Original article

Establishment of cervical lymph node metastasis model of squamous cell carcinoma in the oral cavity in mice

SUN Rui, ZHANG Jian-gang and GUO Chuan-bin

Keywords: *animal model; lymphatic metastasis; squamous cell carcinoma; mouth neoplasm*

Background Oral squamous cell carcinoma (OSCC) is the most prevalent malignant tumor in the head and neck region, comprising more than 90% of all oral malignancies. A feasible approach for an animal model to study OSCC lymph node metastasis was established and biological behaviors of three oral squamous cell carcinoma cell lines were compared.

Methods After implanting three kinds of cell lines (GDC185, Tca8113, Tca83) into three different anatomical sites in nude mice, namely the tongue, floor of the mouth, and axillary fossa, we observed the tumorigenicity and the metastatic capacity, which was confirmed by histopathology under a surgical microscope.

Results The animal model injected with GDC185 cells into the floor of the mouth had the highest rate of neck lymph node metastasis (55.6%) and the cell lines had significantly different biological behaviors.

Conclusions Nude mice injected with GDC185 cells into the floor of the mouth could be used as a feasible animal model to study neck metastasis of oral squamous cell carcinoma.

Chin Med J 2008;121(19):1891-1895

Oral squamous cell carcinoma (OSCC) is the most prevalent malignant tumor in the head and neck region, comprising more than 90% of all oral malignancies.[1] With the exception of distant metastases, nearly 50% of patients with OSCC presented with clinical or pathological evidence of lymph node metastases. The 5-year survival rate is less than 50% for patients with single unilateral lymph node metastasis and less than 25% for patients with bilateral metastases.[2] Despite improvements in diagnoses, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths from cancer result from the progressive growth of metastases that are resistant to conventional therapies.[3] One of the difficulties in studying the metastasis of human OSCC is the lack of an appropriate animal model and OSCC cell line. On the basis of previous research, we initially studied the tumorigenicity and metastasis capacity of three cell lines of OSCC and hereby establish an animal model with an appropriate metastatic rate in the neck region.

METHODS

Mice

Eighty-one BALB/C nude mice, CRL: BALB/cAnNCrl-nuBR, (purchased from Beijing Vital River Ltd., China) aged between 5 to 6 weeks were used. They were maintained in a laminar flow isolated rack under specific pathogen-free conditions at the laboratory for animal experiments of the Peking University Healthy Center. The mice were given autoclaved food and water. All mice were handled with sterile techniques under a laminar flow hood when removed from their cages. The research plan was approved by the Ethical Committee of the Laboratory for Animal Experiments, Peking University Health Science Center.

Cell lines

Three cell lines, GDC185 and Tca8113 and Tca83, derived from human OSCC were used. GDC185 was established from a neck lymph node metastasis of an upper gingival carcinoma from a 63-year-old Chinese female. The Tca8113 cell line, derived from a 21-year-old female patient who was diagnosed with tongue squamous cell carcinoma,[4] was obtained from School of Stomatology, Shanghai Jiao Tong University. Tca83 was also derived from a patient who was diagnosed with tongue squamous cell carcinoma (SCC) and was obtained from Peking University School of Stomatology. These cells were cultured in Eagle's minimum essential medium (Invitrogen Corporation, USA) or 1640 medium (Invitrogen Corporation, USA) respectively, which were all supplemented with 10% heat-inactivated fetal bovine serum (Junyaobio Corporation, Australia), at 37°C in a humidified atmosphere of 5% CO_2 in air.

Technique

The cells were injected into three locations: the tongue, the floor of the mouth (FOM) via submandibular to the space among the muscles of FOM, which include the mylohyoid and geniohyoid muscles, base of tongue and axillary fossa. Every location was injected with 1.0×10^6 viable cells/0.1 ml in normal saline by 1 ml injector when

Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Peking University, Beijing 100081, China (Sun R and Guo CB)
Department of Pathology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, Gansu 730000, China (Zhang JG)
Correspondence to: Prof. GUO Chuan-bin, Department of Oral and Maxillofacial Surgery, School and Hospital of Stomatology, Peking University, Beijing 100081, China (Tel: 86-10-62173402. Fax: 86-10-62173402. Email: ruidoctor@bjmu.edu.cn)
This work was supported by a grant from Capital Development Fund, Beijing (No. 2003-3009).

cells were in the logarithmic growth phase. The mice were sacrificed on the 49th day, or when their weight was reduced to 15 or less, or in the case of the incidental death of the animal. Eighty-one mice were divided into 9 groups based on the cell line inoculated and the site of injection. Each group had 9 mice.

