Original Article

Detection of Tumor DNA in Plasma Using Whole Genome Amplification

Daisuke Nakamoto, Nobuharu Yamamoto, Ryo Takagi, Akira Katakura, Jun-etsu Mizoe* and Takahiko Shibahara

Department of Oral and Maxillofacial Surgery, Tokyo Dental College,
1-2-2 Masago, Mihama-ku, Chiba 261-8502, Japan
* Hospital, Research Center for Charged Particle Therapy,
National Institute of Radiological Sciences (NIRS),
4-9-1 Anagawa, Inage-ku, Chiba 263-8555, Japan

Abstract

Altered microsatellite DNA in the blood of cancer patients may provide a novel means for tumor detection. Such alterations are a major characteristic of many types of tumor especially those associated with head or neck cancer. Moreover, recent evidence suggests that senescent tumor cells release DNA into the circulation, which is subsequently carried by the blood and thus enriched in the serum and plasma. We tested 10 head and neck cancer patients (5 with malignant melanomas (MM) and 5 with adenoid cystic carcinomas (ACC)) by polymerase chain reaction (PCR)-based microsatellite analysis of DNA from white blood cells and paired plasma samples. Our goal was to amplify two microsatellite markers, D1S243 and D19S246, which sometimes show microsatellite alterations in head and neck cancer patients. However amplification of fragments from three loci in the plasma samples proved impossible, probably due to the small amounts of DNA isolated. We used multiple displacement amplification (MDA) to amplify genomic DNA from the plasma samples. Two microsatellite fragments were amplified from whole genome amplified DNA. Among 5 heterozygote samples, 3 showed the same pattern in DNA samples from both blood cells and plasma but 2 showed loss of heterozygosity (LOH). Although further study is necessary to confirm whether the LOH found in this study reflects alteration in circulating tumor cell DNA, application of whole genome amplification may allow DNA analysis from limited amounts of such DNA and provide a minimally invasive diagnostic procedure and useful aid in therapy.

Key words: Malignant melanoma—Adenoid cystic carcinoma—
               Loss of heterozygosity (LOH)—Plasma or serum DNA—
               Circulating tumor DNA

Introduction

A holy grail for many clinical investigators has been the development of a blood test for cancer. Two articles in *Nature Medicine* represent an important advance in this quest

that may lead to a refinement in the way cancer patients are managed. Nawroz *et al.*[18] and Chen *et al.*[5] reported that tumor DNA from head and neck squamous cell carcinomas (HNSCC)[18] and small cell lung cancers (SCLC)[5] was detectable in the plasma of patients with these tumors, and suggested that structural abnormalities in tumor DNA may provide important prognostic information for the treatment of cancer patients.

Because head and neck cancers, if undetected, tend to metastasize to solitary lung tumors, the development of an early, highly sensitive detection method for metastasis is of paramount importance in achieving successful treatment in patients with head and neck cancer. While reliable methods for the prediction of cancer recurrence and metastasis would be of the greatest clinical importance, they are not available at present. The specific clinical signs and symptoms indicative of metastasis are not usually helpful for an early diagnosis as they are usually associated with the more advanced stages of the disease. Nor are the results of diagnostic imaging or imaging studies sufficiently reliable. Moreover, large-scale screening programs for this particular patient population are not feasible in practical terms and would at best only result in the incidental discovery of small tumors[1]. Therefore, development of a noninvasive method for early detection of head and neck cancer and the establishment of more reliable criteria for deciding further therapy would be a major advance in the clinical management of this patient population. Much interest has focused on the potential use of nucleic acid markers in the blood of patients with cancer as they offer material that is easy to access by minimally invasive procedures. Furthermore, microsatellite alterations in the tumor and/or plasma could offer a prognostic indicator. Microsatellite analysis of circulating nucleic acids represents a new approach in the search for tumor markers. Their widespread application and clinical relationship with malignant phenotype will likely give them an increasing clinical importance in the future[14].

Microsatellite analysis is a PCR-based technique that permits the detection of cancer-specific DNA alterations, loss of heterozygosity (LOH), and microsatellite instability (MSI) in neoplastic tissue[16,19]. LOH and MSI have been detected in the circulating DNA of patients with a variety of malignancies such as non-small cell lung cancer[21], renal cell carcinoma[10], bladder cancer[25], breast cancer[20], colon cancer[7], malignant melanoma[23], and oral cancer[11].

