Genetic Diagnosis of Oral Cancer (From Diagnosis to Therapy)

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Abstract:Five-year survival rates of oral cancer are not improving in spite of the progression of multidisciplinary treatment, because distant metastasis is the major cause of death in individuals suffering cancer. Therefore, early detection of metastasis at cervical lymph nodes and choosing appropriate treatment for advanced cases are highlighted for reducing deaths due to oral cancer and keeping QOL after treatment. First, actual condition of the micrometastasis in the cervival lymph nodes was analyzed with semiserial sectioning and its detection rate was improved with genetic diagnosis. SCC antigen mRNA is found to be a suitable marker of oral cancer. Next, I applied the sentinel node navigation surgery using intraoperative real-time genetic diagnosis. In order to further improve diagnostic accuracy, an optimal gene marker set was identified by means of a whole genome-wide microarray gene-expression profiling, which can detect common upregulated and downregulated genes in oral cancer. I believe that the genetic diagnosis is pivotal for choosing appropriate treatment, especially for individuals diagnosed with oral cancer.

Key words: oral cancer, genetic diagnosis, sentinel node navigation surgery, DNA microarray

Introduction

Metastasis is a significant prognostic factor of oral cancer. An early detection of micrometastasis is an important issue, however, radiographic diagnosis which is a macroscopic diagnostic tool can't depict a microscopic variation. Neck lymph nodes have various morphological variations, an assembly of small lymph nodes, ectopic salivary gland, involvement of fatty tissue and dilatation of the lymphatic hillus. These variations may disturb accurate diagnosis of the micrometastasis on the radiography. Then, what is a better method? HE staining of a frozen section is a quick and conventional method, and called as a golden standard in the world, but a micrometastasis at the peripheral portion of the lymph node is omitted. According to a mathematic calculation, one 10-micrometer section covers only less than 0.1% of the volume. Semiserial sectionnings are required to detect a micrometastasis, but it is a cumbersome and impractical method. The third method is a genetic diagnosis which amplify a target gene brought by the metastatic cancer cells. A conventional method detects a target band on the agarose gel, and a quantitative PCR shows a real-time quantification of copy number of a target gene.

HE and immunostaining

First of all, I confirmed the actual frequency of the micrometastasis in oral cancer. More than 550 lymph nodes being given a diagnosis of no metastasis by pathologists were used for the study. Two continuous sections from every 200-um interval were semiserially collected and subjected to HE staining and keratin immunostaining (AE1/AE3). Seventy percents of the micrometastasis located at the periphery of the lymph node, and 30% in the medullary portion. Keratin immunostaing gives easy diagnosis, but it does not contribute to improve the detection rate, because we seldom miss the small cancer nest on the HE staining. Micrometastases were detected in 21.9% of the patients with oral cancer. Of 76 patients, 12.3% showed pN upgrading, i.e., 6 from pN0 to pN1, 1 from pN0 to pN2b, and 2 from pN1 to pN2b. The occult metastasis was also detected in six small lymph nodes less than 5 mm in diameter. Since the average minor axis of the MM was more than 1 mm, we proposed that the lymph nodes should be cut and examined in 1-mm intervals to detect MM and to evaluate the accurate pN classification[1].

Genetic diagnosis of micrometastasis

Since there is no reliable tumor marker for oral cancer, expression levels of several candidate mRNA, cytokeratin family, CD44V6, SCC antigen, MMP family and hTERT, were screened on PCR. CK13 is good for a conventional PCR. SCC antigen seems to be a reliable tumor marker not only for the conventional PCR but also for the quantitative PCR. Two-hundred eleven lymph node are subjected to nested PCR. Pathologic diagnosis was positive in 15 and negative in 196 lymph nodes. Primary tumors and normal mucosa were used as positive control, and neck lymph nodes from non-cancer patients and normal salivary gland were as negative control. Alternative halves of the lymph nodes obtained at the neck dissection were used for CK13, CK19 and SCCA mRNA detection by RT-PCR and also for the histological evaluation. All three genes were positive in the positive control. K13 and SCCA was negative in the control lymph nodes, However, 4 of 10 were positive for CK19. Two of 7 control salivary gland were positive for CK13. CK19, a marker of the glandular epithelium, was also positive in the salivary gland. Of 196 lymph nodes being diagnosed negative by the pathologist, 14% was positive for CK13, 52% for CK19 and 19% for SCCA mRNA. We concluded that CK19 is not a suitable marker due to its illegitimate gene expression in the lymph nodes. SCC mRNA which is positive in the positive control and negative in the negative control seems to be a promising tumor marker of the metastasis[2, 3].