Histological examination

The abdomen and chest were opened at autopsy. The trachea, heart, lungs, pancreas, and stomach were examined for evidence of tumor, under an operating microscope (OPTON, West Germany). The subcutaneous tumors, oral tumors (tongue or FOM), regional lymph nodes and lungs were removed and fixed in 4% formaldehyde solution (1:9 diluted in phosphate buffer saline) at room temperature for 24 hours. All specimens were embedded in paraffin, cut into 3 μm thick sections, stained with hematoxylin and eosin (HE) for microscopic observation, and evaluated for histopathology.

To calculate the volume of the tumor implanted in the three different locations, we measured three axes of the excised tumor, the major axis as length, the minor axis as width and the third axis as height. The volume was calculated using the formula: 1/4 ×length × width × height × π. The unit of volume was cm^3. We used a ruler to measure the tumor size. The scope of the ruler was 25 cm and the precision was 1 mm.

In lymph nodes where metastases were detected, the histological stage of the tumor was graded according to the classification reported by Honma (Table 1),[5] to investigate the progression of the local lymph node metastasis.

Table 1. Histological staging of metastatic tumors*

Grades	Histologic findings
I	Limited to the afferent lymphatic vessels or the marginal sinus
II	Invasion and proliferation from the marginal sinus to the paracortex or the cortical sinus
III	Replacement and disappearance of most of the lymph tissue
IV	Extra-lymphatic invasion and proliferation

*Criteria by Honma.[5]

Statistical analysis

All data were analyzed by the Statistical Package of Social Sciences (SPSS, version 11.5, USA). For tumorigenicity of different cell lines, the Pearson chi-square test was used. As for tumor size, a significant difference was analyzed using the LSD test of one-way analysis of variance (ANOVA) after the number raised to the power 0.2. Fisher's exact probability test was used to analyze tumor metastasis. A P value <0.05 was considered statistically significant. A P value <0.025 was considered statistically significant between groups in the tumor size.

RESULTS

Tumorigenicity of three cell lines

Tumorigenicity comparison of the three cell lines

Table 2 shows that the tumorigenicity of the three cell lines were not significantly different on the whole (P>0.05); but on the FOM, the differences between GDC185 and Tca83 and between Tca8113 and Tca83 were statistically significant (P <0.05). It was noted that Tca83 in the tongue exhibited higher tumorigenicity (6/9) than others, although no statistically significant difference was observed.

Table 2. Tumorigenicity comparison of three cell lines

Locations	GDC185	Tca8113	Tca83
Tongue	3/9	1/9	6/9
Floor of the mouth	9/9*	8/9*	1/9
Axillary fossa	8/9	8/9	5/9
Total	20/27	17/27	12/27

*P <0.05, GDC185 and Tca8113 compared with Tca83 respectively.

Tumor volume comparison of three cell lines

Statistical analysis showed that the tumor volume of the three cell lines was significantly different (P <0.05), although all three cell lines were able to develop into solid tumors (Figure 1). Between groups the differences were also significant (P <0.025). We found that the tumor volume of Tca8113 was the largest of the three cell lines, especially in the axillary fossa (mean±SD: 4.450±4.190). Some solid tumors were so large that the tumors grew out of the back from the axillary fossa. The solid tumors formed by Tca83 were the smallest of the three cell lines (mean±SD: 0.105±0.223) and without surgical microscope analysis could have gone undetected. Tumor volume of GDC185 (mean±SD: 1.696±1.531) was smaller than Tca8113 and larger than Tca83.

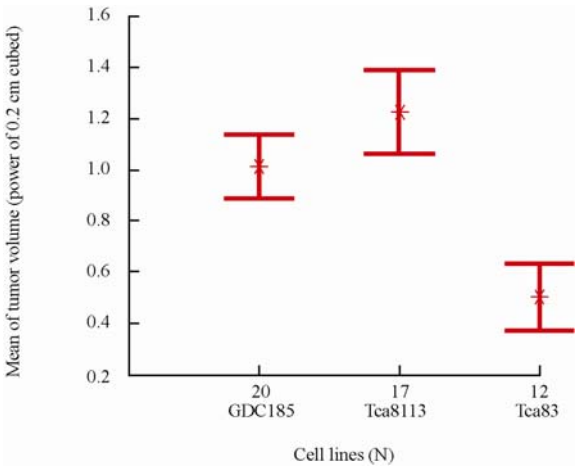

Figure 1. Comparison of tumor volume in the three cell lines. The volume of the implanted tumor was analyzed by one-way ANOVA test (mean±SD). Statistical analysis showed that the tumor volume among the three cell lines had a significant difference (P <0.05), though all the cell lines could develop into solid tumors. The difference was also significant between groups (P <0.025). *represents mean volume; red bars represent standard deviation.

