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Establishment of *in Vivo* Metastasis Model of Human Adenoid Cystic Carcinoma: Detection of Metastasis by PCR with Human β-Globin Gene

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Cells from the human adenoid cystic carcinoma (ACC) line, KOA-1, originating from the minor salivary gland and showing histological features of a dominantly solid pattern of ACC, were transplanted into the subcutaneous tissue of nude mice. The occurrence of lung metastasis was then examined both histologically and by PCR with a human-specific base arrangement of the β-globin gene. Histologic examinations revealed lung metastasis in 5 of 30 mice. On the other hand, PCR showed a higher positive rate: 26 of 67 mice (0/10 at 1 month, 5/15 at 2 months, and 21/42 at 3 months after the transplantation). When some lungs which showed positive results by PCR analysis were transplanted into other mice, KOA-1 tumors developed. The results suggested that this experimental model could be a standard *in vivo* model of metastasis of human ACC.

Adenoid cystic carcinoma (ACC) is a unique malignant tumor of the salivary gland which shows characteristics of slow growth, diffuse invasion, and high frequency of distant metastasis (9, 15). To study the biologic behavior and treatment of cancer metastasis, the *in vivo* animal model is necessary; however, xenografting of human ACC into animals has not been reported.

We have established a new human ACC line (designated KOA-1) that is serially transplantable in nude mice (14). The KOA-1 tumor has also been histologically shown to occasionally metastasize spontaneously to the lung when transplanted subcutaneously into nude mice. The current study was designed to detect lung metastases of KOA-1 tumors in nude mice by the polymerase chain reaction (PCR) method using a human specific base arrangement of the β-globin gene (7), and to discuss the xenografting model of KOA-1 tumors into nude mice as a standard *in vivo* model of the metastasis of human ACC.

MATERIALS AND METHODS

Animal

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

Preparation of KOA-1 tumor

The original tumor was obtained at surgery from a 59-year-old patient with a solid pattern of ACC of the palate (Figs. 1 and 2). The tumor was rinsed three times in phosphate

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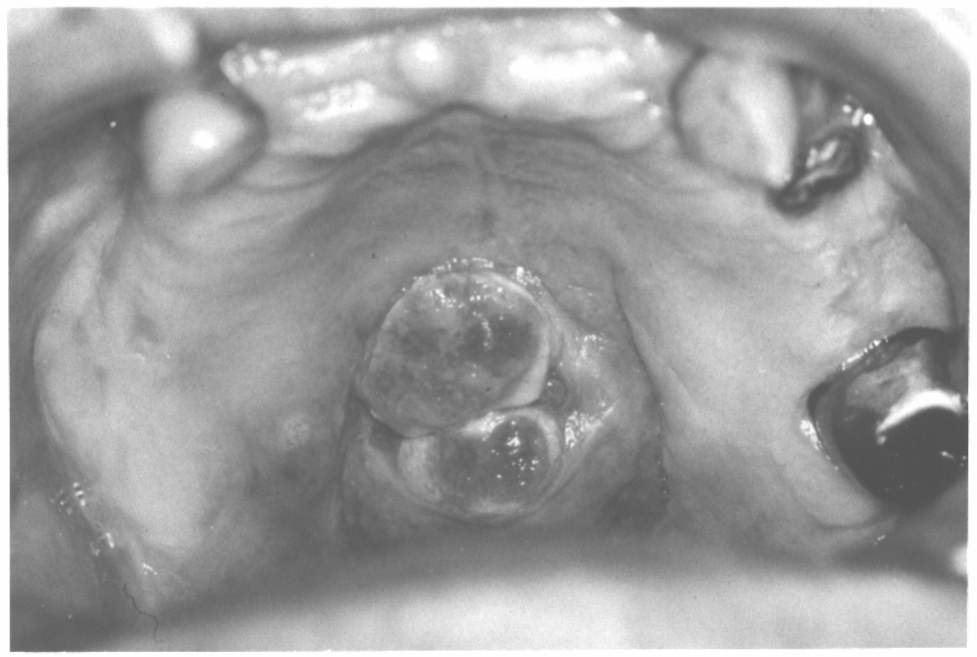

Fig. 1. Macroscopic features of ACC of the palate from which the KOA-1 tumor was obtained.

buffered saline (PBS), cut into about 1 mm^3 pieces and transplanted subcutaneously into the backs of the mice. Passage to other mice was carried out every 3 months. The KOA-1 tumor has been transplanted serially in the backs of nude mice for 61 months and is presently in its 24th generation (Fig. 3).

