Molecular Detection of Cancer Cells in Saliva from Oral and Pharyngeal Cancer Patients

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A key to improve the prognosis of head and neck cancers is an early diagnosis of the disease. No screening method to detect these cancers has been developed yet. Molecular techniques using polymerase chain reaction are a sensitive method to detect a small population of cancer cells among normal cells. We conducted a series of microsatellite analysis to detect cancer cells in saliva from 23 oral and pharyngeal cancer patients. Eight microsatellite markers were selected to test for microsatellite instability (MSI) in the tumor and saliva samples. Of 23 samples, 5 (22 %) had MSI in the tumor samples. In 4 of 5 (80 %) MSI positive samples, we detected the identical MSI in saliva. The possibility of the molecular screening and molecular follow-up is discussed.

Key words : saliva, oral cancer, pharyngeal cancer, microsatellite instability
Abbreviations used : PCR − Polymerase Chain Reaction. MSI − Microsatellite Instability.

INTRODUCTION

Functional defects after a resection of oral and pharyngeal cancers affect the quality of life (QOL) of the patients [1, 2]. Early detection of oral and pharyngeal cancers can improve the prognosis and, furthermore, it minimizes the defects after radical operation and contributes to the QOL after the operation.

The diagnosis of cancers is established by an invasive method of biopsy. No screening method for head and neck cancers has been developed yet. In this study, we applied a molecular method to detect cancer cells in the saliva samples taken from oral and pharyngeal cancer patients. We used microsatellite instability (MSI) as a marker for the cancer cells. MSI can be detected by microsatellite analysis that is a very sensitive polymerase chain reaction (PCR)-based method [3-5].

In the present study, we were able to find the identical MSI in the saliva samples containing a small number of cancer cells as well as in the tumor samples. In one case, we could follow up a relapse-free patient with saliva, which exhibited no microsatellite abnormality after the treatment. The high sensitivity of this method encouraged us in the trials of the molecular follow-up and molecular screening [6].

MATERIALS AND METHODS

Patients and sample collection

Tumors from 23 patients with oral or pharyngeal cancers were enrolled in this study. All tumors were surgically resected at Yamaguchi University Hospital. Tumors included 12 oral, 6 oro-pharyngeal, and 5 hypopharyngeal cancers.

The tumor tissue specimens were fresh frozen and microdissected to remove surrounding normal tissues. Sections were digested with 1 % SDS/proteinase K, and tumor DNA was extracted as previously described [7, 8]. Normal control DNA was obtained from peripheral lymphocytes and processed in the identical manner as the tumor samples.

Saliva samples from the patients were collected before resection by gargling with 10 ml of normal saline or swabbing on

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the tumors. Samples were fresh frozen and processed to extract DNA. The saliva samples were centrifuged at 2500 rpm for 10 min. The cell pellet was digested and DNA was extracted. In some cases, saliva was collected several months after the operation to follow up the mirosatellite alteration.

Five control saliva samples were collected from tumor free subjects with chronic tonsillitis.

Microsatellite analysis

Tumor and control DNA was examined for MSI by PCR-based microsatellite analysis as described previously [4, 6-9]. Eight microsatellite markers were chosen according to the previous reports [4, 10, 11-13] and are listed in Table 1. They have high incidences of microsatellite abnormalities, such as loss of heterozygosity (LOH) or MSI in head and neck cancers. One primer from each primer pair was labeled with [γ-^{32}P] ATP with T4 kinase. PCR was performed in a total volume of 10 μl containing 25 ng genomic DNA, 20 ng of each primer, and 0.5 unit of Taq polymerase. PCR amplification was performed with 35 cycles of denaturation at 95 ℃ for 30 s, annealing at 55-58 ℃ for 60 s, and extension at 72 ℃ for 60 s. PCR products were separated on urea/polyacrylamide/formamide gels followed by autoradiography. MSI was considered positive when a novel band was observed at any one microstellite marker in the sample compared with the normal control DNA. In the MSI-positive cases, the DNA from the saliva sample was analyzed for MSI in the identical manner. The results from the tumor and saliva samples were compared for the identity of MSI.