Comparison of lymph node metastases in three cell lines

Comparison of local lymph node metastasis of all injection sites in the three cell lines

Comparison of local lymph node metastasis of all injection sites in the three cell lines (Table 3) showed that

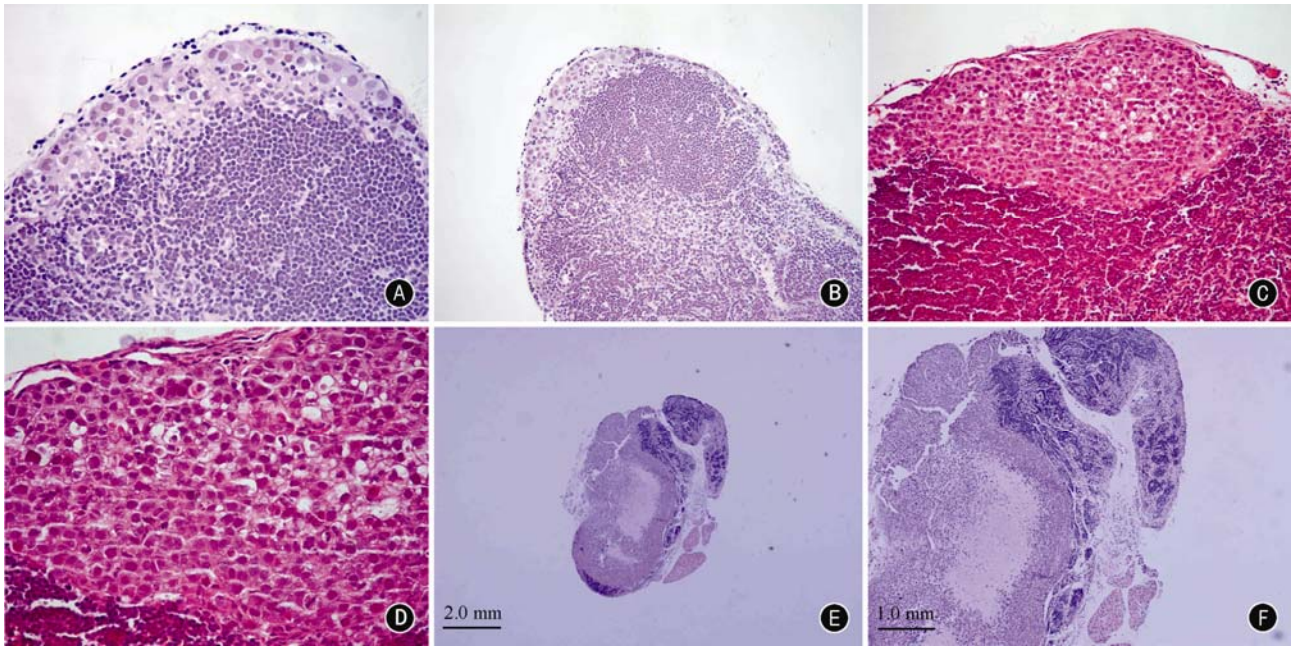

Figure 2. A and **B:** the tumor cell growth, limited in the marginal sinus, grade I (A: original magnification × 400; B: original magnification × 200). **C** and **D:** the tumor cell invasion and proliferation from the marginal sinus to the paracortex or the cortical sinus, grade II (C: original magnification × 200; D: original magnification × 400). **E** and **F:** replacement and disappearance of most of the lymph tissue, grade III (E: original magnification ×40; F: original magnification × 100) (A–F: HE staining).

Tca83 had minimum metastatic capacity, GDC185 had maximum metastatic capacity and Tca8113 was in between. The Pearson chi-square and Logistic regression tests did not show any significant differences in the histological metastatic progression between the GDC185 and Tca8113 groups (P >0.05). However there was a statistically significant difference between these two cell lines and Tca83 (P <0.05).

