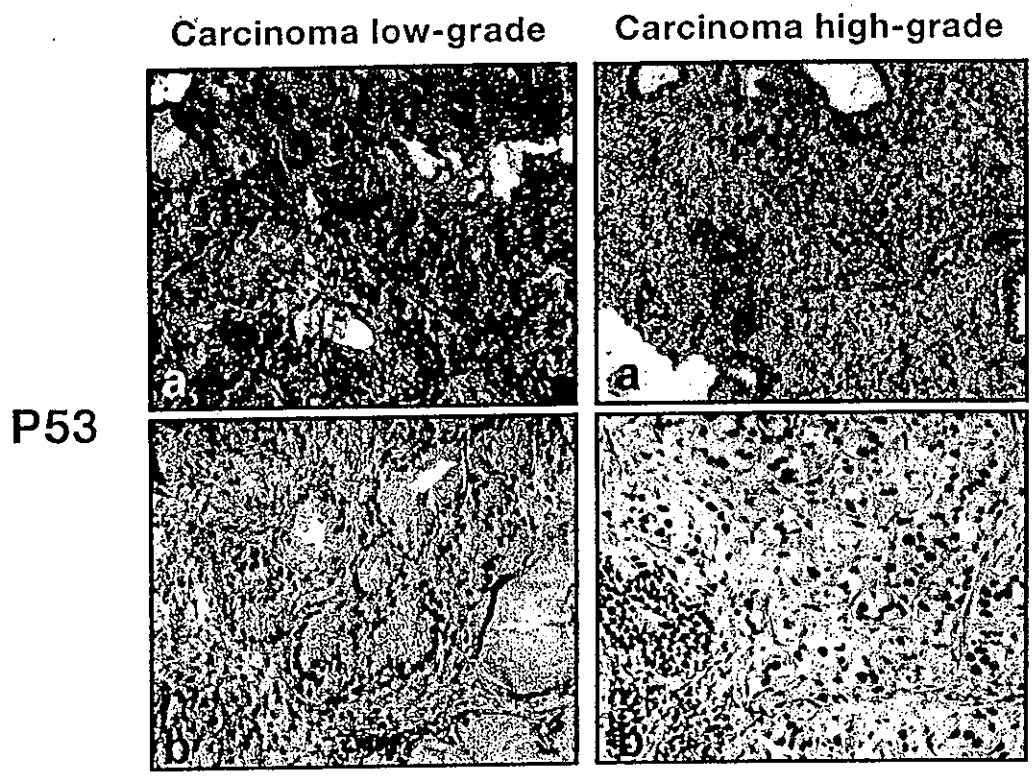

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5

Microsatellite Instability in Cancer: Assessment by High Resolution Fluorescent Microsatellite Analysis

Shinya Oda and Yoshihiko Maehara

Introduction

Microsatellites are one of the most abundant classes of repetitive deoxyribonucleic acid (DNA) sequences dispersed throughout the eukaryotic genome, and they comprise short reiterated motifs varying one to several base pairs. Microsatellites are highly polymorphic in human populations, which suggests that this polymorphism may be derived from relatively high mutation rates in these sequences. However, they appear stable during a relatively short time such as the life span of individuals. Somatic instability of microsatellite sequences has initially been reported in human colorectal cancer (Ionov *et al.*, 1993; Thibodeau *et al.*, 1993) and in the familial cancer-prone syndrome hereditary nonpolyposis colorectal cancer (HNPCC) (Aaltonen *et al.*, 1993; Peltomaki *et al.*, 1993). In 1993, mutations in one of the genes essential for DNA mismatch repair (MMR) were found in HNPCC kindred (Fishel *et al.*, 1993; Leach *et al.*, 1993).

MMR is an important DNA repair system that counteracts base mismatches and strand misalignments that occur during DNA replication and recombination (Modrich and Lahue, 1996). In regions of DNA comprising repeats of simple mononucleotide or dinucleotide motifs, slippage of DNA polymerases occurs frequently, and strand misalignments are formed. If uncorrected by MMR, they are fixed as insertion or deletion of repeat units after a next round of replication. Microsatellites are included in this type of repetitive sequences. Therefore, the phenomenon of unstable microsatellites, microsatellite instability (MSI), is considered to reflect MMR deficiency. MSI is frequently associated with various human malignancies (Arzimanoglou *et al.*, 1998). Because defective MMR is regarded as a risk factor for familial predisposition or second malignancies, analyses of MSI have been prevalent, particularly in the field of oncology. However, in the literature, results of MSI analyses lack consistency (Arzimanoglou *et al.*, 1998).

Although analysis of MSI is now commonplace, a designation of MSI is sometimes difficult. The 1997 National Cancer Institute (NCI) workshop, "Microsatellite Instability and RER Phenotypes in Cancer Detection and Familial Predisposition," concluded that the variety of microsatellites used was a major cause for discrepancies among data in the literature and recommended a panel of five microsatellites as a "working reference panel" (Boland *et al.*, 1998). In addition to selection of markers for analysis, methodologic problems may also account for a part of discrepancies among the data. In many cases, changes in microsatellite lengths are minute—as small as loss

Handbook of Immunohistochemistry and *in situ* Hybridization of Human Carcinomas, Volume 2: Molecular Pathology, Colorectal Carcinoma, and Prostate Carcinoma

or gain of a single repeat unit. In addition, cell populations carrying changes in microsatellites are not always major in a given sample. Accurate analysis of MSI therefore requires highly sensitive, quantitative, and reproducible characters in an assay system. In the most widely used approach for MSI analysis, microsatellite sequences are amplified by polymerase chain reaction (PCR) using radio-labeled primers. PCR products are run in the conventional sequencing gel and imaged by autoradiography using X-ray films. However, polyacrylamide gel electrophoresis is susceptible to migration errors, and autoradiography is known to have biased detection characteristics. PCR itself also has intrinsic problems. The most widely used thermostable DNA polymerase (*Taq*) has a terminal deoxynucleotidyl transferase (TdT) activity, which adds one additional base to PCR products in a sequence-dependent manner. TdT activity of *Taq* polymerase is variably expressed, depending on the conditions used. This property, in addition to slippage of the polymerase, increases the complexity of PCR products. These factors have been present as a major obstacle against an accurate analysis of MSI.

New electrophoresis techniques using fluorescence labeling and laser scanning have recently evolved. In some systems, each fragment is quantitatively detected, and its mobility is standardized accurately. We have applied such a fluorescent technique for MSI analysis to overcome the above-mentioned methodologic problems (Oda *et al.*, 1997). Application of our new assay system, High Resolution Fluorescent Microsatellite Analysis (HRFMA), has made it possible to describe more detailed microsatellite changes and, consequently, elucidated previously unrecognized aspects of MSI in human cancer.

MATERIALS

Enzymes

For PCR, *Taq* polymerase (*TaKaRa Taq*, TakaRa Bio Inc., Tokyo, Japan) was used. Other equivalent *Taq* products are also available. However, because other thermostable polymerases, including *TaKaRa Ex Taq* (Takara Bio Inc.), *Pfu* (Stratagene, La Jolla, CA), *Vent* (New England Biolabs, Inc., Beverly, MA), and so on, behave differently on repetitive sequences (unpublished data), these polymerases are not recommended.

