Detection of Microsatellite Alterations in Plasma DNA of Malignant Mucosal Melanoma Using Whole Genome Amplification

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Abstract

Malignant mucosal melanoma (MMM) still has the poorest prognosis. There is a paucity of molecular biological studies on MMM of the head and neck. We investigated free-circulating DNA microsatellites with loss of heterozygosity (LOH) in the blood of MMM patients. Cancer-related DNA is found in plasma, with cancer patients showing a higher level of free-circulating DNA than normal subjects. However, it is difficult to obtain sufficient amounts of such DNA for PCR analysis. We have searched for ways to improve all stages of such research, and detected new microsatellite alterations by triplicated whole genome amplification (WGA) and triplicated PCR amplification. In order to achieve a better understanding of the extent of the alterations affecting chromosomes we determined the occurrence of LOH at the following gene loci: D1S243, D6S311, D9S161, and D19S246; only 4 out of the 20 microsatellite markers usually used in MMM were used in this study. We determined LOH in 17 MMM patients.

It was possible to confirm LOH on at least one marker in 12 (70.6%) out of the 17 patients. Metastasis or recurrence was confirmed in 3 (17.6%) out of the 17 patients, and all of them were found to have LOH. LOH at microsatellite markers D1S243, D6S311, D9S161 and D19S246 in the plasma of these patients statistically correlated with MMM.

The results of this study suggest that these loci are suitable for identifying cancer-related DNA of MMM, and that analysis of LOH in plasma DNA released into the circulation may be useful as a screening tool.

Key words: Free-circulating DNA—Plasma DNA—Loss of heterozygosity (LOH)—Whole genome amplification (WGA)—Malignant mucosal melanoma
Introduction

Malignant mucosal melanomas (MMM) account for approximately 1% of all malignant melanomas, and exhibit far more aggressive behavior than that of skin melanomas. MMMs in the head and neck regions account for half of them, and occur mainly in the upper respiratory tract, oral cavity and pharynx. They are more prone to metastasising into regional and distant sites. At time of presentation and during clinical course, cervical lymph node metastases have been observed in 50% of such cases.\(^1,4,9,11,14,16,26,27,30\) Free-circulating DNA is reported to be associated with cancer. Tumor cells may release DNA into the circulation, resulting in increased quantities of free DNA in cancer patients.\(^19,31,33,34,39\) Such free-circulating DNA exhibits tumor-related alterations such as microsatellite alterations\(^5,24,33,36,40\), \(K\)-ras mutation\(^3,8\), \(p53\) mutation\(^8,33\), and aberrant promoter hypermethylation of tumor suppressor genes\(^8,10\) in a variety of malignancies, including breast cancer\(^5,33\), pancreatic cancer\(^3\), bladder cancer\(^40\), lung cancer\(^3,24\), and head and neck cancer\(^23,24\). Microsatellite alterations have been discovered in plasma DNA in malignant melanoma patients.\(^12,21,38\) Loss of heterozygosity (LOH) has been observed in certain loci in malignant melanoma\(^12,13,17,19,25,29,32,35,38\). It has been reported that allelic imbalance of microsatellites occurs in PCR amplification using small quantities of DNA\(^6\) and in PCR amplification from plasma, probably due to small amounts of template DNA\(^6\). Therefore, in order to analyze circulating DNA in plasma, it is necessary to establish a reliable method for detecting such small amounts of DNA.

Whole genome amplification (WGA) techniques based on multiple-displacement amplification (MDA) have been used for DNA amplification to obtain a homogeneous and high-quality DNA from small quantities of materials.\(^7\) The availability of this technique for assaying plasma DNA has significantly expanded the scope of the search for limited microsatellite alterations, and may lead to more reliable results. In this study, we searched for ways to improve all stages of such research, and tried to apply this method to the detection of tumor-related microsatellite alterations in patients with MMM of the head and neck using plasma DNA.

Materials and Methods

1. DNA samples from patients and control subjects

Seventeen patients with MMM in the head and neck attending the Hospital of the Research Center for Charged Particle Therapy, National Institute of Radiological Sciences (NIRS) between 2004 and 2005 took part in the study (Table 1). None of the patients underwent blood transfusion or chemotherapy. Peripheral venous blood samples (16 ml) were obtained from patients before radiotherapy in a vacutainer CPT Cell Preparation Tube (Becton Dickinson, Franklin Lakes, NJ). Blood was collected within 2 weeks before radiotherapy. Plasma was immediately separated from cells by centrifugation (500 x g; 15 min), and plasma and white blood cells were stored at \(-80^\circ C\) until further use. Blood samples from 15 healthy subjects were also obtained as controls to study detection of heterozygosity on each marker. Prior informed consent in writing to enrollment in the study was obtained from all patients and also from their families. This study was also approved by the institutional review boards of the Tokyo Dental College and NIRS.