Table 3. Metastatic capacity of three cell lines (n)

Locations	GDC185	Tca8113	Tca83
Tongue	0/9	1/9	1/9
Floor of the mouth	5/9*	0/9	0/9
Axillary fossa	4/9	7/9#	0/9
Total	9/27△	8/27△	1/27

*P <0.05, GDC185 compared with Tca8113 and Tca83. #P <0.05, Tca8113 compared with Tca83. △P <0.05, GDC185 and Tca8113 compared with Tca83 respectively.

Comparison of metastatic capacity of each injection in different cell lines

On the FOM, GDC185 showed typical lymph node metastatic capacity (Table 3), whereby the metastasis was observed in 5 out of 9 animals (55.56%). However, there was no metastasis in Tca8113 and Tca83 specimens. The difference between GDC185 and Tca8113, and between GDC185 and Tca83 were significant (P <0.05) (Table 3).

The three cell lines were observed to have different metastatic rates. On the axillary fossa, the local metastasis rates were observed in 7/9 specimens injected with Tca8113. Metastasis was also observed in both Tca8113 and GDC185. Metastasis was not observed in Tca83 specimens. There was no significant difference between GDC185 and Tca8113, or between GDC185 and Tca83. However, the difference between Tca8113 and Tca83 was statistically significant (P <0.05).

On the tongue, there was no metastasis in GDC185. Though metastasis was observed in both Tca8113 and Tca83, the metastasis rate was very low (1/9). The difference among them was not significant (P >0.05).

Histological stage of metastatic lymph node foci

The histological stage of metastatic tumors in the local lymph nodes was graded according to Honma's classification.[5] Color histological graphs are shown in Figure 2. Figures 2A and 2B show that the growth of tumor cells is limited to the marginal sinus, whereas Figures 2C and 2D show the invasion and proliferation of tumor cells from the marginal sinus to the paracortex or the cortical sinus. Figures 2E and 2F show the regeneration and disappearance of most of the lymph tissue. From Table 4, we can see that the proliferative capacity of Tca8113 was greater than that of the other cell lines.

Table 4. Histological stages of lymph node metastasis foci (n)*

Cell lines	Number of lymph nodes with metastatic tumors	Histologic stage of metastatic tumor*		
		I	II	III
GDC185	9	5	3	1
Tca8113	8	0	1	7
Tca83	1	1	0	0

*Criteria by Honma.[5]

DISCUSSION

In cancer treatment, metastasis is the most formidable problem faced by the clinical oncologist.[6,7] The prognosis

of a patient with cancer metastasis is usually poor because no effective systemic therapy is generally available.[8] *In vivo* tumor models can be used to evaluate new therapeutic methods. Many investigators have used xenograft models of tumor progression to study the development and behavior of human carcinomas. The athymic nude mouse was first described by Pantelouris.[9] Povlsen and Rygaard[10] who first seeded human tumor cells into nude mice, and this model has been used to study the relationship between the host and tumor in a variety of anatomical sites.[11,12] Braakhuis et al[13] and Hier et al[14] particularly used the xenograft model to study head and neck cancers. These studies and others findings are useful in studying the development and propagation of tumors and the development of metastasis.

However, they were not able to describe the phenomena of local lymphatic metastasis. Many researches confirmed that injecting tumor cells into different locations of nude mouse can establish correspondingly variable lymphatic metastatic animal models. Myers et al[15] reported that they implanted the human squamous cell carcinoma of the oral cavity cell lines Tu159, Tu167, and MDA1986 into the submucosa of tongue and this formed solid tumors and produce lymph node metastasis. However, the rate of lymph node metastasis was mostly quite low.

Dinesman et al (1990)[16] initially simulated the physiological environment of the human oral cavity and developed an animal model of lymph node metastasis in nude mice. The metastatic rate is 2/42 (4.76%). In 1998, Simon et al[17] observed one metastasis in 24 animals. In 2002, Zhang et al[18] during their first screening, did not find any lymph node metastasis, but observed a 1/5 (20%) rate of metastasis in the second screening, and 4/9 (44.44%) in the third. Unfortunately, the rate of lymph node metastasis was very low and not suitable for studying lymphatic metastases. Our experiment shows that the metastatic rate was 5/9 (55.6%) in the GDC185 cell line. This result is lower than that reported in the study by Kawashiri et al[19] who obtained a rate of 9/11 (81.82%). However, we believe that our result is more representative of what is observed in a clinical setting.