Oligonucleotides Used for PCR

All the oligonucleotides used as a primer were synthesized and purified by high-performance liquid chromatography (HPLC). The sequences of the oligonucleotide primers are as follows:

D2S123-5′; 5′-AAACAGGATGCCTGCCTTTA, D2S123-3′; 5′-GGACTTTCCACCTATGGGAC, D5S107-5′; 5′-GGCATCAACTTGAACAGCAT, D5S107-3′; 5′-GATCCACTTTAACCCAAATAC, D10S197-5′; 5′-ACCACTGCACTTCAGGTGAC, D10S197-3′; 5′-GTGATACTGTCCTCAGGTCTCC, D11S904-5′; 5′-ATGACAAGCAATCCTTGAGC, D11S904-3′; 5′-GCTGTGTTATATCCCTAAAGTG-GTGA, D13S175-5′; 5′-TGCATCACCTCACATAG-GTTA, D13S175-3′; 5′-GTATTGGATACTTGAA-TCTGCTG.

In the 3′ primers, guanine residues were chosen at the 5′ end of the oligonucleotides, to control TdT activity of *Taq* polymerase (see Discussion). In D11S904, a guanine residue was added artificially at the 5′ end of the 3′ primer.

The 5′ PCR primers were labeled with ROX (6-carboxy-x-rhodamine), HEX (6-carboxy-2′,4′,7′,4,7,-hexachloro-fluorescein), or 6-FAM (6-carboxyfluorescein). Size standards were labeled with TAMRA (N,N,N′,N′-tetramethyl-6-carboxyrhodamine) or ROX.

Preparation of Genomic DNA

1. Tissue specimens.

Tissue specimens were collected immediately after surgery and kept in liquid nitrogen or at −80°C.

2. Digestion buffer: 10 mM Tris-Cl (pH 8.0), 0.1 M ethylenediamine tetra-acetic acid (EDTA) (pH 8.0), 0.5% sodium dodecyl sulfate (SDS).
3. Proteinase K: 20 mg/ml.
4. Buffer-saturated phenol.
5. Chloroform.
6. Ethanol.
7. 1X TE buffer; 10 mM Tris-Cl (pH 7.5), 1 mM EDTA.

Polymerase Chain Reaction

1. 10X PCR buffer; 100 mM Tris-Cl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$.
2. Deoxyribonucleotide-triphosphate (dNTP) mixture; 2.5 mM for each.

Fragment Analysis Using an Automated Sequencer (1): Gel Plate System—ABI373A, 377, etc.

1. Urea.
2. 10X TBE.
3. 30% 19:1 Acrylamide/N,N′-methylene-bis-acrylamide solution.

4. Formamide.
5. Tracking dye; 25% blue dextran, 25 mM EDTA (pH 8.0).
6. Size standard; GeneScan 500 TAMRA (Applied Biosystems, Foster City, CA).

Fragment Analysis Using an Automated Sequencer (2): Capillary System—ABI310, 3100

1. 47 cm × 50 μm capillary (Applied Biosystems).
2. Sample tubes and gaskets (Applied Biosystems).
3. Buffer vials (Applied Biosystems).
4. Glass syringe (Applied Biosystems).
5. Capillary polymer; 310 POP4 (Applied Biosystems).
6. 10X running buffer; Genetic Analyzer Buffer with EDTA (Applied Biosystems).
7. Template suppression reagent (Applied Biosystems).
8. Size standard; GeneScan 500 ROX (Applied Biosystems).

METHODS

Preparation of Genomic DNA from Tissue Specimens

Preparation of high molecular weight DNA from tissue specimens was done, as described elsewhere.

1. Thaw a tissue specimen and cut off a part in a 3 × 3 mm size, using sterile scissors.
2. Mince using scissors in a 1.5 ml microtube.
3. Add 400 μl lysis buffer and 2 μl of 20 mg/ml Proteinase K.
4. Mix gently.
5. Incubate at 55°C for 1 hr, with shaking.
6. Spin briefly, and add 400 μl buffer-saturated phenol.
7. Shake gently at room temperature for 10 min.
8. Spin at 15,000 rpm for 10 min at room temperature.
9. Collect the aqueous phase in a new microtube.
10. Add 400 μl of buffer-saturated phenol/chloroform/isoamylalcohol (25:24:1).
11. Repeat **Steps 7–9**.
12. Add 400 μl chloroform/isoamylalcohol (24:1).
13. Repeat **Steps 7–9**.
14. Add 40 μl of 10 N ammonium acetate, and mix.
15. Add 1 ml 100% ethanol and mix gently, until high-molecular-weight DNA is completely insolublized.
16. Keep at 4°C for 10 min.
17. Spin at 15,000 rpm for 20 min at 4°C.
18. Decant gently the supernatant and remove traces of ethanol, as much as possible.
19. Add 360 μl of 1X TE (pH 8.0) and dissolve the pellet, without shaking. This step normally takes overnight at 4°C.
20. Repeat **Steps 6–18**.
21. Wash the pellet with 70% ethanol.
22. Decant gently the supernatant, and set the tube with the top open under a vacuum until no trace of 70% ethanol is visible. This step normally takes 3–10 min. Do not allow the pellet to dry completely.
23. Dissolve the pellet completely in an adequate volume of 1X TE (pH 8.0), without shaking.
24. Scan the absorbance at from 220 to 320 nm. Calculate the concentration from OD_{260}. The quality of DNA can also be checked by routine agarose gel electrophoresis.
25. Store at 4°C.

Polymerase Chain Reaction

1. Dilute genomic DNA solution to 5 μg/ml with 1X TE (pH 8.0).
2. Prepare the premix (per tube); 5 μl of 10X PCR buffer (100 mM Tris-Cl (pH 8.3), 500 mM KCl, 15 mM $MgCl_2$), 7 μl of dNTP mix, 5 μl for each of primers, 22.5 μl of dH_2O, 0.5 μl (2.5u) of *Taq* polymerase. If an automated sequencer is a gel-plate system (ABI373A, 377, etc.), use HEX (normal) and ROX (cancer)-labeled primers. If a capillary sequencer (ABI310, 3100, etc.) is used, choose HEX (normal) and 6-FAM (cancer) for primer labeling.
3. Aliquot 45 μl of the premix into a 0.2 ml thin-walled PCR tube.
4. Add 5 μl of 5 μg/ml genomic DNA sample.
5. Mix well, and spin down.
6. Set the tube in a thermal cycler, and carry out the following program:
   One cycle:
   Presoaking at 95°C for 4 min.
   35 cycles:
   Denaturing at 95°C for 30 sec.
   Annealing at 55°C for 30 sec.
   Extension at 72°C for 30 sec.
   One cycle:
   Additional extension at 72°C for 10 min.
   Keep at 4°C.
7. Mix well, and spin briefly.
8. Store at 4°C until loaded onto an automated sequencer.

Fragment Analysis Using an Automated Sequencer (1): Gel Plate System—ABI373A, 377, etc.

1. Clean the gel plates with detergent solution and isopropanol. Assemble the gel mold according to the manual provided by the manufacturer. Make sure that there is no dust or stain on the glass plates, especially in the belts scanned by laser light, and if necessary, clean the surface again.

2. Mix 40 g urea, 8 ml of 10X TBE, 12 ml of 30% 19:1 acrylamide/bis solution, and 20 ml dH_2O in a 100 ml beaker. Stir well, heating, until urea has been completely dissolved.

3. Adjust the volume to 100 ml in a messcyllinder.

4. Filtrate the solution using a disposable bottle-top filter (pore size; 0.45 μm), and deaerate in the same filter ware for 10 min after the solution has been completely filtrated.

5. Transfer the solution in a small Erlenmeyer flask.

6. Add 45 μl of TEMED (N,N,N′,N′-tetramethylethylenediamine) and 0.4 ml of 10% APS to the solution and mix by rapid swirling.

