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Detection of Cancer Cells in the Peripheral Blood and Lung of Mice after Transplantation of Human Adenoid Cystic Carcinoma

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Polymerase chain reaction (PCR) targeting human β-globin gene has been reported to be able to detect micrometastasis in an animal model. We attempted to detect cancer cells from the lung and peripheral blood in nude mice, into which a human adenoid cystic carcinoma (ACC) line (KOA-1 and KOA-1L3) had been transplanted.

Positive PCR reaction was observed in the lung of a total of 19 of 47 (40.4%) mice into which KOA-1 tumor cells had been transplanted: none of 10 after 1 month, seven of 18 after 2 months, and 12 of 19 after 3 months or later. The KOA-1L3 tumor showed earlier lung metastasis, that is, two of 24 after 7 days and two of 12 after 14 days.

Positive PCR reaction was confirmed in the blood samples of three of 19 mice. The lungs of these mice were positive PCR results after subcutaneous transplantation of the KOA-1 tumor. Three of four mice in these blood samples were confirmed in Positive PCR reaction after subcutaneous transplantation of the KOA-1L3 tumor similarly. The lungs of these mice showed PCR positive reaction as well as the lungs of transplantation KOA-1 tumor mice. On the other hand, negative PCR reaction observed these lungs of 60 mice after subcutaneous transplantation of the KOA-1 or KOA-1L3 tumor. All of them were showed negative PCR results in the blood samples. This experimental model can be expected to provide a more detailed understanding of ACC, as well as help to develop a therapy for metastasis of ACC.

Adenoid cystic carcinoma (ACC) is a unique malignant tumor which originated from the salivary gland and is characterized by slow growth, diffuse invasion, and high frequency of distant metastasis (10,15,16). We recently established a new ACC line, designated as KOA-1, which is serially transplantable into the subcutaneous tissue of nude mice and spontaneously metastasizes to the lung (4,15). Further, we established a variant of KOA-1 by means of in vivo selection, and designated it KOA-1L3. This KOA-1L3 showed faster growth and developed earlier lung metastasis than the parental KOA-1. Both these tumor lines showed evidence of having retained the unique ACC characteristics of frequent metastasis.

The prognosis for patients with oral cancer, including salivary gland carcinoma, has been remarkably improved over the past several decades. Local cure can be attained for most patients, but occasionally distant metastasis occurs. Diagnosis and treatment of distant

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metastasis in the early stage have therefore become increasingly important. Techniques for detecting small numbers of cancer cells have developed with advances in molecular biology. Some investigators (3,6) have used RT-PCR assays for cytokeratin (CK) or squamous cell carcinoma antigen (SCCA) to detect micrometastasis of the cervical lymph nodes. However, no studies have been published concerning the detection of cancer cells in the blood of oral cancer patients. The purpose of the experiments reported here was to detect micrometastasis in the lung and circulating cancer cells in the peripheral blood by means of nested PCR using transplantation of the KOA-1 and KOA-1L3 human ACC line into an animal model.

MATERIALS AND METHODS

Six-week-old male BALB/c nu/nu nude mice were kept under sterile conditions throughout the experiments. All animal experiments were carried out in compliance with the "Guidelines for Animal Experiments of Kobe University Graduate School of Medicine".

The original KOA-1 tumor was obtained at surgery from a 59-year-old patient with a solid pattern of ACC of the palate. The tumor was rinsed three times in phosphate buffered saline (PBS), cut into about 1mm cubes and transplanted subcutaneously into the backs of the mice. Passage to other mice has been carried out every 3 months for 5 years. The transplantation success rate has been almost 100%.

For the next stage, the KOA-1 tumor was again transplanted into the subcutaneous tissue of nude mice as described above. Three months later, lung metastases were harvested and re-transplanted subcutaneously into other mice. This cycle was repeated three times to yield the tumor line KOA-1L3.

