·Technology and Methods·

Establishment of patient-derived xenotransplantation models for non-small cell lung cancer in immune deficient mice

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[Abstract] **Background and Objective:** Targeted therapies have become a valuable therapeutic option for cancer. Establishment of different animal tumor models has become necessary. This study was to establish xenotransplantation models for patient-derived non-small cell lung cancer (NSCLC) in immune deficient mice. **Methods:** Immune deficient mice, BALB/C-nu, NOD/scid and SCID, 16 in each strain, were used. Sixteen tumor specimens were obtained from patients with NSCLC. Each specimen was subcutaneously transplanted into one mouse from each of the three strains. The tumor formation rate, time to tumor engraftment, tumor volume doubling time were recorded and compared among the three strains of mice. Histology of xenograft tumors was examined. **Results:** The total tumor formation rate was 75% (12/16). The tumor formation rate was the highest in SCID mice (56.25%). Only four tumors were engrafted in SCID mice, and two in BALB/C-nu mice. The tumor formation rate, time to tumor engraftment, and tumor volume doubling time were not significantly different among the three strains of mice. The incidence of tumor size over 1cm in the upper flanks of the mice (56.25%) was significantly higher than that in the lower flanks (25%) (P=0.037). Haematoxylin Eosin staining revealed a high degree of histological similarity between all xenograft and the parental tumors. **Conclusions:** We have established xenotransplantation models for patient-derived NSCLC with a success rate of 75% in BALB/C-nu and SCID mice. The xenograft tumors have the same histological features to those of their parental tumors.

Key words: non-small cell lung cancer, xenograft tumor, immune deficient mice, transplantation

Lung cancer is one of the most common malignant cancers in China. It also ranks the third among the death cause of the malignant cancers in China.[1] The five- year survival rate of lung cancer remains lower than 15%. Therefore, it has important significance to improve the recovery rate and the five-year survival rate of lung cancer. Lung cancer has heterogeneity, so that every patient has its own response to the anti-lung-cancer drugs; studies on the onco-molecular biology reveal that the occurrence and development of the cancer are closely related to the abnormal changes in the intracellular signaling pathway. Researchers have designed drugs for the targeted molecule in different pathways. Targeted drugs are recommended for individualized therapy.[2] We

planed to establish several different animal models corresponding to different lung cancers which have abnormal pathways, so that we could explore suitable drugs for those different pathways. Besides, lung cancer cell lines from Chinese patients are not sufficient, so a group of cell lines were expected to be established using the animal xenotransplantation models. This study aimed to investigate the establishment of patient-derived xenotransplantation models for non-small cell lung cancer (NSCLC) in immune deficient mice, and improve the successful rate of the xenotransplantation models of lung cancer.

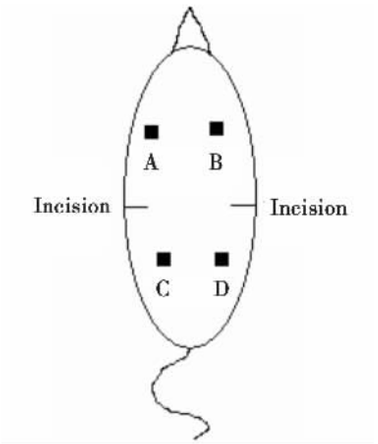

Figure 1 Inoculation sites of non-small cell lung cancer tissues in immune deficient mice

Materials and Method

Laboratory animals and the xenotransplantation models of lung cancer in immune-deficient mice. Four-eight week BALB/C nude mice, SCID mice and NOD/SCID mice, 16 in each strain were used. The number of male and female mice was equal. The mice were weight ranging from 15 to 25g, and were brought from the Institute of Laboratory Animal Sciences, CAMS&PUMC, and were raised in a specific pathogen-free (SPF) condition. The lung cancer tissues were taken from the operating room in the condition of germ free, then put it in the sterile centrifuge tube containing 15ml Hanks fluid, which were moved to the laboratory at 4℃ and washed by 0.01M PBS in the superclean bench for three times. Subsequently, the normal and the necrotic tumor tissues were removed, and cut into 1.0-1.5 mm^3 pieces, which were subcutaneously transplanted into the back of the three strain immune deficient mice by the trocar. Each specimen derived from the patients was transplanted into one mouse from each of the three strains, and each mouse was transplanted in four positions labeled ABCD. Each of the two points on the right was transplanted with one tumor piece (1.0-1.5 mm^3), but that on the opposite side was transplanted with two pieces. Tumor tissues were inoculated into the four points using the trocar (Fig. 1). Tumor tissues from 16 patients were transplanted, and the pathologic types were: four out of them were adenocarcinoma; 11 of them were squamous carcinoma, and only one of them was atypical carcinoid with poor differentiation.