In the present study, we applied whole genome amplification (WGA) by multiple displacement amplification to detect microsatellite alteration in limited amounts of tumor cell-derived DNA.

Materials and Methods

1. Patients

Patients consisted of 10 Japanese persons with head and neck cancer (5 with malignant melanomas and 5 with adenoid cystic carcinomas) who visited the Hospital of the Research Center for Charged Particle Therapy, NIRS over a 4-year period between 2001 and 2004 (Table 1). No patients underwent a blood transfusion. Informed consent was obtained from all patients and also from their families. This study was also approved by the institutional review board of Tokyo Dental College and NIRS. Clinicopathologic staging was determined by the TNM classification of the International Union against Cancer. Of the 10 patients with head and neck cancer, 4 were at stage III and 6 were in stage IV.

2. Plasma sample collection and DNA isolation

Peripheral venous blood samples (16 ml) were obtained from patients before radiotherapy or at 4 weeks after radiotherapy in vacutainer CPT tubes (Becton Dickinson, Franklin Lakes, NJ). Plasma was immediately separated from cells by centrifugation ($500 \times g$; 15 min), and plasma and white blood cells were stored at $-80°C$ until further use. Plasma DNA was extracted using QIAamp Blood Kit

Table 1   Microsatellite analysis of plasma or serum tumor DNA and clinical data concerning in 10 head and neck cancer patients

Patient	Pathological diagnosis	Age	Gender	Site	T	N	Stage	Drawing blood	F/U	Microsatellite alteration	Clinical outcome
1	MM	68	F	Nasal	3	0	III	Before	13	NI	Survival
2	MM	63	M	Nasal	3	0	IV	After	39	LOH (−)	Survival
3	MM	64	M	Paranasal	4	0	IV	After	38	LOH (−)	Survival
4	MM	29	M	Frontal sinus	4	0	IV	After	10	LOH (+)	Death
5	MM	68	F	Nasal	3	0	III	After	8	NI	Death
6	ACC	49	F	Maxillary sinus	3	0	III	Before	21	NI	Survival
7	ACC	55	M	Ethmoidal sinus	4	0	IV	After	24	LOH (+)	Survival
8	ACC	60	M	Nasopharynx	4	0	IV	Before	18	NI	Survival
9	ACC	69	M	Parotid gland	4	0	IV	After	15	LOH (−)	Survival
10	ACC	79	M	Maxillary sinus	4	0	IV	After	21	NI	Survival

MM: Malignant melanoma, ACC: Adenoid cystic carcinoma, M: Male, F: Female, Before: Before irradiation, After: After irradiation, F/U: Follow-up in months, NI: Not informative, LOH: Loss of heterozygosity

(Qiagen, Hilden, Germany) according to the manufacturer's protocol. Control DNA was extracted from white blood cells using Dr. GenTLE™ Systems (TaKaRa, Tokyo, Japan).

3. WGA by multiple displacement amplification (MDA)

WGA was performed with the REPLI-g kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol using plasma DNA. Briefly, $2.5\,\mu l$ plasma DNA was mixed with $4\times$ master mix (containing the reaction buffer, dNTP mixtures and hexamer primers) and phi29 DNA polymerase in $50\,\mu l$ final volume. Reaction was completed after incubation for 16 hr at 30°C, followed by incubation at 65°C for 3 min to inactivate the enzyme.

4. Microsatellite markers, PCR and LOH analysis

LOH analysis of the plasma was performed using two microsatellite markers on two different chromosomes, D1S243 (1p-q) and D19S246 (19q13.3). These were selected based on previous studies on malignant melanomas and adenoid cystic carcinomas[9,26] demonstrating LOH at each loci. All of the primer sets were obtained from Research Genetics (Huntsville, AL). DNA fragments were amplified by polymerase chain reaction (PCR) in a $10\,\mu l$ final volume containing 0.25