Animals were killed between 2 and 6 months after the transplantation of KOA-1, and between 1 and 3 weeks after the transplantation of KOA-1L3. After we collected about 0.3 ml of blood by the central venous, the lungs were excised and rinsed 3 times in PBS. The blood and lung tissues were kept frozen at –70℃ until DNA extraction. Some lungs were also prepared for histologic examination.

DNA was extracted according to the protocol reported elsewhere (4). Two sets of primers were designed for the human β-globin gene (Table 1). The PCR conditions for the first reaction were 92℃ for 90s for 1 cycle, then for 30 cycles at 92℃ for 30s, 54℃ for 30s and 72℃ for 30s. The first primers were designed outside the amplified region by the nested primer. For the second step, 5 μl of the first PCR product was removed and re-amplified using two sets of overlapping inner primers. Conditions for the nested PCR were the same as for the first reaction. The products were then analyzed by electrophoresis on 2% agarose gel (Fig. 1).

Table 1 Oligonucleotide primers used for first and second PCR

Primer		amplified size of DNA
First	GH20(forward): d(GAAGA GCCAA GGACA GGTAC) GH21(reverse): d(GGAAA ATAGA CCAAT AGGCA G)	408bp
Second	KM29(forward): d(GGTTG GCCAA TCTAC TCCCA GG) KM38(reverse): d(TGGTC TCCTT AAACC TGTCT TG)	262bp

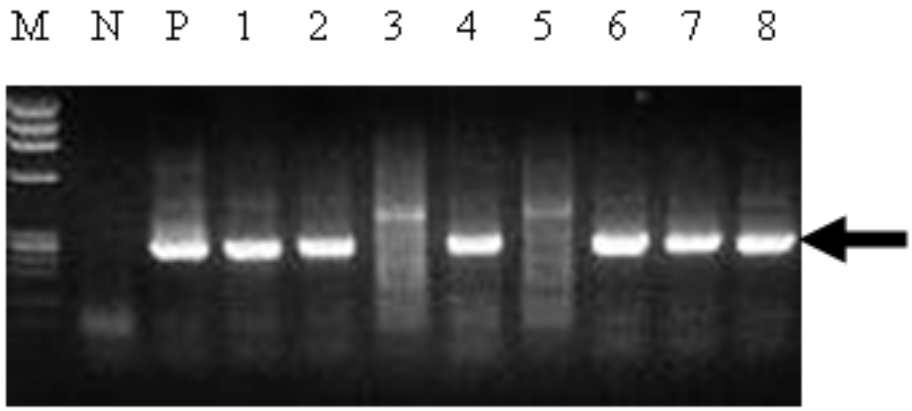

Fig. 1 Detection of β-globin gene in the KOA-1 mice.
A band of 262 bp indicates the presence of human β-globin gene sequences. Positive reaction was shown in mice Nos. 1, 2, 4, 6, 7 and 8. M: Hae III, bp marker. P: positive control, KOA-1 tumor. N: negative control, normal mouse lung.

RESULTS

Positive PCR reaction was observed in the lung of 19 of 47 (40.4%) mice into which the KOA-1 tumor had been transplanted. As for the time of onset of lung metastasis of the KOA-1 tumor, PCR analysis showed negative results in all 10 mice 1 month after the transplantation, positive reaction in seven of 18 mice after 2 months, and in 12 of 19 mice after 3 months or more (Table 2).

The KOA-1L3 tumor showed much earlier lung metastasis, with a PCR-determined positive reaction in two (8.3%) of 24 mice as early as 7 days after transplantation, and in two (16.7%) of 12 mice 14 days after transplantation of the KOA-1L3 cells (Table 2). The metastasis rate after more than 14 days could not be examined because mice bearing the KOA-1L3 tumor had all died within 3 weeks, probably because of cachexia.