Indicators. Tumor formation rate and the tumor volume doubling time. The mice were observed once a week since the tumor tissues were transplanted, and the observation lasts two-six months. When the tumor mass could be observed by naked eyes, the long diameter (b) and the short diameter (a) were measured once a week using the vernier caliper. The tumor was assumed to be ellipsoid, and the volume of the tumor was calculated by the formula $V=1/2a^2b$. Mice were kept alive until the long diameter of the tumor was over 1 cm. The largest tumor from each mouse was chosen, the tumor volume doubling time (Td) was calculated by the formula $Td=lg2\times (t1-t0)/ (lgV1-lgV0)$, where T stands for the days, and V stands for the volume. The tumor formation rate of different animals was calculated (the successful transplantation number/the transplantation number). If the long diameter of the xenotransplantation tumor was over 0.5cm, it meant that the tumor was successfully engrafted. The time to tumor engraftment was from the transplant time to the date when the long diameter was over 0.5cm. The natural mortality of animals of different strains were calculated (The total number of animals which did not get ill or die because of the enlargement of the tumor, and which were put to death after the transplantation because of other

Table 1 Comparison of xenografts formed in different immune deficient mice

Strain	Tumor formation rate	Time to tumor engraftment (d)	Tumor doubling time(d)	Nature mortality
BALB/C-nu	43.75%(7/16)	56.33±25.95	13.17±11.47	0%(0/16)
NOD/scid	25.00%(4/16)	45.25± 7.72	9.64± 8.86	25%(4/16)[a]
SCID	56.25%(9/16)	71.38±28.30	10.01± 6.45	0%(0/16)

[a]$P<0.05$, vs. BALB/C-nu and SCID. Values are presented as mean±SD.

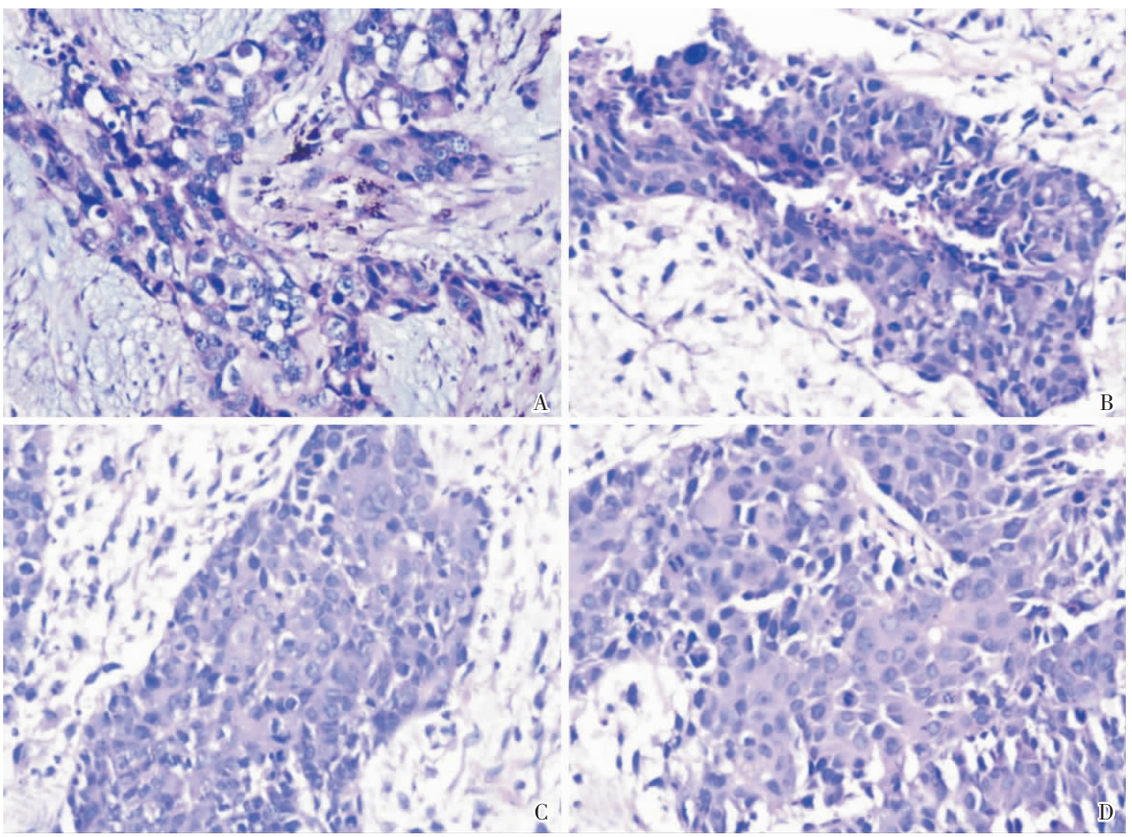

Figure 2 Haematoxylin Eosin staining of the patient-derived non-small cell lung cancer tissues and xenografts(×20)
A: Moderately-poorly differentiated squamous cell carcinoma; B: Xenografts developed in the NOD/scid mouse; C: Xenografts developed in the SCID mouse; D: Xenografts developed in the BALB/C-nu mouse.