RESULTS

Results of the microsatellite analysis are summarized in Table 2. All 23 cases showed LOH in at least one marker, ranging from 1 to 5 markers. Five of 23 cases (21 %) displayed MSI. All of these MSI positive cases

Table 1 Microsatellite markers used in this series

Markers	Location	Repeat sequences	Size
D3S1289	3p21-23	(CA)23	197-215
D3S1300	3p21.1-14.2	(CA)22	217-241
D8S320	8	(AAAG)19	380-440
D8S321	8q24.13-qter	(AAAG)12	221-245
D9S242	9q32-33	(AAAG)26(AAGG)3	148-208
D11S488	11q24.1-25	(AAAG)14(GAAG)10	243-195
D20S82	20	(AAAG)10	250
D20S85	20	(AAAG)12	120

Table 2 Results of the microsatellite analysis

Markers	Informative cases	LOH	MSI
D3S1289	19	13	0
D3S1300	16	11	0
D8S320	14	4	0
D8S321	18	4	2 (2)*
D9S242	20	12	3 (2)*
D11S488	12	5	0
D20S82	13	2	0
D20S85	18	3	0

In 5 out of 23 (2 cases in D8S321 and 3 cases in D9S242), MSI was demonstrated. In 4 cases (asterisk), MSI was also detected in the saliva samples.

had LOH in the other multiple markers with a range of 1-4 markers. The frequency of LOH was not different between MSI-positive and negative groups.

MSI was detected in 3 oral cancers, 1 tonsil cancer, and 1 hypopharyngeal cancer. There was no significant correlation between MSI and clinico-pathological features, such as the site of the tumor, the tumor stage, smoking habit, or having double cancer (data not shown). Among the 5 MSI-positive cases, 4 cases (80 %) had similar MSI in the saliva samples. Representative autoradiographs are shown in Fig. 1-2. The site or the size of the tumor did not affect the results of the saliva samples. Five control saliva samples did not show any LOH or MSI.

In one tongue case (#52), saliva was collected during the relapse-free follow-up period 6 months after the surgery. The preoperatively detected MSI was not found in the follow-up saliva sample (Fig. 3). This implied that the preoperatively detected tumor clone disappeared after the operation. In the other 4 MSI positive cases, we could not collect the follow-up saliva samples because the patients either dropped out from the follow-up study or died of the disease.

DISCUSSION

Microsatellite instability in head and neck cancers

MSI in head and neck cancers was discussed in several reports previously [6, 11, 12, 14, and 15]. In those studies, MSI was found with various incidences ranging from 7 to 57 %. Based on the previous study, we chose 8 microsatellite markers to detect MSI. In one study [6], tetranucletide repeat markers with the (AAAG)n sequences were reported to have high incidence of MSI. The authors used 23 markers and detected MSI in 25 out of 44 (57 %) tumors. The frequency of MSI highly depends on the number and selection of the markers. Proper markers should be selected to detect more MSI.

Previous studies reported that MSI was related to the cancer progression and prognosis of the colon, prostate, pancreatic, and gastric cancers [16-18]. In this study, we found no correlation between MSI and clinicopathological findings. Most of the previous reports on head and neck cancers also showed no correlation between MSI and any clinicopathological features [11, 12, and
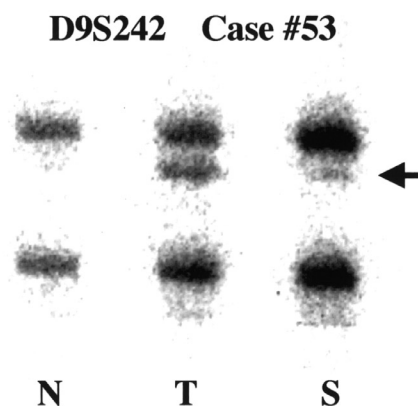

Fig. 1 Microsatellite instability of Case # 53. Tumor sample had microsatellite instability (MSI). MSI was also detected in the saliva sample (S). N, normal lymphocytes; T, tumor; S, mouth washout. Arrow, microsatellite deletion.
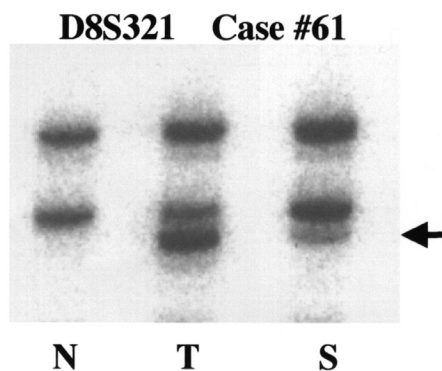

Fig. 2 Microsatellite instability of Case # 61. MSI was detected in the tumor (T) and saliva sample (S). N, normal lymphocytes; T, tumor; S, mouth washout. Arrow, microsatellite deletion.
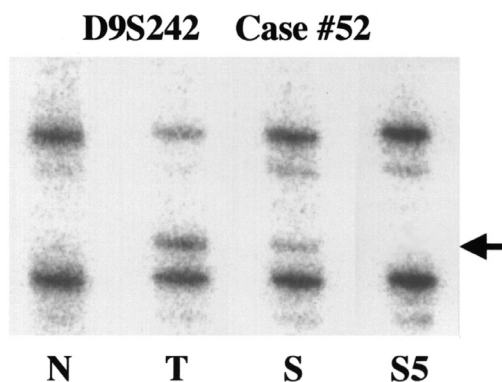

Fig. 3 Microsatellite instability of Case # 52. MSI was detected in the saliva sample before resection (S). MSI had disappeared in the saliva collected during the relapse-free follow-up period (S5). N, normal lymphocytes; T, tumor; S, mouth washout. Arrow, microsatellite insertion.