7. Slowly pour the solution into the gel mold, using a pipette or a small polyethylene-made washing bottle with a flexible nozzle. The solution should be poured in the continuous stream so that air bubbles are not included in the gel.

8. Place the gel mold horizontally and examine the gel carefully. If any of air bubbles are present in the areas where DNA samples track, remake a gel.

9. Insert the 24 well-comb and pour the excess gel solution on the comb. Spare 0.5–1.0 ml of the gel solution in a microtube as an indicator for polymerization.

10. Set the gel for at least 3 hr. To avoid overdyring the gel, place Kimwipe paper soaked with 1X TBE at the top, and, if necessary, the bottom of the gel mold, and wrap the gel mold with SaranWrap. The gel can be stored at room temperature up to 24 hr after polymerization is complete.

11. When polymerization is complete, wipe away dried urea or acrylamide and clean the surface of the gel plate, especially in the belts scanned by laser light. Remove the comb carefully.

12. Set the gel plate to the automated sequencer according to the manual provided by the manufacturer.

13. Turn on the power of the sequencer and the computer. The analytic software "GeneScan ver. 1.2.2" is automatically started. Check the gel plate ("plate check"), as instructed in the user's manual. If the signal baseline is not straight, due to the dust on the glass plate, remove the gel from the apparatus and clean again the belts scanned by laser light with isopropanol. If this symptom is not improved, analyses may be affected in some lanes.

14. Set the buffer chambers and pour 1X TBE. Prerun the sequencer (1500 V, 20 mM, 30 W) according to the instruction in the manual.

15. In the meantime, prepare samples. Mix 12 μl of ROX-labeled PCR product and 3 μl of HEX-labeled PCR product in a microtube. This ratio has been determined according to the difference in signal strength among fluorescence compounds so that signal levels acquired in the sequencer may be in a similar range.

16. Prepare the premix (per tube): 2.5 μl of formamide, 0.5 μl of tracking dye, and 0.5 μl of GeneScan 500 TAMRA size marker. Aliquot 3.5 μl into each microtube.

17. Add 1.5 μl of the PCR product mixture to the aliquoted premix, and mix well.

18. Heat at 95°C for 5 min, and chill immediately on ice. Spin briefly.

19. Before loading, remove urea crystalized in the well completely by flushing the well using a syringe with a fine needle. Load samples onto the gel using a flatted flexible tip.

20. Start electrophoresis and scan in the sequencer.

21. After entering necessary information in the "sample sheet" and setting "preprocess parameters" and "analysis parameters" start data acquisition (press the "collection" button). This is normally done 1 hr after electrophoresis has been started. Data acquisition should be done for at least 6 hrs.

22. Acquired data are automatically analyzed if the mode has been selected beforehand in the sample sheet. However, when opening the "Gel File," check whether the "Channel" traces each band correctly in each lane. If necessary, correct "Channel Selector Line," according to the manual, and then reanalyze the data.

23. Results are seen in the "Electropherogram." In HRFMA by gel plate system, two independent PCR products labeled with HEX and ROX are electrophoresed in each lane. To adjust the signal level of these two products, use "Dye Scale" command in the "View" menu. Change values for each fluorescence so that two profiles are superimposed on the Electropherogram. If the size standard has been selected, the data will be displayed by fragment size (the horizontal axis), but, to obtain correct fragment size, verify size calculations and reanalyze the data.

Fragment Analysis Using an Automated Sequencer (2): Capillary System—ABI310, 3100

1. Mix 5 μl of 6-FAM-labeled PCR product, 5 μl of HEX-labeled PCR product, and 30 μl dH_2O in a microtube.

2. Mix 1 µl of this mixture with 23.5 µl of Template Suppression Reagent and 0.5 µl of GeneScan-500 ROX Size Standard in a new tube.

3. Heat at 95°C for 5 min, and chill immediately on ice. Spin briefly.

4. Transfer the sample into a sample tube.

5. Set and fill the capillary with the POP4 polymer according to the user's manual. Dilute 10X running buffer with dH_2O, and set the buffer and dH_2O on the auto-sampler.

6. Set sample tubes on the sample tray.

7. Close the front cover and start up "GeneScan ver. 3.1.2." Set the temperature at 60°C.

8. Start up the "Data Collection" program. Fill in the "Sample Sheet."

9. Open the "Injection List." After setting the parameters, click the "Run" button.

10. Acquired data are automatically analyzed if the analysis parameters and the size standard file are selected in the "Injection List" beforehand. In case of reanalyzing the data, start the "GeneScan Analysis" program. After setting parameters in the "Analysis Parameters" window and choosing size standard and analysis parameter files in the "Analysis Control Window," click the Analyze button.

11. Results are seen in the Electropherogram, as in the gelplate system. In HRFMA by capillary system, two independent PCR products labeled with 6-FAM and HEX are electrophoresed in each run. To adjust the signal level of these two products, use "Dye Scale" command in the "Settings" menu, as in the gelplate system.

Criteria for MSI

The criterion for MSI that we apply to data obtained using HRFMA is "appearance of novel peaks in at least one of more than five selected dinucleotide microsatellites." In "inseparable heterozygous" allelotypes (see Results and Discussion), significant changes in the ratio between the major peaks of two parental clusters also can be regarded as MSI. However, in this case, the possibility of "loss of heterozygosity (LOH)" is not excluded (Maehara *et al.*, 2001) (Figure 12E and F). We designate this category as MSI/LOH. We further classify MSI into two subtypes, according to the fragment length of newly appeared peaks (see Results and Discussion; Figure 12C, D, G, and H).

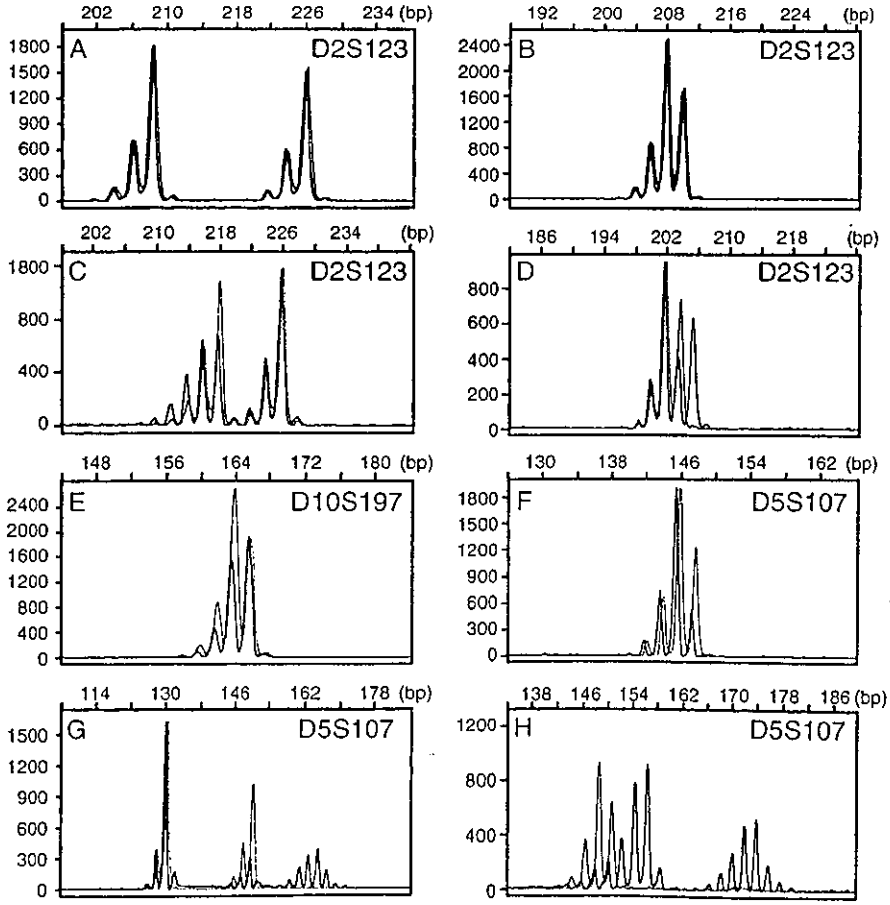

Figure 12 **Microsatellite instability determined using HRFMA. A:** Normal in a heterozygous allelotype. **B:** Normal in an inseparable heterozygous allelotype. **C:** Type A microsatellite instability (MSI) in a heterozygous allelotype. **D:** Type A MSI in an inseparable heterozygous allelotype. **E, F:** Type A MSI indistinguishable from LOH (inseparable heterozygous allelotype). **G:** Type B MSI in a heterozygous allelotype. **H:** Type B MSI in an inseparable heterozygous allelotype.