Sentinel node navigation surgery

Based on the mentioned data, I started the genetic diagnosis for sentinel navigation surgery. Lymphoscintigraphy was performed 2 hrs after radioisotope injection around the primary tumor. A lead plate is placed on the face of patient to protect a shine-through, and 4 positioning markers are put to identify the location of the sentinel node. In order to shorten the examination time, we modified the following issues. The final goal of time-shortening is one and half hours, because the sentinel node biopsy is applied for N0 patients with early cancer and the surgery is finished within that time. First, since the acid guanidium thiocyanate-phenol-

chloroform extraction procedure using an RNA isolation kit takes much time, mRNA was extracted in a RNA queous minicolumn. The procedure finishes within 30 minutes. Second, reverse transcription takes about one hour. And TaqMan PCR we have used takes 2 hours to get the outcome. Therefore, we newly used LightCycler that regulates temperature quickly via air and equips 32 glass capillaries with high surface area-ratio. One-step method in glass capillaries using the LightCycler enabled RT and PCR of 45 cycles within 45 min. The all procedures are finished within 2 hours[4]. Further, this is a real-time monitoring system. When a lymph node contains many number of the target gene copy, micrometastasis is diagnosed on the way of PCR, and it is reported to the surgeon in the operation room.

Real time PCR showed 2 order higher sensitivity than the conventional PCR on a cell spiking test. SCC antigen is also confirmed to be a reliable marker and it was detected even in one cancer cell among lymphatic cells, however, CK13 showed illegitimate gene expression in the lymphatic cells. We conclude that CK family is not suitable for the high-sensitive quantitative PCR.

Outcome of the first 10 cases are shown in Table 1. Of 14 sentinel lymph nodes in these patients, 3 were positive by HE staining, and 1 was added by semiserial sectioning. Six lymph nodes were positive by the genetic diagnosis in total. Up to date, we performed this diagnosis to 26 patients, however, we can not yet associate the significance of the genetic diagnosis with the survival rate simply due to the less number of the cohort.

Optimal multiple tumor markers

Dr. Morton, a founder of sentinel lymph node, showed a definite relationship between survival rate and the number of positive gene markers in the lymph nodes of malignant melanoma[5]. He concluded that molecular method will increase accuracy of micrometastasis detection and improve 5-year survival. He recommends usage of multiple markers. Therefore, an optimal set of multiple gene markers for oral cancer should be identified. What are the additional reliable markers of the oral cancer? I studied the gene expression profile using DNA microarray to look for both common markers of oral SCC and individual specific markers.

Two-hundred and ten genes were upregulated in 5 oral

- Norma oral mucosa
- OSCC-1
- OSCC-2
- OSCC-3
- OSCC-4
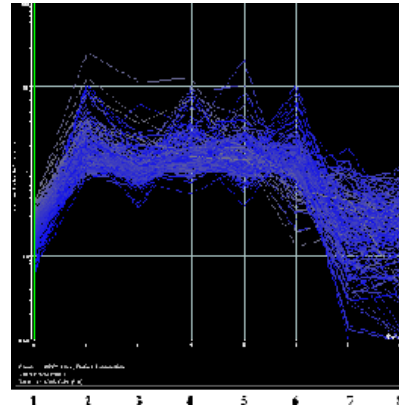- OSCC-lung-meta
- Osteosarcoma
- Malignant melanoma

Fig. 1: Overexpressed genes of oral cancer (ABI 1700 DNA microarray analyzer)

squamous cell carcinoma. These genes specific for oral squamous cell carcinoma are not upregulated in other types of malignant tumor (Fig.1). Further, we notice that the strength of the gene expression varies among the cell lines. These data lead us to the discovery of common marker and individual tumor marker which seems to be available for the personal diagnosis.

Based on the DNA array data, we narrowed down candidate genes for the detection of micrometastasis. Even though the number of examined samples were small, we are now validating the multiple-marker set (Fig.2).

Conclusion

We have developed an intraoperative real-time genetic diagnosis for the sentinel node navigation surgery. SCCA mRNA seems to be a promising tumor marker for oral cancer. A diagnosis with multiple marker genes should be established to improve its accuracy. Individual diagnostic marker system will be developed in the near future.