units HotStarTaq DNA polymerase (Qiagen, Hilden, Germany) in $10\times$ PCR buffer (50 mM KCL, 10 mM Tris-Cl [pH 8.0], 1.5 mM $MgCl_2$), 0.2 mM deoxynucleotide triphosphate, $0.2\,\mu M$ of each primer, 2.5 mM $MgCl_2$ and 2.5 ng genomic DNA. The manufacturer's amplification protocol specified initial denaturation for 15 min at 94°C, 40 cycles of 20 sec each at 94°C, 30 sec at 62°C, 30 sec at 72°C, followed by a final extension step of 72°C for 6 min. PCR products were electrophoresed on 6% polyacrylamid gel containing 7 M urea at 100 V for 2 hr, or 3% denaturing agarose gel (SIGMA: A 6013 Type I: low EEO) containing 6.6% formaldehyde/MOPS at 50 V for 2 hr. After electrophoresis, allelic band intensity was detected with GelStar Nucleic Acid Stain (TaKaRa Bio, Tokyo, Japan), measured by a Molecular Imager FX (Bio-Rad, Hercules, CA, USA), and analyzed by Phoretix software (Phoretix International, UK). This was followed by calculation of allelic ratios in each of the plasma samples (tumors) and white blood cell samples (normal controls). Our goal was to amplify plasma DNA in triplicate WGA and amplify DNA fragments in triplicate PCR reactions for each whole genome amplified product. Finally, WGA products (plasma DNA) and white blood cell samples were amplified 9 times each by PCR. Allelic ratios were compared between white blood cell and
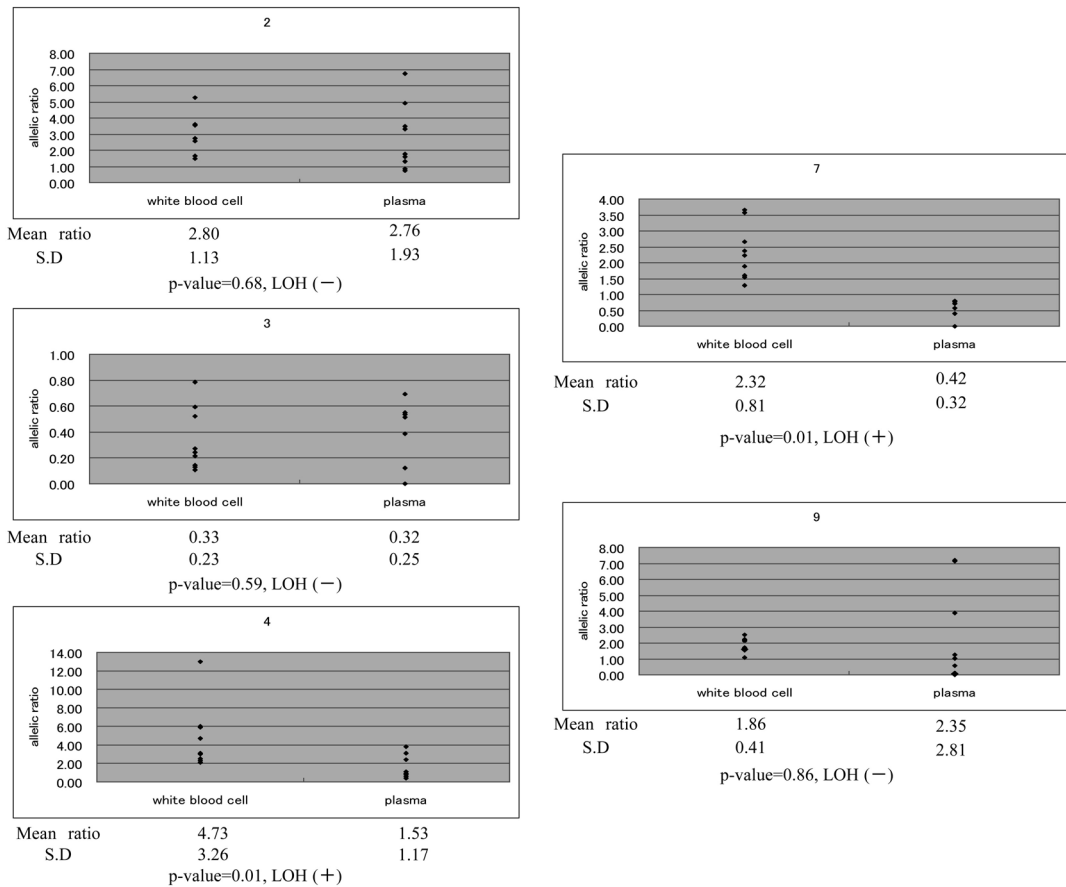
Fig. 1   Comparison of allelic ratios between white blood cell and plasma DNA, from three patients (2, 3, and 4) diagnosed with malignant melanoma on D1S243, and two patients (7 and 9) diagnosed with adenoid cystic carcinoma on D19S246

patient 2: LOH ($-$), patient 3: LOH ($-$), patient 4: LOH ($+$) (p$=$0.01), patient 7: LOH ($+$) (p$=$0.01), and patient 9: LOH ($-$)

plasma DNA.