RESULTS AND DISCUSSION

Selection of the Microsatellite Markers

Selection of microsatellites is always controversial. A wide variety of microsatellites has been used as a marker for MSI analysis. This circumstance raised a considerable confusion in this field. In 1997, the National Cancer Institute (NCI) sponsored a workshop to review and unify this field (Boland *et al.*, 1998). In this workshop, it was concluded that the diversity in data derives mainly from the variety of microsatellites used, a panel of five microsatellites was recommended as a "working reference panel." This panel consists of two mononucleotide microsatellites and three dinucleotide microsatellites.

In selecting markers for MSI assay, repeat length and unit size appear important. Repeat length determines the susceptibility of DNA polymerases to slippage (Greene and Jinks-Robertson, 1997; Tran *et al.*, 1997), and unit size determines workability of an MMR system on repeat units looping out of the strand (Sia *et al.*, 1997). Indeed, the rare alteration of microsatellite sequences in *Drosophila* cells is thought to derive from their shortness (Schug *et al.*, 1997), and eukaryotic MMR is known to work mainly on relatively short repeat units varying from one to several bases (Genschel *et al.*, 1998). We have selected five dinucleotide microsatellites located in five independent chromosomes, which contain different repeat lengths varying from 16 to 58 (see Materials). To confer validity on this set of markers, we tested whether they show instability in cells deficient in MMR. Oki *et al.* (1999) have shown that these sequences were highly unstable in cells with a known mutation in *hMSH2* or *hMLH1* and that even cells defective in *hMSH6* (*GTBP*) exhibit a low level of MSI. In addition to MutSα (i.e., hMSH2/hMSH6 heterodimer) MutSβ comprising hMSH2 and hMSH3 functions on nucleotides looped out of the strand in eukaryotic MMR (Genschel *et al.*, 1998; Marsischky *et al.*, 1996). Therefore, we concluded that this set of markers is highly sensitive to changes derived from defective MMR.

In the NCI workshop mentioned earlier two mononucleotide microsatellites were included in the working reference panel. However, as discussed later in this chapter, use of mononucleotide microsatellites appears problematic. Behavior of *Taq* polymerase on this variety of repeats is unknown because this problem has not been addressed using an artificially synthesized template. In addition, the effect of TdT activity in *Taq* polymerase will be more critical in mononucleotide repeats. Although mononucleotide repeats were known to be a sensitive marker for MSI, correlation between instability in mononucleotide microsatellites and mutation in MMR genes has not been confirmed (Percesepe *et al.*, 1998).

In the NCI workshop, MSI phenotypes were classified into two categories: MSI-H and MSI-L (Boland *et al.*, 1998). The first is defined as ones showing microsatellite alterations in "the majority (40%) of markers" and the second exhibiting changes only in "a minority (<40%) of markers." In this workshop, the number of markers required for microsatellite analysis was intensively discussed because the sensitivity of assay may depend on the number of markers examined. Indeed, recent studies using more than 10 microsatellites are not rare. However, to answer this problem, it may be more pertinent to test whether a given set of markers exhibits instability in established cell lines deficient in MMR. As mentioned earlier, our five dinucleotide markers detected changes even in cells defective in hMSH6 (Oki *et al.*, 1999), which may indicate that at least five markers are required for a sensitive assay. Indeed, the NCI workshop recommended five markers.

Electrophoretic Profiles of Amplified Microsatellite Sequences

Microsatellite sequences amplified by PCR show a complicated cluster of fragments with different lengths and amounts, due to modifications by polymerases used. In addition, microsatellite sequences are highly polymorphic in human populations, which implies that, in many cases, cells are heterozygous for the length of each microsatellite allele. These facts have been in part an obstacle against an accurate microsatellite analysis. *Taq* polymerase is a major thermostable bacterial DNA polymerase used for PCR. This enzyme has TdT activity, in addition to the template-dependent 5′-3′ DNA polymerase activity. Modification of PCR products by TdT activity has been reported in detail (Hu, 1993). TdT activity of *Taq* polymerase adds one additional base to the 3′ end of synthesized strands in a sequence-dependent manner. In many cases, this activity is variably expressed, which leads to a variety with a one-base pair difference in PCR products. This phenomenon is known as a "stuttering" in microsatellite analysis. In addition, *Taq* polymerase is highly susceptible to slippage on repetitive sequences. Slippage of *Taq* polymerase also confers a repeat unit-pitched variety to PCR products of microsatellite sequences.

To analyze *Taq*-dependent modifications in PCR products of microsatellite sequences, we used artificially synthesized microsatellite sequences (Oda *et al.*, 1997). The 105-bp region of an artificially synthesized D13S175 human dinucleotide microsatellite was

amplified as a complex of several fragments with different lengths. The highest peak corresponded to 108 bp and was accompanied by shorter peaks different by one base. Because heterogeneity of templates was excluded in this case, this multiplicity of peaks is derived from slippage and TdT activity of *Taq* polymerase. As shown in Figure 13, the major PCR products were fragments with an insertion of one additional repeat unit, and next to these products fragments of the correct size were present. Those lacking one repeat unit were also detectable. In addition to these modifications by polymerase slippage, TdT activity adds one additional base to PCR products, the result being a cluster of fragments the size of which varies from −2 to +3. Thus, when *Taq* polymerase is used, we observe microsatellite alterations through these modifications.

Slippage of *Taq* polymerase on repetitive sequences is not avoidable, although it is known that this activity can be partially controllable by altering Mg^{++} concentration

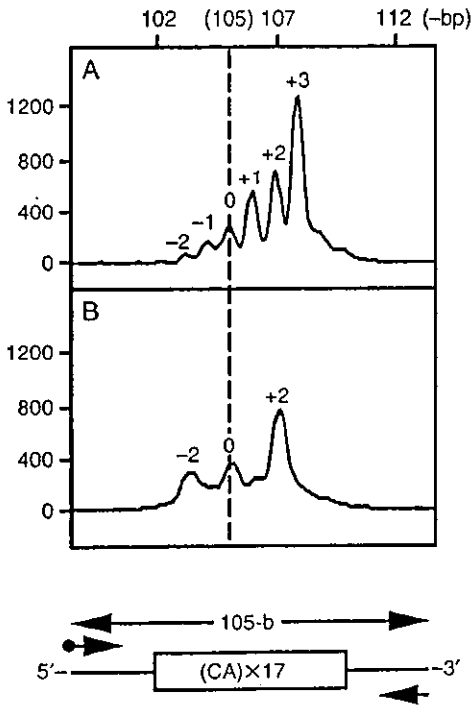

Figure 13 *Taq* polymerase-dependent modification of polymerase chain reaction (PCR)-amplified microsatellite sequences. Dinucleotide microsatellite, D13S175, which contains 17 repeats of CA, was artificially synthesized and its 105-bp region was amplified by PCR using *Taq* polymerase. Six peaks, +3, +2, +1, 0, −1, and −2, were observed in A. The 3′ exonuclease activity of T4 DNA polymerase removed 3′-protruded nucleotides from the PCR products and simplified them into three peaks, +2, 0, and −2, which were generated by polymerase slippage in B. (Adapted from Maehara *et al.*, 2000, with permission.)