References

1. Hamakawa H, Takemura K, Sumida T, Kayahara H, Tanioka

Table 1: Outcome of first 10 patients underwent sentinel node navigation surgery SCCA mRNA is used as a marker gene.

No. of patient	Primary site	cTNM	No. and location of sentinel LN	H(+)/ semiserial H(+)	G(+)	final pN
1	maxillary gingiva	T1N0M0	1 (SMLN)	0/0	0	pN0 (sn)
2	tongue	T2N0M0	2 (SMLN, MIJLN)	0/0	0	pN0 (sn)
3	tongue	T2N0M0	1 (MIJLN)	0/0	0	pN0 (sn)
4	maxillary gingiva	T2N0M0	1 (UIJLN)	1/0	1	pN1 (sn)
5	maxillary gingiva	T2N0M0	1 (SM LN)	0/0	0	pN0 (sn)
6	floor of the mouth	T1N0M0	1 (SMLN)	0/0	0	pN0 (sn)
7	maxilary gingiva	T2N0M0	2 (contra-lateral UIJLN)	1/0	2	pN2c (sn)
8	floor of the mouth	T1N0M0	1 (SMLN)	0/0	0	pN0 (sn)
9	tongue	T2N0M0	2 (UIJLN)	1/0	2	pN2b (sn)
10	maxillary gingiva	T2N0M0	2 (SMLN, UIJLN)	0/1	1	pN1 (sn)
			14	3/1	6	

SMLN: submandibular lymph node, UIJLN: upper internal jugular lymph node, MIJLN: middle internal jugular lymph node. G(+): number of metastatic LN by genetic diagnosis, H(+): number of metastatic LN by one section through the maximal diameter of LN, Semiserial H(+): additional number of metastatic LN that was detected by semiserial sections

H, Sogawa K. Histological study on pN upgrading of oral cancer. Virchows Arch 2000: 437:116-121.

2. Hamakawa H, Fukizumi M, Bao Y, Sumida T, Onishi A, Tanioka H, Sato H, Yumoto E. Genetic diagnosis of micrometastasis based on SCC antigen mRNA in cervical lymph nodes of head and neck cancer. Clin Exp Metastasis 1999; 17: 593-599.

3. Hamakawa H, Fukuzumi M, Bao Y, Sumida T, Kayahara H, Onishi A, Sogawa K. Keratin mRNA for detecting micrometastasis in cervical lymph nodes of oral cancer. Cancer Lett 2000; 160: 115-123.

4. Hamakawa H, Onishi A, Sumida T, Terakado N, Hino S, Nakashiro KI, Shintani S. Intraoperative real-time genetic diagnosis for sentinel node navigation surgery. Int J Oral Maxillofac Surg 2004; 33: 670-675.

5. Takeuchi, H., Morton, D. L., et al.: Prognostic Significance of Molecular Upstaging of Paraffin-Embedded Sentinel Lymph Nodes in Melanoma Patients. J Clin Oncol, 22: 2671-2680, 2004.
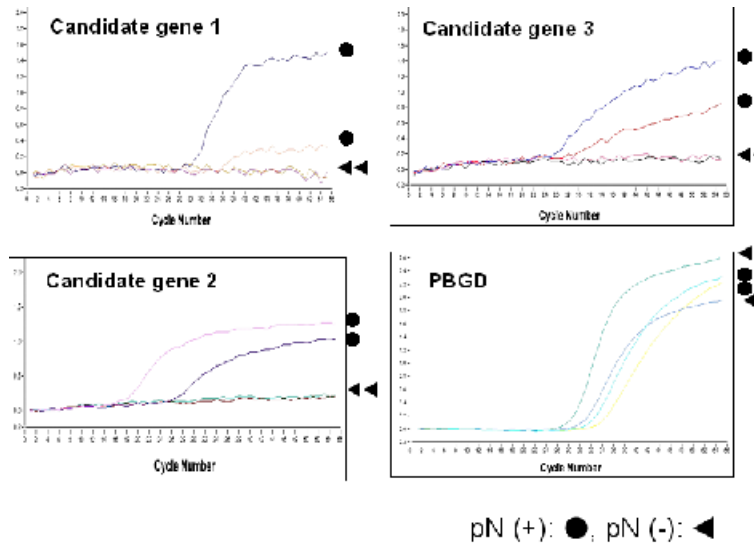

Fig. 2: Three candidate marker genes chosen from the data of ABI 1700 are commonly expressed in metastatic lymph nodes.