Xenografting into the nude mice

The lungs of mice that developed tumors were excised 1, 2, and 3 months after the transplantation. Some of the lungs were divided in two: one half was fixed in 10% formalin, embedded in paraffin, cut into 4-μm sections in the center of the lung and stained with hematoxylin and eosin; the other half was rinsed 3 times in PBS, frozen at -70°C and analyzed by nested PCR. The other lungs were also divided in two: one half for PCR analysis and the other half was transplanted directly into another mouse and examined histologically after 3 months to determine tumorigenicity.

Table I. Oligonucleotide primers used for first and second PCR.

Primer		amplified size of DNA
first	GH 20 (forward): d (GAAGA GCCAA GGACA GGTAC) GH 21 (reverse): d (GGAAA ATAGA CCAAT AGGCA G)	408bp
second	KM 29 (forward): d (GGTTG GCCAA TCTAC TCCCA GG) KM 38 (reverse): d (TGGTC TCCTT AAACC TGTCT TG)	262bp

PCR analysis

DNA was extracted from the lungs of the mice by SepaGene (SANKO JUN-YAKU, Co. Ltd., Japan) according to the manufacture's protocol. Two sets of primers were designed for

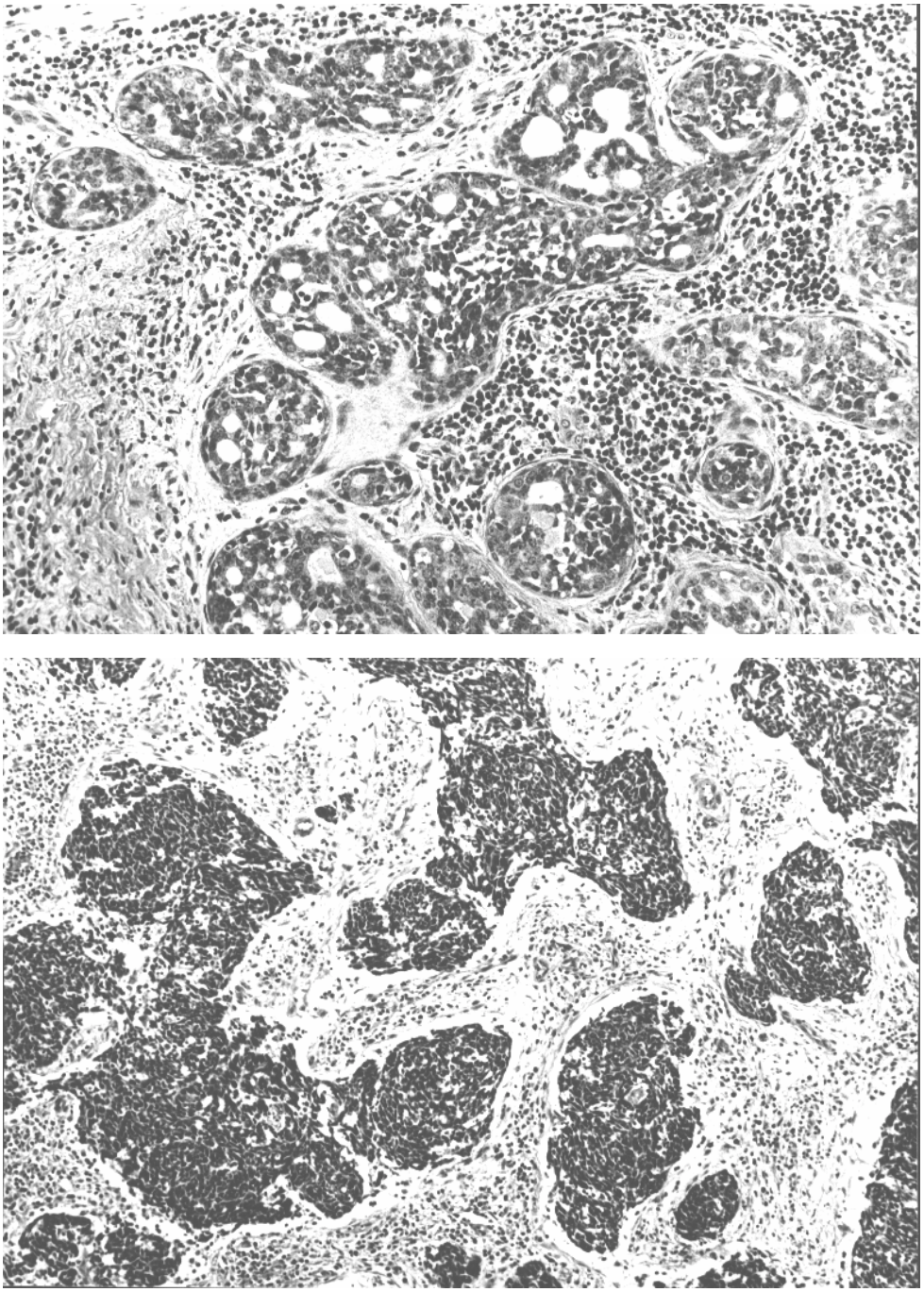

Fig. 2. Histological features of the original tumor showing cribriform (A, upper pannel) and solid (B, lower pannel) patterns of ACC. (HE stain, x100).

the human β-globin gene (10) (Table I). The PCR conditions (Table Ⅱ) for the first reaction were 92°C for 90s for 1 cycle, then for 30 cycles at 92°C for 30s, 54°C for 30s and 72°C for 30s. The first primers were designed outside the amplified region by the nested primer. In the second step, 5μl 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.

About 50μg DNA was extracted from 100mg of the KOA-1 tumor. In a preliminary experiment, the human β-globin gene was detectable in 5^{-4} diluted DNA water by the nested PCR method.

Table Ⅱ. PCR condition.

General reaction mixture for PCR (total 50μl)	
TaKaRa Taq ™ (5 units/μl)	0.3μl
10 X PCR Buffer	5μl
dNTP Mixture(2.5mM each)	4μl
Template	<1μg
forward primer	0.5μM
reverse primer	0.5μM
Sterilized distilled water	up to 50μl