in PCR. TdT activity of *Taq* polymerase is considered to be a major cause for the low reproducibility seen in PCR products of microsatellite sequences, because this activity is easily altered not only by primer sequences but also by reaction conditions. Efforts to control TdT activity of *Taq* polymerase have been reported. Brownstein *et al.* (1996) have reported that modifications at the bottom sequence of PCR primers are useful to control TdT activity, whereas use of 3′ exonuclease activity in some DNA polymerases to remove additional nucleotides has also been reported (Ginot *et al.*, 1996; Oda *et al.*, 1997). We once used T4 DNA polymerase. However, enzymatic treatment after PCR may appear complicated. According to the report by Hu *et al.* (1993), Oki *et al.* (1999) found that addition of single guanine residue at the 5′ end of 3′ (nonlabeled) primer induces TdT activity completely at the 3′ end of labeled strands and consequently removes "stuttering" efficiently (unpublished data). In this case, all of the labeled strands are one base longer than their real lengths. At present, we use this method. Thus, as shown in Figure 13, the most simplified profile of amplified dinucleotide microsatellite sequences is a two bases-pitched cluster of peaks showing an increasing pattern; the amount of fragments increases in proportion to their length. When using genomic DNA template, two clusters of peaks derived from paternal and maternal alleles are seen in many cases (i.e., heterozygous) (Figure 12A, C, and G; *green lines*) and, in some cases, they cross (i.e., "inseparable" heterozygous) (Figure 12B, D–F, and H, *green lines*) or coincide (i.e., homozygous). In MSI analysis, it is essential that this basic pattern is always being obtained in the normal control.

In addition to clusters of peaks included in the basic pattern, additional peaks may be seen in some cases. The sources of these artifacts are mainly secondary structures in the electrophoresed DNA fragments and incorrect calculation files in the analysis program. Artifacts derived from secondary structures in PCR products often form a cluster of peaks shorter than their real lengths. In this case, more stringent denaturing before loading is necessary. Immediate chilling after denaturing is also important. In case the artifacts do not disappear by these procedures, the optical system in a sequencer, particularly the laser axis, should be checked. The ranges of laser emission by various fluorescence compounds used in this system cross each other. Therefore, the analysis program requires a calculation file in which overlapped emission is subtracted in each fluorescence. This calculation file is named "Matrix File" and originally is installed by the manufacturer, according to the combination of fluorescence labels used. However, in some cases, trails by other fluorescence labels, particularly ones used in a

coelectrophoresed size standard, form small peaks that are indistinguishable from peaks that appear as a result of MSI. In this case, a new Matrix File should be originally prepared according to the manual. In MSI analysis, an assay system should be completely free from these artifacts.

Advantages in High Resolution Fluorescent Microsatellite Analysis

As discussed earlier, PCR products of a dinucleotide microsatellite sequence are composed of fragments the size of which varies from −2 to +2 bp. In microsatellite-unstable cells, this sequence fluctuates by 2 bp in some populations. Therefore, in MSI analysis, an accurate electrophoresis is needed. However, in the conventional assay system, sequencing gel electrophoresis has been used. Affected by various conditions, migration of DNA fragments is error-prone in a sequencing gel. Use of an automated sequencer for microsatellite analysis is on the increase. However, in many cases, labeling is done using a single fluorescence and samples are run on separate lanes. In some systems, migration of each DNA fragment is standardized, using a coelectrophoresed size marker with a different fluorescence. However, even in such systems, it appears difficult to standardize migration absolutely because there is no calculation file that corrects mobility differences among fluorescence compounds. To exclude migration errors completely, it appears more pertinent to run two samples labeled with different fluorescent labels in one lane. We have established this dual fluorescence coelectrophoresis system. We examined electrophoretic mobilities and specific intensity of DNA fragments labeled with various fluorescence compounds and found the combinations and the ratios in which their electrophoretic profiles match completely (Figure 12A and B). This improvement has obviously facilitated a precise comparison of two independent PCR products.

Application of fluorescent labeling to microsatellite analysis has another advantage. In the conventional MSI assay, PCR products are radio-labeled and imaged by autoradiography. However, X-ray films often used in autoradiography have biased detection characteristics, which leads to wrong estimate of the signal magnitude and, sometimes, to a loss of bands, particularly in ones with a low signal. From this point of view, laser scanning of fluorescent-labeled fragments seems to be more feasible for microsatellite analysis because it has highly linear detection characteristics in addition to a high sensitivity for detection (Oda *et al.*, 1997). This should lead to a correct detection of DNA fragments both in number and in signal magnitude. This property of the detection system is essential because, in designating MSI, changes in signal magnitude in each peak are important as are changes in the number of peaks (see Methods) (Maehara *et al.*, 2001). This quantitative character of the detection system has also lead to a sensitive assay of MSI.

In detecting minute changes, one may be concerned about the reproducibility in results. To test whether results obtained using HRFMA are highly reproducible, we electrophoresed the same PCR products more than 10 independent times. We also performed PCR of normal tissue DNA more than 10 independent times, and eletrophoresed them. Results were highly reproducible. In all of these data, no additional peak was observed and the electrophoretic profiles were identical (data not shown). These findings imply that in HRFMA, appearance of novel peaks can be regarded as MSI. The ratio between two neighboring peaks did not vary more than 5% in independent PCR products, which implies that changes in the signal magnitude in each peak can be interpreted as a change in template DNA. When mixing genomic DNA samples with different microsatellite lengths in various ratios, the system detected the existence of template with a different microsatellite length at 10% (Oda *et al.*, 1997). These data may guarantee sensitive detection of MSI in HRFMA.

Qualitatively Different Subtypes of Microsatellite Instability in Human Cancer

Development of a sensitive and quantitative MSI assay has shed light on qualitative differences in MSI in human cancer. Using HRFMA, we have observed two qualitatively different subtypes of MSI in various human malignancies. We define Type A alterations as length changes of ≤6-base pairs (Figure 12C and D). Type B changes are more drastic and involve alterations of ≥8-base pairs, and they sometimes appear as if a "third" allele is present in addition to the parental alleles (Figure 12G and H). In our panel of more than 100 patients with colorectal cancer, the frequencies of Type A and Type B MSI were approximately 30% and 10%, respectively. Inspection of published data reveals that microsatellite changes thus far reported in various tumors, including ones in HNPCC, are largely Type B, possibly related to the fact that in the conventional microsatellite assay it is less difficult to detect Type B changes. More subtle Type A MSI might have remained undetected in many cases.