5. Assessment of LOH

Microsatellite regions for which heterozygosity was detected from the white blood cell samples were used for assessment of LOH. Homozygotes were considered to be not informative (NI). The data obtained in the nine amplification runs from PCR were assessed using the Wilcoxon signed-rank test. The Wilcoxon signed-rank test was used to compare the WBC and plasma allelic ratios calculated with the Phoretix software. When a significant difference was detected at a confidence level of p<0.05 the result was

taken to represent a LOH ($+$) and when no significant difference was detectable the result was taken to represent LOH ($-$).

Results

DNA fragments were amplified by PCR using $1.0\,\mu$l isolated DNA (2.5 ng as DNA). It was possible to detect PCR products from white blood cell DNA, but not from plasma DNA. PCR amplification was therefore performed after WGA in plasma. When WGA was performed using $2.5\,\mu$l plasma DNA it became possible to detect PCR products.
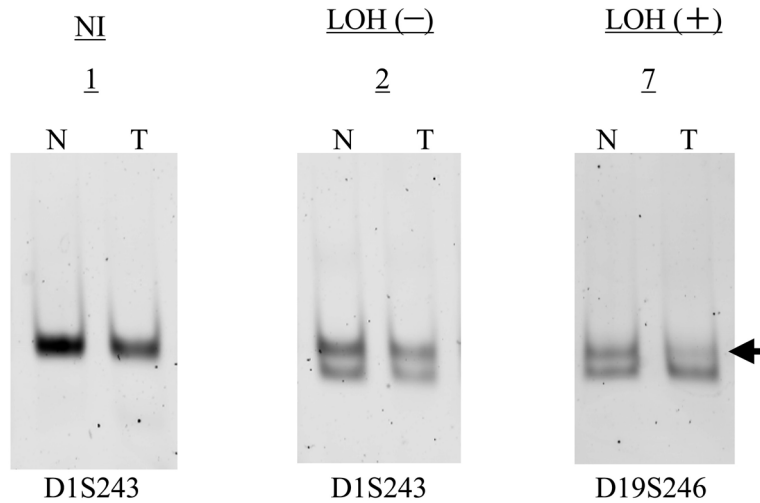
Fig. 2 Typical examples of the microsatellite analysis of plasma or serum (T), and white blood cell (N) DNA of head and neck cancer patients. Patients numbers are shown above, and microsatellite markers are designated below each block.

Paired normal (N) and tumor (T) samples suggesting loss of the upper allele (LOH (+), patient 7), showing no evidence of LOH (LOH (−), patient 2), and not informative (NI, patient 1).
N: Normal DNA (white blood cell), T: Tumor DNA (plasma), LOH: Loss of heterozygosity, NI: Not informative

Heterozygote genotypes were obtained in 3 out of 5 malignant melanomas at the D1S243 locus and 2 out of 5 adenoid cystic carcinomas at the D19S246 locus and LOH was confirmed in one of the former and in two of the latter (Fig. 1).

In this study, plasma and serum DNA alterations showed no correlation with clinical parameters such as age, TNM stage, or lymph node status. Typical examples of the results of microsatellite analysis are shown in Fig. 2.

Discussion

Clinical trials of carbon ion radiotherapy for various cancers were initiated in June 1994 at the Hospital of the Research Center for Charged Particle Therapy, NIRS in Chiba, Japan, using the world's first Heavy Ion Medical Accelerator in Chiba (HIMAC) dedicated to medical purposes[24]. Heavy charged particles such as carbon and neon ions excel in their physical dose distribution and high biological effectiveness. Preliminary results of phase II clinical trials have shown extremely favorable therapeutic results in the treatment of tumors otherwise intractable with conventional photon radiation[15,17]. Malignant melanoma and adenoid cystic carcinoma are two malignant tumors with the poorest prognosis even today.