Positive PCR reaction was confirmed in the blood samples of some animals (Table 3). Three (6.4%) of 47 mice with positive PCR results in the lung after transplantation of the KOA-1 tumor showed positive PCR products also in the blood. The positive rate in the blood was higher in mice bearing the KOA-1L3 tumor, with 3 (75%) of 4 mice showing positive results in the lung, although the number examined was small. On the other hand, 60 mice which showed negative PCR results in the lung after transplantation of either the KOA-1 or KOA-1L3 tumor also had negative PCR results in the blood samples.

Table 2 Lung metastasis rate of KOA-1 and KOA-1 L-3 by each period

Time after the transplantation	PCR analysis
KOA-1	
1 month	0 / 10 (0%)
2 month	7 / 18 (38.8%)
3 month	12 / 19 (63.1%)
KOA-1 L-3	
7 days	2 / 24 (8.3%)
14 days	2 / 12 (16.7%)

Table 3 The results of β-globin of the lung and blood by PCR

Lung metastasis /Circulating tumor cells			numbers
KOA-1			
○	/	○	3
○	/	×	16
×	/	○	0
×	/	×	28
		Total	47
KOA-1 L-3			
○	/	○	3
○	/	×	1
×	/	○	0
×	/	×	32
		Total	36

○ positive reaction for PCR
× negative reaction

DISCUSSION

Adenoid cystic carcinoma (ACC) of the salivary gland shows little histological evidence of cellular atypia and its doubling time is very long compared to that of other human malignant neoplasms (15). However, ACC invades the surrounding tissue more diffusely than any other type of head and neck cancer, and frequently metastasizes to the lung, although lymph node metastasis is very rare (10,13,15). ACC shows strong affinity for the basement membrane of vessels and peripheral nerves, which may explain ACC's unique characteristics (1). The prognosis for patients with ACC depends mainly on the control of distant metastasis rather than on the success of loco-regional cure. The blood vessels are thought to be the major route for dissemination of ACC cells, since the frequency of lymph node metastasis of ACC is much lower than that of squamous cell carcinoma of the oral cavity.

We recently established an in vivo model for metastasis of human ACC. Some investigators have established ACC cell lines by means of cell culture, but no previous studies have been reported involving heterotransplantation of human ACC into animals (5,11,12). We believe that the KOA-1 and KOA-1L3 tumors which we established are unique since they are serially transplantable into the subcutaneous tissue and spontaneously metastasize to the lung when tranplanted into the subcutaneous tissue of nude mice.

Micrometastasis, which is not detectable by routine histologic examinations, can now be identified by genetic methods. Detection of small numbers of cancer cells in lymph nodes, distant organs, or peripheral blood has been recently attempted using various genetic methods (2,7). As for oral cancers, some investigators reported that micrometastasis to the lymph nodes was detectable by using SCC antigen mRNA or expression of a spliced transcript of type II keratin K5, but detection of circulating cancer cells in peripheral blood has not been successful in either patients with oral cancer or in animal models (8).

In our study, the PCR product for the human β-globin gene could be detected for identification of metastasis in the lung and blood. When this gene is detected in nude mice, metastasis is thought to be present, although there may be a false positive reaction because PCR may amplify DNA fragments derived from dead cancer cells. The gene was detected

in the lungs of 40.4% the mice bearing the KOA-1 tumor and 11.1% of those bearing the KOA-1L3 tumor. Moreover, human β-globin gene was detected in the blood of three of 47 mice bearing the KOA-1 tumor, and of three of 36 mice bearing the KOA-1L3 tumor. To the best of our knowledge, the study presented here is the first to confirm the presence of circulating tumor cells in the blood in an animal experiment involving ACC.

Pages et al. reported the detection of cancer cells in the blood of mice after transplantation of colon cancer cells and prior to formation of distant metastasis (9). Our study showed that all mice in which circulating ACC cells were detected also had positive PCR findings in the lung. The low incidence of the positive rate in blood compared to that in the lung may be due to the number of ACC cells in the blood being too small to be detected with this method. The finding that all mice without lung metastasis detected by genetic assay did not show positive results in their blood samples either seems to indicate a close relationship between ACC cells in blood and those in distant organs. The current study showed that cancer cells could be detected in peripheral blood by means of the nested PCR method using β-globin gene. This experimental model of KOA-1 and KOA-1L3 tumors can be expected to provide a more detailed understanding of ACC, as well as help develop a therapy for metastasis of ACC.