When dinucleotide MSI in cancer was first reported by Thibodeau *et al.* (1993), the patterns of alterations were classified into two categories: Type I mutation with "a significant increase or decrease in the apparent

fragment size" and Type II mutation with "minor alteration" such as changes within 2 bp. Type A MSI appears similar to their Type II mutation. On the other hand, Type I mutations may correspond to our Type B instability. Microsatellite changes determined using HRFMA in human or mouse cells with a known defect in MMR genes were within 6 bp (Oki *et al.*, 1999 and unpublished data), which implies that MSI observed in these cells are Type A. Indeed, examination of published microsatellite changes in cells of MMR gene-knock out mice clearly indicates that most changes are of Type A. These findings strongly suggest that Type A is a direct consequence of defective MMR. Nevertheless, Type B MSI is noted in various tumors, including HNPCC. The problem is that mutations in MMR genes have been reported in tumors displaying this type of instability. However, the reported frequencies of mutation in the two major MMR genes, *hMSH2* and *hMLH1*, in HNPCC kindred are not always high. Additional and previously unrecognized molecular abnormalities may underlie Type B instability. Thus, application of a sensitive and quantitative technique has elucidated qualitative differences in MSI in human cancer. Such techniques will precisely distinguish tumors with different molecular backgrounds.

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A phase II study of cisplatin and docetaxel administered as three consecutive weekly infusions for advanced non-small-cell lung cancer in elderly patients

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Background: To evaluate the efficacy and safety of treatments for advanced non-small-cell lung cancer in elderly patients aged 75 years or older, we conducted a phase II study of cisplatin and docetaxel administered in three consecutive weekly infusions.

Patients and methods: The eligibility criteria for the study included the presence of chemotherapy-naive advanced non-small-cell lung cancer, age ≥75 years, Eastern Cooperative Oncology Group performance status of 0 or 1, a measurable lesion, adequate organ functions and signed informed consent. The chemotherapy regimen consisted of cisplatin (25 mg/m^2) and docetaxel (20 mg/m^2) on days 1, 8 and 15 every 4 weeks.

Results: Between February 2000 and March 2002, 34 elderly patients with non-small-cell lung cancer were enrolled in the study and 33 patients were treated. Two complete responses and 15 partial responses were obtained for an objective response rate of 52% in 33 treated patients. The median survival period was 15.8 months, and the 1-year survival rate was 64%. Toxicities were mild with no grade 4 toxicities. Only grade 3 leukopenia (6%), neutropenia (12%), anemia (3%), hyponatremia (3%) and nausea/vomiting (3%) were observed.

Conclusion: Cisplatin and docetaxel administered in three consecutive weekly infusions was safe and effective for the treatment of elderly patients with chemotherapy-naive non-small-cell lung cancer.

Key words: cisplatin, docetaxel, elderly patients, non-small-cell lung cancer, weekly administration

Introduction

Lung cancer is one of the most common carcinomas not only in Japan, but also in the United States and Europe. More than 55 000 patients die from lung cancer each year, and the mortality rate is still increasing in Japan [1, 2]. In particular, the number of elderly lung cancer patients is increasing in Japan [1, 2]. Surgery is the most effective curative treatment for early stage non-small-cell lung cancer (NSCLC); however, only 30% of patients with NSCLC receive a curative resection [3]. Cisplatin-based chemotherapy offers a survival benefit and symptom relief for patients with inoperable NSCLC [4]. However, we have demonstrated that classic standard cisplatin-based chemotherapy regimens such as cisplatin (80 mg/m^2) on day 1 with etoposide (100 mg/m^2) on days 1–3 or cisplatin (80 mg/m^2) on day 1 with vindesine (3 mg/m^2) on days 1 and 8 cause severe myelotoxicity in elderly NSCLC patients aged ≥75 years [5]. We used a very restricted eligibility criteria to select patients who could tolerate the cisplatin-based standard chemotherapy. Among 34 elderly patients, only 10 fitted the eligibility criteria. In spite of granulocyte colony-stimulating factor (G-CSF) support, nine of the 10 eligible patients experienced grade 4 neutropenia and six had infectious episodes [5]. Thus, we hypothesized that the recommended dose for elderly patients aged ≥75 years should be determined in a specific phase I study only for elderly patients.

Docetaxel has demonstrated antitumor activity in NSCLC patients with chemotherapy-naive lesions and tumor progression after receiving cisplatin-based regimens [6–10]. Docetaxel with cisplatin is one of the most promising chemotherapy regimens for NSCLC [11]. The commonly used dose and schedule of docetaxel is 60–100 mg/m^2 every 3 weeks; however, moderate to severe neutropenia is frequently observed [6–11]. Recent studies have shown that weekly administration of docetaxel produces a higher dose intensity and less myelotoxicity [12–14]. Thus, we conducted two independent phase I studies for elderly and non-elderly patients with NSCLC to determine the recommended dose for phase II studies and to evaluate the safety and efficacy of cisplatin and docetaxel administered as three consecutive weekly infusions in both non-elderly (≤74 years) and elderly (≥75 years) patients

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[15]. Different recommended doses of docetaxel were obtained for non-elderly and elderly patients [15]. The recommended doses were 25 mg/m^2 cisplatin and 35 mg/m^2 docetaxel on days 1, 8 and 15 for non-elderly patients, and 25 mg/m^2 cisplatin and 20 mg/m^2 docetaxel on days 1, 8 and 15 for elderly patients.

Two phase II studies of cisplatin and docetaxel administered as three consecutive weekly infusions for non-elderly and elderly patients were conducted. The results of the phase II study for non-elderly patients with NSCLC have been reported elsewhere; the objective tumor response was 30% [95% confidence interval (CI) 15% to 46%] and the median survival time was 12.8 months [16]. Here, we report the promising results of a phase II study for elderly patients with NSCLC.

Patients and methods

Patient selection

Patients with histologically and/or cytologically documented NSCLC were eligible for the study. Each patient was required to meet the following criteria: clinical stage IV or IIIB (including only patients with no indications for curative radiotherapy), an Eastern Cooperative Oncology Group performance status (PS) of 0 or 1, age ≥75 years, no prior chemotherapy, measurable lesions, adequate hematological function [white blood cell count (WBC) 4000–12 000/mm^3; neutrophils ≥2000/mm^3; platelets ≥100 000/mm^3; hemaglobin ≥9.0 g/dl], adequate hepatic function (total bilirubin <1.1 mg/dl, aspartate aminotransferase and alanine aminotransferase <60 IU/l), and adequate renal function (creatinine ≤1.2 mg/dl, creatinine clearance ≥60 ml/min). Patients with active infection, severe heart disease, uncontrollable hypertension or diabetes mellitus, active concomitant malignancy and pleural and/or pericardial effusion requiring drainage were excluded. The study was approved by the Institutional Review Board at the National Cancer Center, Yokohama Municipal Citizen's Hospital and Niigata Cancer Center. Written informed consent was obtained from each patient.

Patient evaluation

The pretreatment evaluation consisted of complete blood cell count, differential count, routine chemistry measurements, a chest radiograph, a chest computed tomography (CT) scan, abdominal ultrasound or CT scan, whole-brain magnetic resonance imaging or CT scan, and an isotope bone scan. Complete blood cell count, differential, count and routine chemistry measurements were carried out at least twice a week during the first course of chemotherapy.

Treatment schedule

All patients were admitted to hospital during the first course of chemotherapy. Chemotherapy consisted of cisplatin (25 mg/m^2) on days 1, 8 and 15 and docetaxel (20 mg/m^2) on days 1, 8 and 15 every 4 weeks. Docetaxel was infused over 30 min with 16 mg dexamethasone and 3 mg granisetron administered just before the docetaxel infusion. Ninety minutes after the completion of the docetaxel infusion, 25 mg/m^2 cisplatin were administered over 15 min with 1500 ml normal saline over 3.5 h. The prophylactic administration of G-CSF was not permitted. Administration of G-CSF was permitted in patients with grade 4 neutropenia and/or leukopenia or grade 3 febrile neutropenia. The administration of both cisplatin and docetaxel were skipped on day 8 and/or day 15 if the patients met the following criteria: WBC <2000/mm^3 and/or platelets <50 000/mm^3. No dose modifications were carried out on days 8 and/or day 15 of the cisplatin and docetaxel administrations. Treatment was carried out for at least two courses, unless unacceptable toxicity or disease progression occurred.