Identifying free DNA from tumor cells would provide a very effective means of diagnosing early metastasis and assessing the outcome of therapy. The presence of tumor DNA in the bloodstream may be due to lysis of circulating cancer cells or micrometastatic cells, because of DNA leakage resulting from tumor necrosis or apoptosis[2,4,5,8,18]. In this study measurement of DNA concentrations after extracting plasma DNA showed that it was undetectable spectrophotometrically in samples and PCR amplification of microsatellites was unsuccessful. Recently, multiple displacement amplification (MDA) has been developed as a technique for WGA from very small amounts of DNA and has been reported to yield large quantities of high quality DNA[6,13]. We performed microsatellite

analysis using plasma DNA amplified by MDA. Research on LOH in various head and neck cancers has shown evidence of tissue type-specific microsatellite alterations.

Analysis of DNA isolated from plasma revealed that 2 of the 10 patients had LOHs in one of two loci. It has already been reported that the LOH at the D1S243 locus occurred in malignant melanoma patients, and that LOH at the D19S246 locus occurred in adenoid cystic carcinoma patients[9,26]. Although we could not confirm whether the LOH found in this study could also be detected in tumor tissues, it is possible it was derived from tumor DNA considering the aforementioned reports. In one LOH (+) patient, LOH was detected 4 weeks after carbon ion radiotherapy. If detection of such LOH from plasma suggests incomplete removal or destruction of tumor tissues by surgery or other treatment then determination of LOH would provide an important indicator for prognosis.

Bergen *et al.*[3], Hanson and Ballantyne[12], and Sun *et al.*[22] reported that use of small amounts of DNA as starting materials for WGA sometimes resulted in allelic imbalance or allele drop following PCR amplification. Therefore, we can not exclude the possibility at the moment that the LOH found in this study was induced by WGA.

In order to apply WGA to the clinical diagnosis and therapy of cancer patients it is necessary to prove that LOH in microsatellite markers detected using whole genome amplified DNA from plasma always reflects the status of the tumor. Further studies investigating more microsatellite markers will be required to clarify the usefulness of this procedure in the noninvasive screening of cancer.

Acknowledgements

References

1) Akiyama H, Tsurumaru M, Udagawa H, Kajiyama Y (1997) Esophageal cancer. Curr Probl Surg 34:767–834.

2) Anker P, Lefort F, Vasioukhin V, Lyautey J, Lederrey C, Chen XQ, Stroun M, Mulcahy HE, Farthing MJG (1997) K-ras gene mutations in the plasma of colorectal cancer patients. Gastroenterology 112:1114–1120.

3) Bergen AW, Qi Y, Haque KA, Welch RA, Chanock SJ (2005) Effects of DNA mass on multiple displacement whole genome amplification and genotyping performance. BMC Biotechnol 5:24.

4) Chen XQ, Bonnefoi H, Diebold-Berger S, Lyautey V, Lederrey C, Faltin-Traub E, Stroun M, Anker P (1999) Detecting tumor-related alterations in plasma or serum DNA of patients diagnosed with breast cancer. Clin Cancer Res 5:2297–2303.

5) Chen XQ, Stroun M, Magnenat JL, Nicod LP, Kurt AM, Lyautey J, Lederrey C, Anker P (1996) Microsatellite alterations in plasma DNA of small cell lung cancer patients. Nature Med 2:1033–1035.

6) Dean FB, Hosono S, Fang L, Wu X, Faruqi AF, Bray-Ward P, Sun Z, Zong Q, Du Y, Du J, Driscoll M, Song W, Kingsmore SF, Egholm M, Lasken RS (2002) Comprehensive human genome amplification using multiple displacement amplification. Proc Natl Acad Sci USA 99:5261–5265.

7) Diep CB, Thorstensen L, Meling GI, Skovlund E, Rognum TO, Lothe RA (2003) Genetic tumor markers with prognostic impact in Dukes' stages B and C colorectal cancer patients. J Clin Oncol 21:820–829.

8) Eisenberger CF, Schoenberg M, Enger C, Hortopan S, Shah S, Chow NH, Marshall FF, Sidransky D (1999) Diagnosis of renal cancer by molecular urinalysis. J Natl Cancer Inst (Bethesda) 91:2028–2032.

9) El-Rifai W, Rutherford S, Knuutila S, Frierson HF Jr, Moskaluk CA (2001) Novel DNA copy number losses in chromosome 12q12-q13 in adenoid cystic carcinoma. Neoplasia 3:173–178.

10) Gonzalgo ML, Eisenberger CF, Lee SM, Trock BJ, Marshall FF, Hortopan S, Sidransky D, Schoenberg MP (2002) Prognostic significance of preoperative molecular serum analysis in renal cancer. Clin Cancer Res 8:1878–1881.