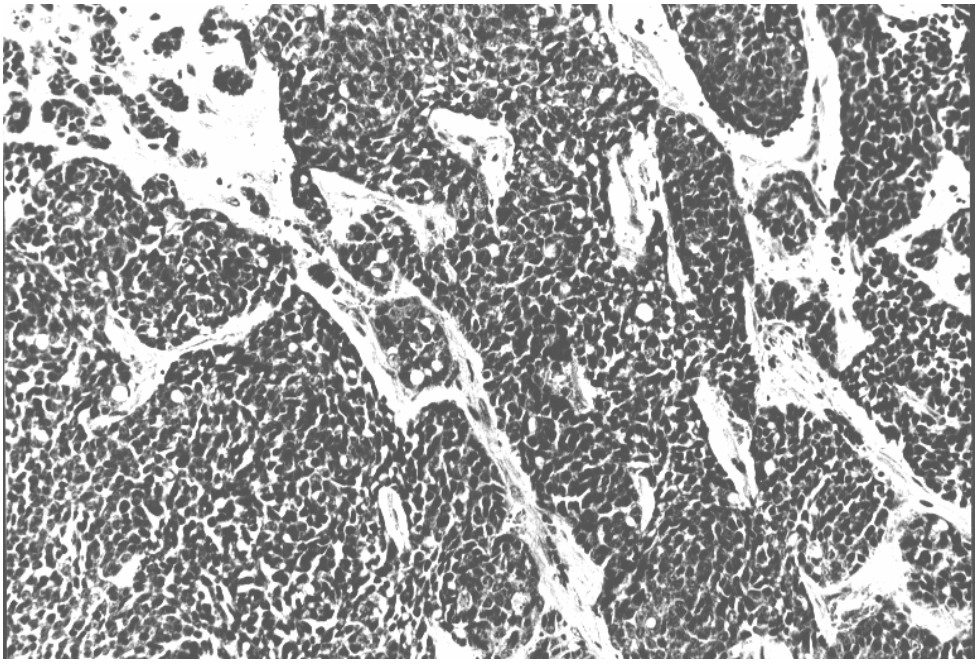

Fig. 3. Histological features of KOA-1 tumor showing well-demarcated, predominantly solid pattern of ACC in the subcutaneous tissue of mice. (HE stain, x100).

RESULTS

Of 18 mice examined histologically, 5 (27.8%) exhibited lung metastasis 3 months after the transplantation (Fig. 4 and Table Ⅲ). Small to medium nests of cancer cells identical to the subcutaneously transplanted KOA-1 cells were scattered in the lung. The metastatic nodes not detected macroscopically were observed only by histological examination.

Contrasting with relatively low rate of metastasis detected by histologic examination, PCR analysis showed a high positive rate of 50.0% (21/42) 3 months after the transplantation (Table Ⅲ). As for the onset time of lung metastasis, PCR showed no metastasis results in all the 10 mice 1 month after the transplantation, but showed a positive reaction in 5/15 (33.3%) after 2 months. Histologically, however, no positivity was revealed at 2 months (Table Ⅲ). The final PCR amplification patterns are shown in Figure 5.

Furthermore, in 6 of 37 mice, KOA-1 tumors began to proliferate among the transplanted lung tissue in other mice (Fig. 6 and Table Ⅲ). The 6 lungs showing tumorigenicity also demonstrated positivity by PCR. As for the relationship between PCR analysis and tumourigenicity, 6 of 8 lungs which showed positive results by PCR analysis proliferated in other mice, and none which showed negative results by PCR analysis proliferated in other mice.

Table Ⅲ. The incidence of lung metastasis.

Time after the transplantation	Metastasis rate		
	Histologic examination	PCR analysis	Transplantation to other mice
1 month	0/5 (0%)	0/10 (0%)	0/5 (0%)
2 months	0/7 (0%)	5/15 (33.3%)	0/8 (0%)
3 months	5/18 (27.8%)	21/42 (50.0%)	6/24 (25.0%)

DISCUSSION

Metastasis is one of the most characteristic biological behaviors of cancer. Early diagnosis and treatment of metastasis may lead to an improvement in the survival rate of cancer patients. When metastasis is detected clinically, however, the cancer cells have often already disseminated to the multiple organs, and the patient may be at an incurable stage. The diagnosis of metastasis using molecular biological techniques has been attempted with various tissues including blood (5, 8), pancreatic juice (8), ascites, lymph nodes (4, 16); the significance of these methods is, however, still controversial.

Adenoid cystic carcinoma (ACC), a rare malignant tumor occurring in the major and minor salivary glands, has some unique characteristics such as slow growth, diffuse invasion and high incidence of pulmonary metastasis (9). Lung metastasis finally appears in many patients with ACC regardless of the success or failure of local control, and their prognosis depends upon the control status of distant metastasis as well as of the local condition (15). No effective therapy for metastasis of ACC has been developed to date, however.