Response and toxicity evaluation

The patients' responses were evaluated according to the World Health Organization criteria [17]. A complete response (CR) was defined as the complete disappearance of all clinically detectable tumors for at least 4 weeks. A partial response (PR) was defined as a reduction of ≥50% in the product of the largest perpendicular diameters of one or more clearly measurable lesions or as a >50% reduction in evaluable malignant disease lasting for >4 weeks with no new areas of malignant disease. No change included: the regression of indicator lesions that were insufficient to meet the criteria for PR, <25% increase in any measurable lesion and no new lesions of malignant disease. Progressive disease was defined as an increase in any measurable lesion by >25% or a new lesion of malignant disease. Survival times from the start of treatment were calculated using the Kaplan–Meier method. The toxicity grading criteria of the Japan Clinical Oncology Group (JCOG) were used to evaluate toxicity [18]. Most detailed gradings for individual organ toxicity in the JCOG Toxicity Criteria are identical to those of the National Cancer Institute Common Toxicity Criteria proposed in 1988. The only differences in the definitions used in the present study were that neutrophils were used instead of granulocytes and the definitions for nausea and vomiting were combined.

Statistical analysis

According to the minimax two-stage phase II study design by Simon [19], the treatment program was designed to refuse response rates of 20% and to provide a significance level of 0.05 with a statistical power of 80% in assessing the activity of the regimen as a 40% response rate. The upper limit for first-stage drug rejection was four responses among 18 evaluable patients; the upper limit of second-stage rejection was 10 responses among 33 evaluable patients. Overall survival was defined as the interval between enrolment in this study and death or the last follow-up visit. Median overall survival was estimated using the Kaplan–Meier analysis method [20].

Results

Patient characteristics

Between February 2000 and March 2002, 34 elderly patients with NSCLC were enrolled and 33 were treated in this study (Table 1). One patient did not receive the protocol treatment because the PS of the patient decreased before the start of the treatment and the patient no longer met the eligibility criteria. All treated patients were assessed for response, survival and toxicity. The median age of the patients was 77 years (range 75–86). The gender, PS and histology of the patients were as follows: 26 males, seven females; seven patients with PS 0, 26 patients with PS 1; 20 patients with adenocarcinoma, nine patients with squamous cell carcinoma, three patients with large cell carcinoma and one patient with NSCLC. Twenty-four patients had no prior treatment, five patients had undergone surgery, three patients had received radiotherapy for brain and/or bone metastases, and one patient had undergone both surgery and radiotherapy as prior treatments.

Treatment received and dose intensity

The total number of treatment cycles was 101 and the median was 3 (range 1–15). Two patients received only one course because of a decrease in their PS. Of the 33 treated patients, 12 patients received two courses, 13 received three and six received four or more. One patient received 15 courses; however, he received

Table 1. Characteristics of treated patients

No. of entered patients	34
No. of treated patients	33
Sex	
Male	26
Female	7
Age (years)	
Median	77
Range	75–86
PS (ECOG)	
0	7
1	26
Histology	
Adenocarcinoma	20
Squamous-cell carcinoma	9
Large-cell carcinoma	3
Non-small-cell	1
Stage	
IIIA	1
IIIB	9
IIIB with effusion	3
IV	17
Relapse	6
Prior treatment	
None	24
Radiotherapy	4
Surgery	6

PS (ECOG): performance status (Eastern Cooperative Oncology Group).

treatments on only days 1 and 15 of the fifth to fifteenth courses. Between the first and fourth cycles, 77–100% of the patients received treatments on days 8 and 15 treatment (Table 2). Of the 303 planned administrations, 272 (90%) were carried out.

The median actual dose intensities of docetaxel and cisplatin were 13.4 mg/m^2 (range 8.9–16.4) and 16.7 mg/m^2 (range 11.1–20.4) per week, whereas the projected dose intensities were 15.0 and 18.8 mg/m^2 per week for docetaxel and cisplatin, respectively.

Objective tumor response and overall survival

The objective tumor response is shown in Table 3. Two CRs and 15 PRs occurred for an objective response rate of 52% (95% CI 31% to 67%) in 33 treated patients. The overall survival periods of all treated patients are shown in Figure 1. The median survival time of the 33 treated patients was 15.8 months with a median follow-up time for 11 censored patients of 18.1 (15.2–35.5) months. The 1-year and 2-year survival rates were 64% and 26%, respectively.

Table 2. Treatment received

No. of treatment cycles	No. of patients	Treatment received on	
		Day 8	Day 15
1	33	31 (94%)	32 (97%)
2	31	28 (90%)	24 (77%)
3	19	19 (100%)	17 (89%)
4	6	5 (83%)	5 (83%)
5	2	1 (50%)	1 (50%)

Toxicity

The worst grades of hematological and non-hematological toxicities experienced by each patient are listed in Table 4. Both hematological and non-hematological toxicities were relatively mild. No grade 4 hematological or non-hematological toxicities were observed. Only grade 3 leukopenia (6%), neutropenia (12%), anemia (3%), hyponatremia (3%) and nausea/vomiting (3%) were observed. None of the patients received G-CSF. Renal toxicity was also relatively mild: grade 2 renal toxicity was observed in only one of 33 patients.

Discussion

We previously reported that classic standard cisplatin-based chemotherapy regimens cause severe myelotoxicity in elderly patients aged ≥75 years [5]. Based on that previous study of elderly patients with NSCLC, we conducted phase I studies in which cisplatin and docetaxel were administered as three consecutive weekly infusions in both non-elderly and elderly patients with NSCLC using the same eligibility criteria, except for age, and the same definitions of dose-limiting toxicity and maximum-tolerated dose [15]. Our hypothesis was that the recommended dose for elderly patients aged ≥75 years would differ from that for non-elderly patients. In the previous phase I studies, we demonstrated a difference in the recommended dose of docetaxel combined with cisplatin between non-elderly and elderly patients [15]. The recommended doses of docetaxel with 25 mg/m^2 cisplatin were 35 and 20 mg/m^2 on days 1, 8 and 15 for non-elderly and elderly patients, respectively. We also conducted phase II studies for non-elderly and elderly patients with NSCLC using each recommended dose and the same eligibility criteria, except for age. The

Table 3. Response rate

No. of patients	CR	PR	NC	PD	NE	Response rate (95% CI)
33	2	15	13	2	1	52% (31% to 67%)

CI, confidence interval; CR, complete response; NC, no change; NE, not evaluable; PD, progressive disease; PR, partial response.