11) Hamana K, Uzawa K, Ogawara K, Shiiba M, Bukawa H, Yokoe H, Tanzawa H (2005) Monitoring of circulating tumour-associated DNA as a prognostic tool for oral squamous cell carcinoma. Br J Cancer 92:2181–2184.

12) Hanson EK, Ballantyne J (2005) Whole genome

amplification strategy for forensic genetic analysis using single or few cell equivalents of genomic DNA. Anal Biochem 346:246–257.

13) Hosono S, Faruqi AF, Dean FB, Du Y, Sun Z, Wu X, Du J, Kingsmore SF, Egholm M, Lasken RS (2003) Unbiased whole-genome amplification directly from clinical samples. Genome Res 13:954–964.

14) Johnson PJ, Lo YMD (2002) Plasma nucleic acids in the diagnosis and management of malignant disease. Clin Chem 48:1186–1193.

15) Kamada T, Tsujii H, Tsuji H, Yanagi T, Mizoe J, Miyamoto T, Kato H, Yamada S, Morita S, Yoshikawa K, Kandatsu S, Tateishi A (2002) Efficacy and safety of carbon ion radiotherapy in bone and soft tissue sarcoma. J Clin Oncol 20:4466–4471.

16) Mao L, Lee DJ, Tockman MS, Erozan YS, Askin F, Sidransky D (1994) Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci USA 91:9871–9875.

17) Miyamoto T, Yamamoto N, Nishimura H, Koto M, Tsujii H, Mizoe J, Kamada T, Kato H, Yamada S, Morita S, Yoshikawa K, Kandatsu S, Fujisawa T (2003) Carbon ion radiotherapy for stage I non-small cell lung cancer. Radiother Oncol 66:127–140.

18) Nawroz H, Koch W, Anker P, Stroum M, Sidransky D (1996) Microsatellite alterations in serum DNA of head and neck cancer patients. Nature Med 2:1035–1037.

19) Sidransky D (1997) Nucleic acid based methods for the detection of cancer. Science 278:1054–1058.

20) Silva JM, Dominguez G, Garcia JM, Gonzalez R, Villanueva MJ, Navarro F, Provencio M, San Martin S, Espana P, Bonilla F (1999) Presence of tumor DNA in plasma of breast cancer patients: clinicopathological correlations. Cancer Res 59:3251–3256.

21) Sozzi G, Musso K, Ratcliffe C, Goldstraw P, Pierotti MA, Pastorino U (1999) Detection of microsatellite alterations in plasma DNA of non-small cell lung cancer patients: a prospect for early diagnosis. Clin Cancer Res 5:2689–2692.

22) Sun G, Kaushal R, Pal P, Wolujewicz M, Smelser D, Cheng H, Lu M, Chakraborty R, Jin L, Deka R (2005) Whole-genome amplification: relative efficiencies of the current methods. Leg Med (Tokyo) 7:279–286.

23) Taback B, O'Day SJ, Boasberg PD, Shu S, Founier P, Elashoff R, Wang HJ, Hoon DS (2004) Circulating DNA microsatellites: molecular determinants of response to biochemotherapy in patients with metastatic melanoma. J Natl Cancer Inst 21:152–156.

24) Tsujii H, Morita S, Miyamoto T, Mizoe J, Kamada T, Kato H, Tsuji H, Yamada S, Yamamoto N, Murata K (2002) Experiences of carbon ion radiotherapy at NIRS, Proc. of 7th International Meeting on Progress in Radio-Oncology ICRO/OGRO 7, Kogelnik HD, Sedlmayer F eds., pp. 393–405, Monduzzi Editore, Austria.

25) Utting M, Werner W, Dahse R, Schubert J, Junker K (2002) Microsatellite analysis of free tumor DNA in urine, serum, and plasma of patients: a minimally invasive method for the detection of bladder cancer. Clin Cancer Res 8:35–40.

26) Walker GJ, Palmer JM, Walters MK, Hayward NK (1995) A genetic model of melanoma tumorigenesis based on allelic losses. Genes Chromosomes Cancer 12:134–141.

Reprint requests to:
    Prof. Takahiko Shibahara
    Department of Oral and
    Maxillofacial Surgery,
    Tokyo Dental College,
    1-2-2 Masago, Mihama-ku,
    Chiba 261-8502, Japan
    E-mail: sibahara@tdc.ac.jp