diseases, and which died from a natural death / the total number of transplanted animals); the tumor formation rate of the left or right transplantation points (tumors over the size of 1 cm in the left or right tumor formation points /tumors in the left or right transplanted points); the incidence of tumors of the size of over 1cm in the upper flanks or the lower flanks (the tumors whose size in the upper flanks or the lower flanks were over 1cm/the tumors could be seen in the upper flanks or the lower flanks).

Histology. The mice were killed by excess pentobarbital administration when the long diameter of the immune-deficient mice was over 1cm. The tumor was dissected and observed by naked eyes. Tumors were then fixed in 10% formalin, embedded with paraffin and sliced, then stained with Haematoxylin-Eosin (H&E) and performed keratin immunohistochemisty staining, finally observed under the microscope. The keratin primary antibody was monoclonal mouse anti-human CK (cytokeration pan) (Cell Signaling Technology, USA). The primary antibody was replaced by PBS as the negative control. The SP(UltraSensitive SP kit of Fuzhou Maixin) method, AEC coloration, haematoxylin counterstaining were performed. Keratin-positive was defined if red particles were observed in the

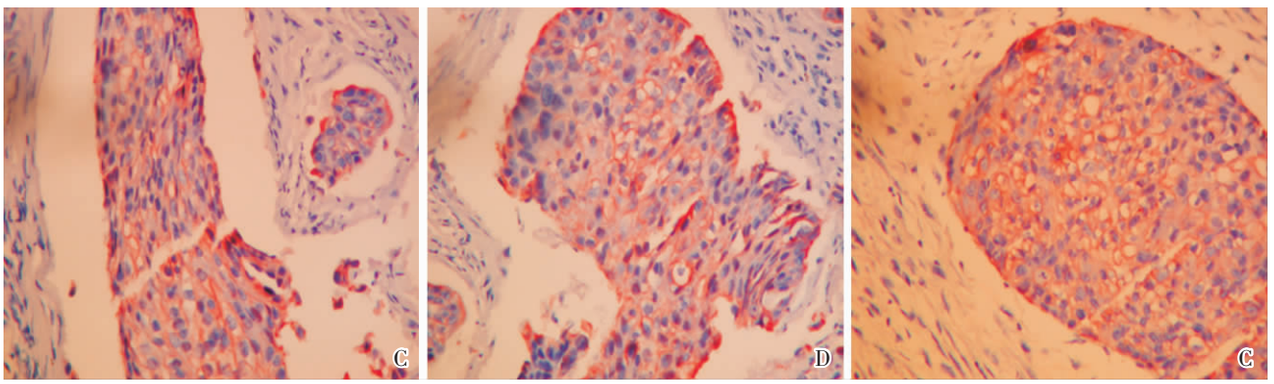

Figure 3 Expression of keratin in tissues of xenografts of non-small cell lung cancer (IHC ×20)
Positive expression of keratin (red) in the cytoplasm of tumor cells in xenografts formed in the NOD/scid mouse (A), the BALB/C-nu mouse (B), and the SCID mouse (C).

intracytoplasm of the tumor cells under the microscope.

Statistical analysis. All data were expressed as mean± SD or the percentage, and analyzed by the SPSS 13.0 Software package. $p<0.05$ was considered statistically significant. Variance analysis, quadruple tabular form data and χ^2 analysis of the line*list data were adopted.

Results

Tumor formation rate and the tumor volume doubling time. Twelve out of 16 tumor specimens were engrafted finally. Only four of them were engrafted in SCID mice, and only two in BALB/C-nu mice, none in the NOD/SCID mice. The total tumor formation rate was 75%. The pathologic types contained four adenocarcinoma and eight squamous carcinoma. The tumor formation rate, time to tumor engraftment, tumor volume doubling time and the natural mortality of the different immune-deficient mice are shown in the Table1. The natural mortality of the different strains of mice had statistical significance (p=0.013). The tumor formation rate of the right side where only one piece was engrafted was 18.75% (18/96), and that of the left side where two pieces were engrafted was 26.04% (25/96), however, the difference between them had no statistical significance (p=0.22). The incidence of the tumor size of over 1cm in the upper flanks of the mice was 56.52% (13/23), and that in the lower flanks was 25% (5/20),the difference between these two had statistical significance (p=0.037).