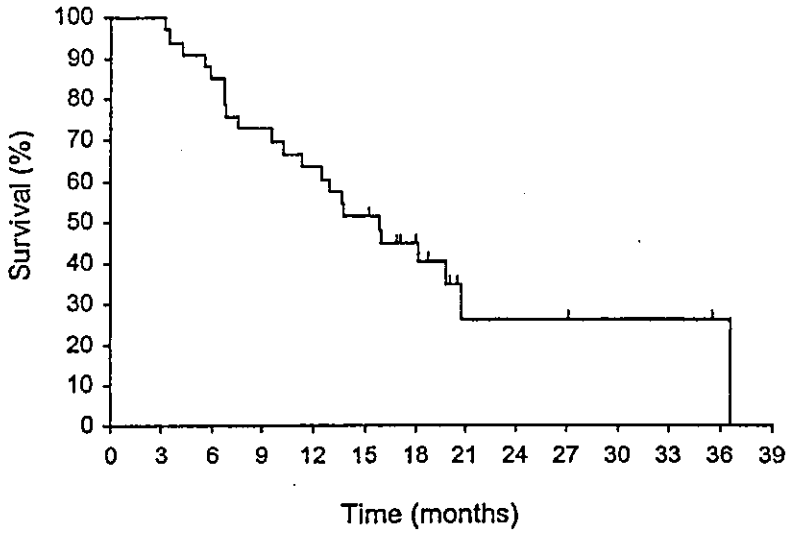

Figure 1. Overall survival time. The median survival time of the 33 treated patients was 15.8 months, and the median follow-up time for 11 censored patients was 18.1 (15.2–35.5) months. The 1-year and 2-year survival rates were 64% and 26%, respectively.

Table 4. Maximum toxicity grades associated with weekly docetaxel and cisplatin in 33 treated patients

	Grade (Japan Clinical Oncology Group)					Grade ≥3
	0	1	2	3	4	
Leukopenia	13	6	12	2	0	6%
Neutropenia	16	5	8	4	0	12%
Anemia	9	8	15	1	–	3%
Thrombocytopenia	30	2	1	0	0	0
Nausea/vomiting	12	10	10	1	–	3%
Hyponatremia	22	8	2	1	0	3%
Diarrhea	23	6	4	0	0	0
Infection	32	1	0	0	0	0
Fever	27	4	2	0	0	0
Bilirubin	25	–	8	0	0	0
Transaminase	25	8	0	0	0	0
Creatinine	28	4	1	0	0	0
Fatigue	26	6	1	0	0	0

results of the phase II study for non-elderly patients with NSCLC have been reported elsewhere [16]. Among the 33 evaluable patients, an objective tumor response of 30% (95% CI 15% to 46%) and a median survival time of 12.8 months were observed [16]. In the current study, we observed an objective tumor response of 52% (95% CI 31% to 67%) and a median survival time of 15.8 months for elderly patients with NSCLC. In spite of the lower dose of docetaxel, the efficacy of the treatment did not seem to be diminished.

Italian oncology groups have conducted randomized trials for elderly patients aged ≥70 years [21–23]. In these studies, non-platinum-based single or double chemotherapy regimens, such as vinorelbine alone or vinorelbine plus gemcitabine were used for elderly patients with NSCLC [21–23]. These chemotherapy regimens might not be adequate for non-elderly patients with a good PS because the cisplatin plus vinorelbine regimen was significantly superior to vinorelbine alone with regard to both the response rate and the survival [24, 25]. Kubota et al. [26] reported that the frequency of grade 4 leukocytopenia in the elderly (≥70 years of age) group was significantly greater than in the non-elderly group and that no difference in overall survival was observed between the two groups. Langer et al. [27] reported that advanced age alone

Table 5. Chemotherapy for elderly patients with non-small-cell lung cancer

Study	Chemotherapy	Age (years)	No. of patients	PS 2 (%)	Stage III (%)	RR (%)	MST
ELVIS [21]	None	≥70	78	24	28	–	21 weeks
	VNR 30 mg/m² days 1, 8 q3 weeks		76	24	26	20	28 weeks
	VNR 30 mg/m² days 1, 8 q3 weeks		233	19	29	18	36 weeks
MILES [22]	GEM 1200 mg/m² days 1, 8 q3 weeks	≥70	233	18	30	16	28 weeks
	GEM 1000 mg/m² + VNR 25 mg/m² days 1, 8 q3 weeks		232	19	31	21	30 weeks
SICOG [23]	VNR 30 mg/m² days 1, 8 q3 weeks	≥70	60	22	42	15	18 weeks
	GEM 1200 mg/m² + VNR 30 mg/m² days 1, 8 q3 weeks		60	27	40	22	29 weeks
MPCRN [29]	DTX 36 mg/m² weekly × 6 q8 weeks	≥65*	39	41	31	18	5 months
Current study	CDDP 25 mg/m² + DTX 20 mg/m² days 1, 8, 15 q4 weeks	≥75	33	0	29	52	15.8 months (69 weeks)

*Or poor candidates for combination chemotherapy due to coexistent medical illness.
ELVIS, The Elderly Lung Cancer Vinorelbine Italian Study; MILES, Multicenter Italian Lung Cancer in the Elderly Study; SICOG; Southern Italy Cooperative Oncology Group; MPCRN, Minnie Pearl Cancer Research Network.
CDDP, cisplatin; DTX, docetaxel; GEM, gemcitabine; VNR, vinorelbine.
MST, median survival time; PS, performance status; RR, response rate.

should not preclude appropriate NSCLC treatment, although elderly patients aged ≥70 years have more co-morbidities and can expect a higher incidence of leukopenia and neuropsychiatric toxicity. In the United States, upper age limits are not included in eligibility criteria to avoid age discrimination. In contrast, most Japanese studies have upper age limits because Japanese government guidelines recommend that elderly patients, >75 years, should not be accrued in common clinical trials [28]. This recommendation was made in concern for the safety of elderly patients. In Japan, most clinical trials include patients aged ≤74 years, and the full-dose chemotherapy is administered. Clinical trials for elderly patients have generally been conducted as specific trials focusing on the treatment of elderly patients in Japan. However, the definition of 'elderly' is still unclear. Thus, the use of platinum-based chemotherapy in elderly patients with NSCLC remains controversial because no randomized phase III studies have been conducted to resolve this question.

Several chemotherapy trials for elderly patients with NSCLC have been reported [21–23, 29] (Table 5). Of the subjects in these trials, 18–41% were PS 2 patients. Eligible patients were 70 or 65 years or older. The response rates of the non-platinum-based single or double chemotherapy regimens ranged from 15% to 22%, and the median survival times ranged from 18 to 36 weeks [21–23, 29]. In the current study, however, PS 2 patients were excluded and only patients aged ≥75 years were included. The objective response rate of 52% (95% CI 31% to 67%) and the median survival time of 15.8 months (69 weeks) in our trial were extremely better than those of previous trials. We considered that the main reason for the better results was the exclusion of PS 2 patients. However, cisplatin chemotherapy might be important not only for non-elderly, but also for elderly patients with NSCLC.

We divided the cisplatin and docetaxel dosages on days 1, 8 and 15 because full-dose cisplatin is too toxic for elderly patients. The weekly administration of docetaxel produces a higher dose intensity and less myelotoxicity [12–14]. Moreover, a weekly schedule may be safer than a 3-weekly schedule because treatment on day 8 and/or day 15 can be omitted if severe toxicity is observed. In the current study, the toxicity, including nausea/vomiting and renal toxicity, was relatively mild, and 90% of the planned administrations were carried out. The dose-limiting toxicities of docetaxel administered in six consecutive weekly infusions were reported to be fatigue and asthenia [12–14]. In the previous phase I study, two out of six patients refused chemotherapy on day 15 because of fatigue and asthenia at level 2: 25 mg/m² cisplatin and 25 mg/m² docetaxel [15]. However, fatigue and asthenia were relatively mild in the current study because of the relatively low-dose of docetaxel (20 mg/m²).

We conclude that cisplatin and docetaxel administered as three consecutive weekly infusions is very effective and safe for elderly patients with chemotherapy-naive NSCLC. The JCOG is conducting a phase III study of cisplatin and docetaxel versus docetaxel alone, administered as three consecutive weekly infusions, for elderly patients with NSCLC to examine the role of cisplatin in the treatment of elderly patients with NSCLC.

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