14]. In one study, a correlation between MSI and non-smoking cancer patients was suggested [15]. Although we could not draw a definite conclusion, MSI seems to have little significance in the tumorigenesis and clinical features in head and neck cancers.

Molecular detection of cancer cells in the body fluid

We found the significance of MSI as a molecular marker for cancer cells. Molecular detection of cancer cells has been reported in several types of body fluids such as urine [5, 19, 20], bronchoalveolar lavage [4], and stool [21] to detect bladder cancer, lung cancer, and colon cancer, respectively. Using the saliva sample, a sensitive method must be established to detect cancer cells among massive epithelial cells from the normal mucosa. Boyle *et al*. used p53 mutation as molecular markers to detect cancer cells in the saliva [22]. They used p53 mutant specific oligomer probe and achieved a high sensitivity. It could detect one cancer cell in 1,000 to 10,000 normal cells [4, 22]. However, it is a mutant specific method that cannot be used for a screening purpose. We used microsatellite analysis according to the method described in the recent paper [6]. Microsatellite analysis can detect one cancer cell among about 200 normal cells [4]. Although it is not as sensitive as p53 mutation specific method, its technique is simple and can be used for screening.

Molecular screening and molecular follow-up

No sensitive screening method has been established for the detection of head and neck cancers. In this study, we were able to detect a small population of the cancer cells in saliva. The incidence of the MSI was not high enough for a practical use so far. If markers with a high MSI incidence are chosen, molecular screening with saliva samples would be feasible [6]. Especially, the high-risk groups such as heavy smokers and heavy drinkers should be the subjects of screening.

We could collect a saliva sample from one tongue cancer patient during the relapse-free period. The previously found MSI-positivity disappeared in the follow-up sample. No follow-up study using saliva has been presented elsewhere. In a bladder cancer follow-up study, a molecular method succeeded in detecting a relapsed tumor earlier than the cystoscopy [5]. We did not have MSI-positive tumor that relapsed after the treatment. If MSI was detected in the follow-up saliva sample, a sensitive molecular follow-up method would have been established. In addition, markers with a high MSI incidence are chosen, molecular follow-up can be applied to a practical use.

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REFERENCES

1) F. W. Deleyiannis, E. A. Jr Weymuller, M. D. Coltrera. Quality of life of disease-free survivors of advanced (stage III or IV) oropharyngeal cancer. Head Neck 19: 466–73, 1997.

2) S. N. Rogers, L. Hannah, D. Lowe, P. Magennis. Quality of life 5-10 years after primary surgery for oral and oro-pharyngeal cancer. J Craniomaxillofac Surg 27: 187–91, 1999.

3) L. Mao, D. J. Lee, M. S. Tockman, Y. S. Erozan, F. Askin, D. Sidransky. Microsatellite alterations as clonal markers for the detection of human cancer. Proc Natl Acad Sci U S A 91: 9871–9875, 1994.

4) S. A. Ahrendt, J. T. Chow, L. H. Xu, S. C. Yang, C. F. Eisenberger, M. Esteller, J. G. Herman, L. Wu, P. A. Decker, J. Jen, D. Sidransky. Molecular detection of tumor cells in bronchoalveolar lavage fluid from patients with early stage lung cancer. J Natl Cancer Inst 91: 332–339, 1999.

5) G. Steiner, M. P. Schoenberg, J. F. Linn, L. Mao, D. Sidransky. Detection of bladder cancer recurrence by microsatellite analysis of urine. Nat Med 3: 621–624, 1997.

6) M. F. Spafford, W. M. Koch, A. L. Reed, J. A. Califano, L. H. Xu, C. F. Eisenberger, L. Yip, P. L. Leong, L. Wu, S. X. Liu, C. Leronimo, W. H. Westra, D. Sidransky. Detection of head and neck squamous cell carcinoma among exfoliated oral mucosal cells by microsatellite analysis. Clin Cancer Res 7: b607–612, 2001.