2. DNA isolation and microsatellite analysis

Genomic DNA was extracted from whole blood of control subjects and white blood cells and plasma of MMM patients using Dr.GenTLE (TAKARA Bio Co., Shiga, Japan). DNA concentration was calculated using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies Inc., DE, USA). To evaluate quality, 100 ng isolated DNA from white blood cells and plasma was subjected to electrophoresis in 0.8% agarose gel at 100 CV for 30 min and stained using ethidium bromide to determine content. Twenty micro-
satellite markers on 8 different chromosomes were selected for analysis based on previous studies on malignant melanomas reporting LOH

\[\text{D1S243 (1p36.33), D1S1597 (1p36.21), D1S214 (1p36.31), D3S1293 (3p24.3), D6S474 (6q21), D6S305 (6q26), D6S262 (6q23.2), D6S264 (6q27), D6S311 (6q24.3), D9S942 (9p21.3), D9S157 (9p21.2), D9S161 (9p21.2), D9S156 (9p22.3), D10S2327 (10q22.3), D10S1213 (10q26.13), D11S897 (11q23.1), D11S990 (11q24.2), D11S900 (11q22.1), D13S796 (13q33.3), D19S246 (19q13.33) (Sigma-genosys, Tokyo, Japan).}

PCR amplification of these loci was performed in a 10 μl final volume containing 0.25 units HotStarTaq DNA polymerase (Qiagen Inc., Hilden, Germany) in 1× PCR buffer (50 mM KCl, 10 mM Tris-Cl [pH8.0], 1.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphate, 0.2 µM each primer, 2.5 mM MgCl₂, and 2.5 ng genomic DNA. Amplification protocol specified initial denaturation for 15 min at 94°C, 40 cycles of 20 s each at 94°C, 30 s at 62°C, 30 s at 72°C, followed by a final extension step of 72°C for 6 min according to the manufacturer’s protocol. PCR products were subjected to electrophoresis on a 3% denaturing agarose gel (SIGMA: A 6013 Type I: low EEQ, Sigma-Aldrich, Tokyo) containing 6.6% formaldehyde/MOPS at 50 V for 2 h each. After electrophoresis, the allelic band was visualized using GelStar Nucleic Acid Stain (TaKaRa Bio Co., Shiga, Japan).

3. DNA isolation from plasma of MMM patients and WGA (MDA method)

Because the quality and quantity of DNA isolated from plasma by the above-mentioned protocol was not satisfactory for microsatellite analysis, we changed isolation method and applied WGA to detect LOH with a PCR-based technique. DNA was re-extracted using QIAamp Blood Kit (Qiagen Inc., Hilden, Germany) from 1 ml plasma according to the manufacturer’s protocol and eluted in 50 μl elution buffer. WGA (REPLI-g Kit; Qiagen Inc., Hilden, Germany) was performed according to the manufacturer’s pro-

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M: Male, F: Female, *: After radiotherapy
tocol using plasma DNA of MMM. Briefly, 2.5 μl (<10 ng) DNA was mixed with 4× master mix (containing the reaction buffer, dNTP mixtures and hexamer primer) and phi29 DNA polymerase in a final volume of 50 μl. Reaction was completed after incubation for 16 h at 30°C, followed by incubation at 65°C for 3 min to inactivate the enzyme. Amounts of whole genome amplified DNA were quantified using the PicoGreen dsDNA Quantitation Assay Reagent Kit (Molecular Probes, Inc., Eugene, Oregon, USA).

4. Determination of LOH in plasma DNA of MMM patients

We selected 7 of the microsatellite markers which showed a high rate of heterozygosity out of the 20 markers, as described above for the WGA DNA samples. Because determination of DNA concentration by absorbance at 260 nm (A260) using NanoDrop ND-1000 Spectrophotometer was easily affected by the interference of contaminants, we changed the method of estimating DNA concentration. DNA concentration was estimated by the PicoGreen dsDNA Quantitation Assay Reagent Kit for both WGA DNA and DNA from white blood cells. DNA fragments of microsatellites were amplified by PCR in a 10 μl final volume containing 2.5 ng of whole-genome amplified DNA and genomic DNA isolated from white blood cells. PCR products were subjected to electrophoresis on 3% denaturing agarose gel containing 6.6% formaldehyde/MOPS. Electrophoresis and staining of PCR products was performed as described above. Allelic band intensity was measured with a Molecular Imager FX (Bio-Rad, Hercules, CA, USA), and analyzed using Phoretix software (Phoretix International, UK), followed by calculation of allelic ratios (long fragment/short fragment ratio or short fragment/long fragment ratio) in each of the plasma DNA and white blood cell DNA samples. We performed WGA in triplicate and PCR amplification in triplicate for each sample. Therefore, 9 kinds of each PCR product were obtained from WGA products of plasma DNA and white blood cell DNA. Allelic ratios were compared between allelic band obtained for white blood cells and plasma DNA. Only the microsatellite loci showing heterozygotic alleles in patients were used for LOH assessment, and all other loci were considered as being not informative (NI).