Animal models of cancer metastasis are necessary for the study of treatment methods as well as of the biological behaviors of the disease. Since the first *in vivo* metastasis model described in 1973 with a highly metastatic cell line separated from B16 melanoma (1), various other experimental models have been reported. These may be divided into three categories according to the transplantation method: subcutaneous, intravascular, and orthotopic. The first represents various steps of metastatic processes: local invasion, attachment to vessels, intravasation, dissemination, extravasation, growth in distant organs, and escape from host immunity; however the rate of metastasis is generally too low for use as a routine animal model of cancer metastasis (2). Intravascular transplantation shows the

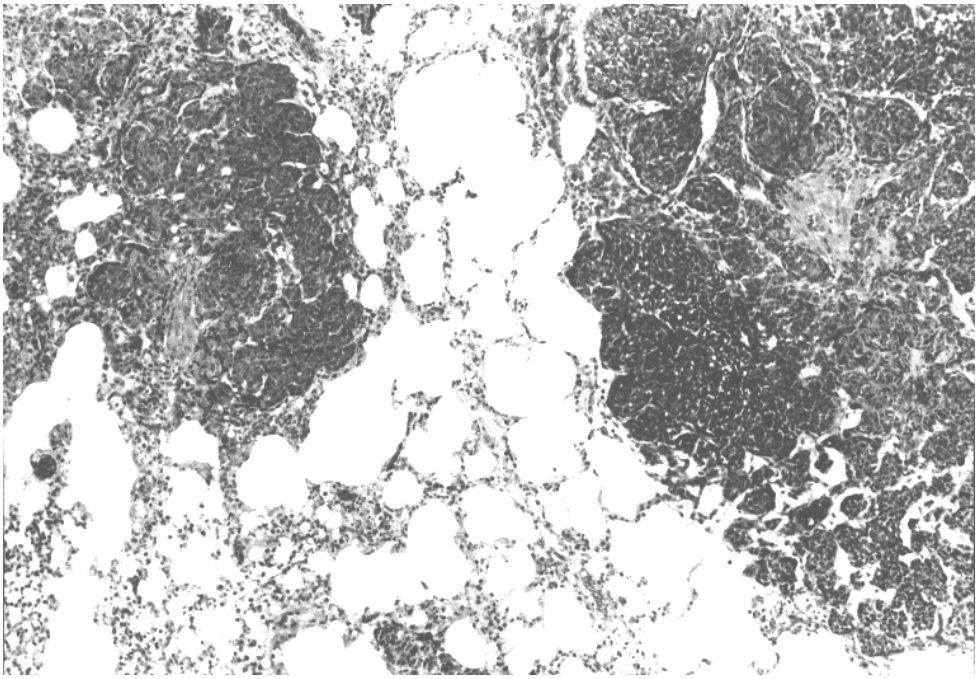

Fig. 4. Multiple micrometastases were observed histologically in the lung 3 months after the transplantation. (HE stain, x100).

highest rate of metastasis because cancer cells are injected directly in blood vessels; however, it dose not represent other steps of metastasis such as local invasion, attachment to a vessels, or intravasation. Metastasis often occurs in animals when cancer cells are transplanted into the same organ they were derived from (2). It occurs when oral cancers are transplanted orthotopically into the tongues of nude mice (14), but this model is not suitable for long-term observation because the intraoral tumors grow rapidly and animals often die of eating disturbance before metastasis can develop.

Cell lives of human ACC have been successfully established (3, 6, 12, 13), but to our knowledge the present study is the first *in vivo* animal model of the metastasis of ACC. It is well known that cancer cells transplanted subcutaneously into nude mice show well-demarcated growth and do not generally metastasize. It is notable that in our study the KOA-1 tumor showed spontaneous metastasis to the lung even when transplanted subcutaneously, indicating that this tumor line retains the characteristics of human ACC, the high frequency of metastasis, in the nude mice.

