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.

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...
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 microsatellite 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 $\gamma^{32}$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 °C for 30 s, annealing at 55-58 °C for 60 s, and extension at 72 °C 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 microsatellite 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.

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>
<thead>
<tr>
<th>Table 1</th>
<th>Microsatellite markers used in this series</th>
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<tr>
<td>Markers</td>
<td>Location</td>
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<tr>
<td>D3S1289</td>
<td>3p21-23</td>
</tr>
<tr>
<td>D3S1300</td>
<td>3p21.1-14.2</td>
</tr>
<tr>
<td>D8S320</td>
<td>8</td>
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<tr>
<td>D8S321</td>
<td>8q24.13-qter</td>
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<tr>
<td>D9S242</td>
<td>9q32-33</td>
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<tr>
<td>D11S488</td>
<td>11q24.1-25</td>
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<tr>
<td>D20S82</td>
<td>20</td>
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<tr>
<td>D20S85</td>
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<table>
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<tr>
<th>Table 2</th>
<th>Results of the microsatellite analysis</th>
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<tr>
<td>Markers</td>
<td>Informative cases</td>
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<tr>
<td>D3S1289</td>
<td>19</td>
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<tr>
<td>D3S1300</td>
<td>16</td>
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<td>D8S320</td>
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<td>D20S85</td>
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</table>

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], tetranucleotide 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 clinico-pathological findings. Most of the previous reports on head and neck cancers also showed no correlation between MSI and any clinico-pathological 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 a molecular marker 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**