5. Statistical analysis

The data obtained in the 9 amplification runs from PCR were divided into 3 groups for which WGA was performed, and among those 3 groups the Kruskal-Wallis test was performed to determine tendency to dispersion of PCR. For those groups for which no significant difference was discovered at a confidence level of p<0.05, the Wilcoxon signed-rank test was used to compare white blood cell allele ratio and plasma allele ratio, calculated with Phoretix software. When no reproducible allelic balance was obtained among these 3 runs the result was taken as inaccurate. When a significant difference was detected at a confidence level of p<0.05, the result was taken as LOH (+), and when no significant difference was detectable, the result was taken as LOH (–).

Results

1. Microsatellite analysis of control samples and MMM patients

PCR amplification was performed using 2.5 ng DNA for control samples and the PCR products were compared using 3% denaturing agarose gel. Heterozygote genotypes were obtained in 7 out of the 20 loci that were used. These were 1 out of 15 samples for D1S214, 6 out of 15 for D1S243, 3 out of 15 for D3S1293, 5 out of 15 for D6S311, 2 out of 15 for D9S942, all for D9S161 and 8 out of 15 for D19S246.

Because 4 of these loci (D1S243, D6S311, D9S161, and D19S246) showed a higher rate of heterozygosity, we decided to use these loci for further analysis to detect LOH from 17 MMM patients. We initially amplified the D1S243 locus using DNA isolated from the plasma of the patients. However, it was diffi-
cult to detect PCR amplification products in the plasma DNA of MMM. Therefore, we examined whether PCR inhibitor was present in these samples. PCR amplification was carried out by dividing the template DNA into 3 groups: white blood cell DNA, mixed white blood cell DNA plus plasma DNA, and plasma DNA. While PCR products were detected after amplification of white blood cell DNA, no PCR products were detectable after amplification of the mixed white blood cell DNA plus plasma DNA, or plasma DNA. This suggested that the plasma DNA might contain PCR enzyme inhibitors.

We also investigated whether the same amounts of spectrophotometrically quantitated DNA from white blood cells and plasma showed similar band intensities in agarose gel electrophoresis. The intensity of the band in the plasma sample was substantially fainter than that of white blood cells. These results suggested that the plasma did not contain sufficient amounts of DNA for microsatellite analysis. Therefore, we decided to apply whole genome amplification for further analysis. Application of whole-genome amplified DNA greatly improved the PCR amplification efficiency of the microsatellite markers (Fig. 1).

2. Determination of LOH in plasma DNA of MMM patients

For the 17 patients with MMM in the head and neck, LOH allelic ratio was evaluated at 4 microsatellite loci with higher heterozygosity. With respect to the microsatellite loci for which heterozygotic patterns were detected, a Kruskal-Wallis test was applied to make a comparison among the 3 WGA experiments for the respective microsatellite markers. Results that were considered inaccurate were found in only 4 (12.1%) out of 33 loci. Typical examples of the results of Kruskal-Wallis test are shown in Fig. 2. When allelic ratios indicated statistically significant differences based on the Wilcoxon signed-rank test ($p<0.05$), LOH was scored. Typical examples of the results of microsatellite analysis using the Wilcoxon signed-rank test are shown in Fig. 3. LOH was confirmed in 1 (20.0%) out of 5 patients at the D1S243 loci (1p36.3), in 2 (50.0%) out of 4 patients at the D6S311 loci (6q24.3), in 11 (64.7%) out of 17 patients at the D9S161 loci (9p21.2), and in 1 (33.3%) out of 3 patients at the D19S246 loci (19q13.3) (Table 2).

Examinations to determine the presence of LOH resulted in the detection of LOH in 12 (70.6%) out of the total of 17 patients. In 3 out of the 17 patients, recurrence or metastas-
sis occurred, and all of them were found to have LOH. Out of the remaining 14 patients not found to have recurrence or metastasis, 9 had LOH. LOH was detected in 2 cases classified as T2, in 2 out of 4 cases classified as T3, in 6 out of 9 cases classified as T4, and in 2 cases with post-operative recurrence (Table 3).

Fig. 2 Comparison among three groups with WGA replication, using the Kruskal-Wallis test (p<0.05)
When a significant difference was detected at a confidence level of p<0.05 the result was taken as inaccuracy.
Case 4: inaccuracy (p<0.03), case 11: inaccuracy (p=0.05). The data are expressed as the mean ± SD of three independent experiments with samples in triplicate, =<: PCR1, =>: PCR2, ≥: PCR3.