Due to Simon's results[17] of lower lymphatic metastatic rate, we selected different sites of injection. In the FOM, we transcervically inoculated tumor cells into the space of the FOM muscles. The space should be adjacent to or in close proximity to the oral mucosal floor. However, in Simon's experiment, they transcervically inoculated tumor cells into the subcutaneous tissue to a depth superficial to the mylohyoid muscle underlying the FOM. Moreover, their studies emphasized the invasion of the tumor, but not its lymph node metastasis.

In order to find the right type of animal model and study the mechanism of lymphatic metastasis, we should select three appropriate cell lines with which to compare them. As we know, human tumors are heterogeneous[20] and metastases of human tumors are likely to result from a selection among subpopulations of the primary tumor. By screening the population, the selected tumor cells were of higher potent metastatic capacity. GDC185 was established from a neck lymph node metastasis of upper gingival squamous cell carcinoma and has been selected *in vivo*, which means the lymph node metastatic potential is higher. Tca8113 and Tca83 are from a primary solid tumor of the tongue that was also diagnosed as squamous cell carcinoma.

The present experimental results indicated that inoculating GDC185 cells into the FOM produces an appropriate animal model of lymph node metastasis, and the metastatic pattern was representative of lymph node metastasis of human oral squamous cell carcinomas in the clinical setting. All three cell lines were able to form solid tumors; albeit their tumor volumes were significantly different, indicating that the proliferative capacity of the three cell lines was different. Tca8113 had the greatest proliferative capacity, Tca83 had the least, and GDC185 was in the middle. This difference could also be observed in the histological staging of lymph node metastasis. Considering the three injection sites, GDC185 and Tca8113 had significantly higher metastatic capacity than Tca83. When the metastatic capacity of various injection sites was calculated separately, there was a difference between GDC185 and Tca8113. Although GDC185 and Tca8113 produced similar lymph node metastases in the axillary fossa, the former had a higher rate of metastasis in the FOM compared to the latter. Carefully studying the tumorigenicity and lymph node metastasis of the three cell lines, we found that of all the injection sites, Tca8113 had the strongest metastatic capacity, which coincided with its proliferative capacity in axillary fossa. Secondly, Tca83 demonstrated the lowest proliferative capacity, whereby its tumor volumes in sites of injection were negligible. Surprisingly, its rate of tumorigenicity was higher than other cell lines in the tongue. Thirdly, the proliferation capacity of GDC185 was in between that of Tca83 and Tca8113, but its metastatic capacity was higher in the FOM. There is either a connection or a conflict between cell differentiation and proliferation in the evolutionary development of the cell. Invasion and metastasis are important biological behaviors of carcinoma.[21] Our study showed a relationship between the metastatic and proliferative capacity of the Tca8113 cell line. The latter enhanced invasion, although invasive capacity is not necessarily correlated with lymphatic metastatic capacity. Our viewpoint is in accordance with Gao's notion.[22]

Fidler et al (2007),[23] concluded that orthotopic implantations result in rapid growth of local tumors, and in several model systems produce distant metastases. This phenomenon may be explained by the organ microenvironment hypothesis. However, we discovered that Tca83 has a higher tumorigenicity (6/9) and lower metastatic rate (1/9) in the tongue. This observed

phenomenon appears to conflict with what is observed in the clinical setting, as tongue carcinomas have a higher metastatic rate according to Fidler's theory. The GDC185 cell line demonstrated malignant tumor characteristics, which were reflected in lymphatic metastatic capacity, that were higher than those of the other cell lines. The animal model is consistent with cases in the clinical setting; therefore we propose it to be an optimal animal model for studying oral carcinoma lymph node metastases. There are several hypotheses as to why GDC185 could have higher lymphatic metastasis rate in the FOM. First, the mechanical pressure produced by the muscles of FOM and rapidly proliferating neoplasms may force cords of tumor cells along the tissue planes of least resistance and may also increase cell motility capacity.[24] However, one can question why the three cell lines respond differently under the same inoculation conditions. We propose that the expression of metastasis-related genes (e.g., E cadherin, matrix metalloproteinases) may contribute to this phenomenon.

In conclusion, the results of this study showed that the GDC185, Tca8113 and Tca83 cell lines demonstrate different biological capacities in proliferation and metastasis in the context of various injection sites. These findings suggest that inoculating tumor cells in the FOM in nude mice is a reasonable model that imitates lymph node metastases of human OSCC.