REFERENCES

1. **Franca C.M., Jaeger M.M., Jaeger R.G. et al.** 2000. The role of basement membrane proteins in the expression of neural cell adhesion molecule (N-CAM) in an adenoid cystic carcinoma cell line. Oral Oncol **36**:248-252.
2. **Ghossein R.A., Rosai J.** 1996. Polymerase chain reaction in the detection of micrometastases and circulating tumor cells. Cancer **78**:10-16.
3. **Hamakawa H., Fukizumi M., Bao Y. et al.** 1999. Genetic diagnosis of micrometastasis based on SCC antigen mRNA in cervical lymph nodes of head and neck cancer. Clin Exp Metastasis **17**:593-599.
4. **Komatsubara H., Umeda M., Komori T.** 2002. Establishment of in vivo metastasis model of human adenoid cystic carcinoma: detection of metastasis by PCR with human β-globin gene. Kobe J Med Sci **48**:145-152.
5. **Li SL** 1990. Establishment of a human cancer cell line from adenoid cystic carcinoma of the minor salivary gland. Zhonghua Kou Qiang Yi Xue Za Zhi **25**:29-31.
6. **McDonald L.A., Walker D.M., Gibbins J.R.** 1998. Cervical lymph node involvement in head and neck cancer detectable as expression of a spliced transcript of type II keratin K5. Oral Oncol **34**:276-83.
7. **Nomoto S., Nakao A., Kasai Y. et al.** 1996. Detection of ras gene mutations in perioperative peripheral blood with pancreatic adenocarcinoma. Jpn J Cancer Res **87**:793-797.
8. **Pajonk F., Schlessmann S., Guttenberger R. et al.** 2001. Epithelial cells in the peripheral blood of patients with cancer of the head and neck: incidence, detection and possible clinical significance. Radiother Oncol **59**:213-217.
9. **Pages J.C., Sordat B., Bautista D. et al.** 1996. Detection of rare circulating human colon tumor cells in a nude mouse xenograft model. Cancer Lett **106**:139-144..
10. **Perzin K.H., Gullane P., Clairmont A.C.** 1978. Adenoid cystic carcinomas arising in salivary glands: A correlation of histological and clinical features. Cancer **42**:265-268.
11. **Shirasuna K., Watatani K., Furusawa H. et al.** 1990. Biological characterization

of pseudocyst-forming cell lines from human adenoid cystic carcinomas of minor salivary gland origin. Cancer Res **50**:4139-4145.

12. **Sobue M., Takeuchi J., Niwa M. et al.** 1989. Establishment of a cell line producing basement membrane components from an adenoid cystic carcinoma of the human salivary gland. Virchows Archiv B Cell Pathol **57**:203-208.
13. **Spiro R.H., Huvos A.G., Strong E.W.** 1979. Adenoid cystic carcinoma. Factors influencing survival. Am J Surg **138**: 579-583.
14. **Umeda M., Komatsubara H., Nishimatsu N. et al.** 2002. Establishment and characterization of a human adenoid cystic carcinoma line of the salivary gland which is serially transplantable and spontaneously metastasises to the lung in nude mice. Oral Oncol **38**:30-34.
15. **Umeda M., Nishimatsu N., Masago H., et al.** 1999. Tumor doubling time and onset time of pulmonary metastasis from adenoid cystic carcinoma of the salivary gland. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **88**:473-478.
16. **Umeda M., Nishimatsu N., Yokoo S. et al.** 2000. The role of radiotherapy for patients with adenoid cystic carcinoma of the salivary gland. Oral Surg Oral Med Oral Pathol Oral Radiol Endod **89**:724-729.