Micrometastasis, which is not detectable by routine histological examinations, can now be identified by genetic methods. We used human-specific base arrangements of the β-globin gene, which when amplified by PCR in the lungs of nude mice was identified as metastasis from transplanted KOA-1 tumors. These genetic examinations confirmed the presence of the human β-globin gene in 26 (38.8%) of 67 mice. It is known that the PCR method can detect a cancer cell among 10^6 to 10^7 normal cells; on the other hand, there are sometimes false-positive reactions probably because PCR analysis may amplify DNA fragments derived from dead cancer cells (5, 11). To determine whether the results obtained by PCR indicate micrometastasis or were simply false-positive reactions, we transplanted were transplanted a part of the lung tissue to another mouse. As a result, 6 lungs demonstrating

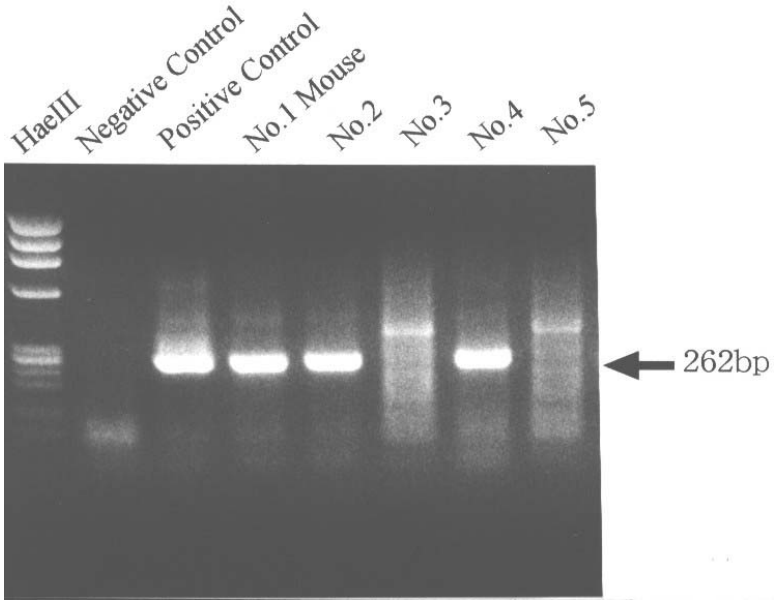

Fig. 5. Detection of β-globin gene in the KOA-1 mice. A band of 262 bp indicates the presence of human β-globin gene sequences. Positive reaction was shown in mouse No. 1, 2 and 4. Hae III, bp marker. Positive control, KOA-1 tumor. Negative control, normal mouse lung.

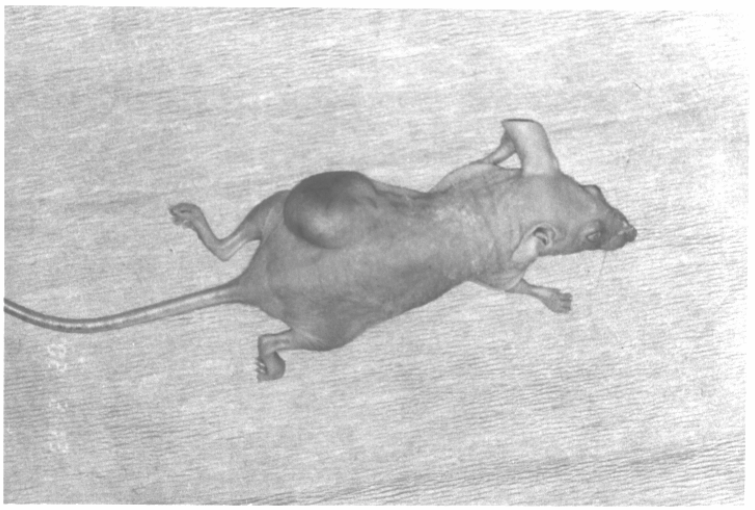

Fig. 6. The lung showing positive PCR analysis proliferated in the skin of another mouse.

tumourigenicity yielded positive results by PCR analysis, which suggested that detection of the human β-globin gene in the mouse lung correctly identified lung metastasis from the KOA-1 tumor. It was shown that percentage of lung metastasis was as high as 50% at 3 months after the transplantation when analyzed by PCR analysis. These findings suggest that this experimental model is useful for further study of the biological behaviour as well as of the treatment methods for metastasis of human ACC.

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