7) P. van der Riet, H. Nawroz, R.H. Hruban, R. Corio, K. Tokino, W. Koch, D. Sidransky. Frequent loss of chromosome 9p21-22 early in head and neck cancer progression. Cancer Res 54: 1156-1158, 1994.

8) J. Califano, P. van der Riet, W. Westra, H. Nawroz, G. Clayman, S. Piantadosi, R. Corio, D. Lee, B. Greenberg, W. Koch, D. Sidransky. Genetic progression model for head and neck cancer: implications for field cancerization. Cancer Res 56: 2488-2292, 1996.

9) K. Okami, L. Wu, G. Riggins, P. Cairns, M. Goggins, E. Evron, N. Halachmi, S. A. Ahrendt, A. L. Reed, W.

Hilgers, S. E. Kern, W. M. Koch, D. Sidransky, J. Jen. Analysis of PTEN/MMAC1 alterations in aerodigestive tract tumors. Cancer Res 58: 509–511, 1998.

10) M. P. Rosin, J. B. Epstein, K. Berean, S. Durham, J. Hay, X. Cheng, T. Zeng, Y. Huang, L. Zhang. The use of exfoliative cell samples to map clonal genetic alterations in the oral epithelium of high-risk patients. Cancer Res 57: 5258–5260, 1997.

11) C. S. Ishwad, R. E. Ferrell, K. M. Rossie, B. N. Appel, J. T. Johnson, E. N. Myers, J. C. Law, S. Srivastava, S. M. Gollin. Microsatellite instability in oral cancer. Int J Cancer 64: 332–335, 1995.

12) S. Piccinin, D. Gasparotto, T. Vukosavljevic, L. Barzan, S. Sulfaro, R. Maestro, M. Boiocchi. Microsatellite instability in squamous cell carcinomas of the head and neck related to field cancerization phenomena. Br J Cancer 78: 1147–1151, 1998.

13) L. H. Xu, J. Chow, J. Banacum, C. Eisenberger, S. A. Ahrendt, M. Spafford, L. Wu, S. M. Lee, S. Piantadosi, M. S. Tockman, D. Sidransky, J. Jen. Microsatellite instability at AAAG repeat sequences in respiratory tract cancers. Int J Cancer 91: 200–204, 2001.

14) A . El-Naggar, K. Hurr, V. Huff, G. L. Clayman, M. A. Luna, J. G. Batsakis. Microsatellite instabiliry in preinvasive and invasive head and neck squamous carcinoma. Am J Pathol 148: 2067–2072, 1996.

15) J. K. Field, H. Kiaris, P. Howard, E. D. Vaughan, D. A. Spandidos, A. S. Jones. Microsatellite instability in squamous cell carcinoma of the head and neck. Br J Cancer 71: 1065–1069, 1995.

16) S. N. Thibodeau, G. Bren, D. Schaid. Microsatellite instability in cancer of the proximal colon. Science 260: 816–819, 1993.

17) H. Suzuki, A. Komiya, S. Aida, S. Akimoto, T. Shiraishi, R. Yatani, T. Igarashi, J. Shimazaki. Microsatellite instability and other molecular abnormalities in human prostate cancer. Jpn J Cancer Res 86: 956–961, 1995.

18) H. J. Han, A. Yanagisawa, Y. Kato, J. G. Park, Y. Nakamura. Genetic instability in pancreatic cancer and poorly differentiated type of gastric cancer. Cancer Res 53: 5087–5089, 1993.

19) L. Mao, M. P. Schoenberg, M. Scicchitano, Y. S. Erozan, A. Merlo, D. Schwab, D. Sidransky. Molecular detection of primary bladder cancer by microsatellite analysis. Science. 271: 659–662, 1996.

20) D. Sidransky, A. Von Eschenbach, Y. C. Tsai, P. Jones, I. Summerhayes, F. Marshall, M. Paul, P. Green, S. R. Hamilton, P. Frost, B. Vogelstein. Identification of p53 gene mutations in bladder cancers and urine samples. Science. 252: 706–709, 1991.

21) D. Sidransky, T. Tokino, S. R. Hamilton, K. W. Kinzler, B. Levin, P. Frost, B. Vogestein. Identification of ras oncogene mutation in the stool of patients with curable colorectal tumors. Science 256: 102–105, 1992.

22) J. O. Boyle, L. Mao, J. A. Brennan, W. M. Koch, D. W. Eisele, J. R. Saunders, D. Sidransky. Gene mutations in saliva as molecular markers for head and neck squamous cell carcinomas. Am J Surg. 168: 429–432, 1994.