REFERENCES

1. Massano J, Regateiro F, Januario G, Ferreira A. Oral squamous cell carcinoma: review of prognostic and predictive factors. Oral Surg Oral Med Oral pathol Oral Radiol Endod 2006; 102: 67-76.
2. Som PM. Detection of metastasis in cervical lymph nodes: CT and MRI criteria and differential diagnosis. Am J Radiol 1992; 158: 961-969.
3. Langley RR, Fidler IJ. Tumor cell-organ microenvironment interactions in the pathogenesis of cancer metastasis. Endocr Rev 2007; 28: 297.
4. He RG, Xu XQ, Zhou XJ, Zhang XL, Qiu WL, Zhang XZ, et al. The establishment and some biological characteristics of a squamous cell line of the human tongue (Tca8113). Cancer (Chin) 1983; 3: 97-100.
5. Honma Y. A study on metastasis to cervical lymph node of oral cancer. Jpn J Oral Maxillofac Surg 1982; 28: 1667-1684.
6. Shida H, Ban K, Matsumoto M, Masuda K, Imanari T, Machida T, et al. Prognodtic significance of location of lymph node metastasis in colorectal cancer. Dis Colon Rectum 1992; 35: 1046-1050.
7. Maurel J, Launoy G, Grosclaude P, Gignoux M, Arveux P, Mathieu-Daude H, et al. Lymph node harvest reporting in patients with carcinoma of the large bowel: a rench population-based study. Cancer 1998; 82: 1482-1486.
8. Tsutsumi S, Kuwano H, Morinaga N, Shimura T, Asao T. Animal model of para-aortic lymph node metastasis. Cancer Lett 2001; 169: 77-85.
9. Pantelouris EM. Absence of thymus in a mouse mutant. Nature 1968; 217: 370-371.
10. Povlsen CO, Rygaard J. Heterotransplantation of a human malignant tumor to nude mice. Acta Pathol Microbial Scand 1972; 80: 713-717.
11. Naito S, von Eschenbach AC, Giavazzi R, Fidler IJ. Growth and metastasis of tumor cells isolated from a human renal cell carcinoma implanted into different organs of nude mice. Cancer Res 1986; 46: 4109-4115.
12. Naomoto Y, Kondo H, Tanaka N, Orita K. Novel experimental models of human cancer metastasis in nude mice: lung metastasis, intraabdominal carcinomatosis with ascites, and liver metastasis. J Cancer Res Clin Oncol 1987; 113: 544-549.
13. Braakhuis BJ, Sneeuwloper G, Snow GB. The potential of the nude mouse xenograft model for the study of head and neck cancer. Arch Otorhinolaryngol 1984; 239: 69-79.
14. Hier MP, Black MJ, Sadeghi N, Kataoka H. A murine model for the immunotherapy of head and squamous cell carcinoma. Laryngoscope 1995; 105: 1077-1080.
15. Myers JN, Holsinger FC, Jasser SA, Bekele BN, Fidler IJ. An orthotopic nude mouse model of oral tongue squamous cell carcinoma. Clin Cancer Res 2002; 8: 293-298.
16. Dinesman A, Haughey B, Gates GA, Aufdemorte T, Hoff DDV. Development of a new *in vivo* model for head and neck cancer. Otolaryng Head Neck 1990; 103: 766-774.
17. Simon C, Nemechek AJ, Boyd DD, O'Malley BW, Goepfert H, Flaitz CM, et al. An orthotopic floor of the mouth cancer model allows quantification of tumor invasion. Laryngoscope 1998; 108: 1689-1691.
18. Zhang X, Liu YN, Glicrease M, Yuan XH, Clayman GL, Chen Z, et al. A lymph node metastatic mouse model reveals alterations of metastasis-related gene expression in metastatic human oral carcinoma sublines selected from a poorly metastatic parental cell line. Cancer 2002; 95: 1663-1672.
19. Kawashiri S, Kumagai S, Kojima K, Harada H, Yamamoto E. Development of a new invasion and metastasis model of human oral squamous cell carcinomas. Oral Oncol Eur J Cancer 1995; 31B: 216-221.
20. Nowell PC. The clonal evalution of tumor cell populations. Science 1976; 194: 23-28.
21. Gao J. Carcinoma Invasion and Metastasis. Beijing: Science Press; 2003: 1.
22. Gao J. Carcinoma Invasion and Metastasis. Beijing: Science Press; 2003: 28
23. Fidler IJ, Kim SJ, Langley RR. The role of the organ microenvironment in the biology and therapy of cancer metastasis. J Cell Biochem 2007; 101: 929.
24. Onn A, Fidler IJ. Metastatic potential of human neoplasms. In Vivo 2002; 16: 423-429.

(Received May 7, 2008)
Edited by LIU Dong-yun