Fig. 3 Comparison of allelic ratios between white blood cell and plasma DNA
When allelic ratios indicated statistically significant differences based on a Wilcoxon signed-rank test (p<0.05), LOH was scored. Case 1: LOH (+) (p=0.01), case 8: LOH (+) (p=0.05), case 12: LOH (−), case 15: LOH (−).
In this study, we detected microsatellite alterations in plasma DNA in 17 MMM patients. Some problems in terms of accuracy and reproducibility were encountered in determining LOH. To solve these difficulties, the first step was to verify the validity of our LOH detection method. Coulet et al. found allelic imbalance in their studies on plasma DNA using PCR amplification from samples with 2.0 ng or less DNA. Initially, we performed amplification using 2.5 ng DNA isolated from the whole blood of control subjects. Investigator...
tions of the PCR products obtained in both denaturing polyacrylamide gel and denaturing agarose produced stable results for 2.5 ng DNA amplification. With the 6% TBE urea polyacrylamide gel, it was not possible to amplify two allelic fragments of paternal and maternal origin, because many non-specific bands were observed after staining, so the tests were performed again with agarose gel.

In our initial microsatellite detection experiments using normal samples, heterozygosity was detected in 7 out of the 20 loci. We selected 4 markers with a higher heterozygosity from these 7 markers in the present study. One of the reasons many loci showed low heterozygosity in this study may have been the use of agarose gel. Some research institutions have found that DNA sequencers allowed a higher degree of separation in the detection of microsatellites. It may, therefore, be necessary to use such DNA sequencers for future studies.

We next performed PCR amplification using plasma DNA. However, no PCR products were obtained. This might be partly due to the presence of anticoagulants in the test tubes, the extraction method, or the low concentration of DNA in plasma. The anticoagulants included heparin, which has been reported to inhibit PCR enzyme reaction. Therefore, we considered how to eliminate inhibitory substances, and introduced a separating column for refining in the plasma DNA extraction process. Substitution of Dr. GenTLE with the QIAamp blood kit yielded a better result. However, it has also been reported that refining in a separating column carries the risk of DNA fragmentation. This means that another method of extraction is required. As we anticipated only a small amount of plasma DNA, it was necessary to check this amount. One hundred ng each of both white blood cell DNA and plasma DNA were, therefore, subjected to electrophoresis. Comparison of the intensity obtained by electrophoresis of plasma DNA with the intensity obtained by electrophoresis of white blood cell DNA showed that the lane intensity from plasma DNA electrophoresis had a smaller signal strength and a smaller concentration than had been determined by quantitative assay. The results revealed smaller concentration values than the calculated values for plasma DNA. Errors in concentration measurements frequently occur when non-DNA impurities are present or when DNA quantities are too small. At this stage, however, it was not possible to determine DNA concentration with any greater level of accuracy.

In recent years, WGA based on MDA has been used as a method of obtaining homogeneous, high-quality amplification using small quantities of DNA. Lu et al. have reported that they achieved favorable results in their SNP detection experiments using plasma DNA amplified with the MDA technique. Furthermore, Dean et al. have also carried out comparative studies on WGA methods and found that MDA was more reliable than primer extension preamplification (PEP) or degenerate oligonucleotide primed PCR (DOP-PCR). In this study, we also performed WGA using MDA prior to PCR. We used plasma DNA to detect microsatellite alterations, and obtained an average of 30 μg DNA from extremely small amount of samples. Although only a limited amount of DNA could be obtained from the plasma, this method made it possible to search for a larger range of microsatellite locations.

Many studies have utilized free DNA in the serum and plasma to establish a prognosis. Some studies have found that the incidence of LOH increased with progress in tumor growth. In this study, we investigated microsatellite alterations by using WGA from a different perspective than that in these previous reports. All of the patients diagnosed as having metastasis or recurrence were found to have LOH.

Some reports have pointed out the unreliability of results associated with PCR amplification from plasma DNA. To overcome this problem we conducted a final assessment based on triplicate WGA and triplicate PCR amplification. The PCR experiments were conducted 3 times using white blood cell DNA. The results demonstrated
that there were no significant differences in the allelic ratios. Although a certain amplification bias (inaccuracy) was discovered in the process of WGA, the values obtained were generally appropriate. Many researchers warn of allele-drop\textsuperscript{2,15,37}. However, our investigations with this method lead us to believe that it allows adequate evaluation of LOH analysis in plasma DNA. In order to evaluate the reproducibility of WGA in this kind of study, comparison of the status of microsatellites between in DNA isolated from tumor tissues and in whole-genome amplified DNA from plasma should be conducted in future study.

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**References**


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