Histological observation. The tumors were seen subcutaneously, with the globoid or ellipse shape. Some of them appeared to be lobular, and had several round nodules. The envelope was complete without adhesions to the skin; the texture of the surfaces was hard, and the incised surface appeared gray; a few of the tumors were accompanied with hemorrhage and necrosis. The H&E staining revealed that the histological morphologies between all xenograft tumors and the parental tumors had a high degree of histological similarity, all of which were adenocarcinoma or squamous carcinoma as the parental tumors; the cells of the tumor were round or ellipse shapes; the volume was comparatively large, appeared as irregular masses; the endochylema was abundant, but the nucleus did not have the same size, and was anachromasis; the karyokinesisi could be seen frequently, and the connective tissues were found in the mesenchyme (Fig. 2). The keratin immunohistochemisty staining of the transplanted tumor were all positive, and red particles were seen in all of the intracytoplasm of the tumor cells (Fig. 3).

Discussion

The full name of BALB/C nude mice is CAnN.Cg-Foxn1^{nu}/CrlVR, with T lymphocyte-deficiency; the full name of NOD/SCID mice is NOD/LtSzPrkdcscid/J, with T lymphocyte-, B lymphocyte- and natural killer

cell-deficiency; the full name of SCID mice is BALB/cByJSmnPrkdc (scid)/J, with T lymphocyte- and B lymphocyte-deficiency. Our study was to use these immune deficient mice to find out which strain was suitable to establish specimen-derived subcutaneous xenotransplantation models for NSCLC. Though the results displayed that the formation rate among these three kinds of nude mice were not statistically significant, the formation rate of the BALB/C and the SCID nude mice was higher than that of the NOD/SCID mice; moreover, only four tumors were engrafted in SCID mice, and two in BALB/C-nu mice, but none in NOD/SCID mice. Besides, the natural mortality of the NOD/SCID mice was higher than that of the other two strains, which was 25% after being transplantated for 2-6 months. The reason is due to the low activity of the NK cells and the NOD-phile leukemia provirus gene "Emv30" carried by the No.11 chromosomes in NOD/SCID mice, so that the mice were liable to the early fetal breast adenolymphoma, whose mean life-span was less than eight months.[3] Because of the long latency period and the low tumor formation rate, also in order to save the cost, we did not consider the NOD/SCID mice for the research of NSCLC xenograft tumors. The natural mortality of the BALB/C and the SCID nude mice had little difference, and both of them could form tumors alone. SCID had a higher tumor formation rate; the skin of BALB/C mice was hairless, more suitable for observing the growth of the tumor. Our suggest that both the SCID and BALB/C nude mice could be used in the first generation to improve the successful engraftation rate, but in the second generation the BALB/C mice could be adopted for observing in the intervention experiment.

It has a long history to use the immune-deficient mice for the tumor research. However, most of them were engrafted by cell lines instead of the specimen, due to the lack of patient-derived specimens and the low successful engraftation rate. Fichtner et al.[4] transplanted 102 NSCLC tissues subcutaneously into the NOD/SCID mice; each specimen was engrafted into three-six mice. Finally 25 of the mice had tumor, with a successful rate of 24.5% . Anderson[5] transplanted 37 lung cancer tissues subcutaneously into the SCID mice, and the successful cases were 17 (46%). We established the model by engrafting one or two pieces in four points by the trocar. The total success rate could reach up to 75%. Even if the specimen was only transplanted into the SCID mice, the rate could also reach 56.25%. We engrafted each specimen into both BALB/C and the SCID mice, even without anesthesia and wound saturation. Most researchers tended to implant using anesthesia and the saturation methods,[6,7] and more embedding tissues are required. Generally, the weight of the mass transplanted into two different positions was about 225mg each.[5] We just used about 100mg for each one. Our method is easy and convenient to operate, needs less tissue, and yields a high tumor formation rate. Therefore it is recommended to transplant the fresh patient-derived tumor specimens subcutaneously. Simultaneously, we found that the successful rate between the two ways, one is to engraft one piece and the other one is engraft two pieces subcutaneously on the back; but the difference was not statistically significant. To improve the tumor formation rate, we also suggest that two pieces should be engrafted in the model. We revealed that the incidence of tumors whose size were over 1cm in the upper flanks of the mice was significantly higher than that in the lower flanks in the same growth. It was indicated that the tumor grew faster in the upper one than in the lower one, maybe because of the abundant blood transportation in the upper flanks. Du et al.[8] indicated that the growth speed of the engrafted tumor in the middle of the consequent was faster than that in the middle of the back and the forelimb axilla, which was similar to ours. H&E staining revealed that the xenograft tumors had the same histological features to those of parental tumors, and the tumors were keratin-positive, illustrating that the xenograft tumors had the epithelial origin.

In summary, we established xenotransplantation models from patient-derived NSCLC tissues in immune-deficient BALB/C-nu and SCID mice by engrafting two pieces of the tumor tissues in

four points by the trocar.

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