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# A liver-metastatic model of human primary gastric lymphoma in nude mice orthotopically constructed by using histologically intact patient specimens

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**[Abstract] Background and Objective:** In recent years, incidence and mortality of lymphoma are markedly increasing worldwide. However, the pathogenesis and mechanism of invasion and metastasis for lymphoma are not yet fully clarified. It is mainly due to the lack of ideal animal models, which can precisely simulate the invasion and metastasis of lymphoma in the human body. So, it is very necessary to establish a highly metastatic nude mouse model of human lymphoma. This study developed a liver-metastatic model of primary gastric lymphoma in nude mice by using orthotopic surgical implantation of histologically intact patient specimens into the corresponding organs of the recipient small animals. **Methods:** A histologically intact fragment of liver metastasis derived from a surgical specimen of a patient with primary gastric lymphoma was implanted into the submucosa of the stomach in nude mice. Tumorigenicity, invasion, metastasis, morphologic characteristics (via light microscopy, electron microscopy, and immunohistochemistry), karyotype analysis, and DNA content of the orthotopically transplanted tumors were studied. **Results:** An orthotopic liver metastatic model of human primary gastric lymphoma in nude mice (termed HGBL-0304) was successfully established. The histopathology of the transplanted tumors showed primary gastric diffuse large B-cell lymphoma. CD19, CD20, CD22, and CD79 $\alpha$  were positive, but CD3 and CD7 were negative. The serum level of lactate dehydrogenase (LDH) was elevated [(1010.56  $\pm$  200.85) U/L]. The number of chromosomes ranged from 75 to 89. The DNA index (DI) was 1.45  $\pm$  0.25 (that is, heteroploid). So far, the HGBL-0304 model has been passed on for 45 generations of nude mice. A total of 263 nude mice were used for the transplantation. Both the growth and resuscitation rates of liquid nitrogen cryopreservation of the transplanted tumors were 100%. The transplanted tumors autonomically invasively grew and damaged a whole layer in the stomach of nude mice. The metastasis rates of liver, spleen, lymph node, and peritoneal seeding were 100%, 94.3%, 62.6%, and 43.5%, respectively. **Conclusions:** The study successfully establishes an orthotopic liver metastatic model of human primary gastric lymphoma in nude mice. The HGBL-0304 model can completely simulate the natural clinical process of primary gastric lymphoma and provides an ideal animal model for the research on the biology of metastasis and antimetastatic experimental therapies of primary gastric lymphoma.

**Key words:** Neoplasm of the stomach, lymphoma, neoplasm transplantation, metastasis, nude mice model

Primary gastric lymphoma is a type of extranodal gastrointestinal lymphoma, which is a malignant primary gastric cancer originating from the lymphoid tissue in the submucosa. Primary gastric lymphoma makes up 5%–11% of malignant gastric carcinomas, with the highest incidence of malignant cancer except for gastric carcinoma<sup>[1,2]</sup>. Recently, the incidence

of non-Hodgkin's lymphoma, especially of extranodal origin, has increased<sup>[3,4]</sup>. However, the pathogenesis and mechanisms of lymphoma are not fully clarified. It is mainly due to the lack of ideal in-vivo and in-vitro animal models. Previous studies used the subcutaneous transplantation of lymphoma cell lines to establish lymphoma models in nude mice. However, due to the genetic instability, microenvironmental variations, and mesenchymal deficiencies of in-vitro cell lines, the model of subcutaneous transplantation of lymphoma cell lines in nude mice did not perfectly simulate the whole processes of growth, invasion, and metastasis of human lymphoma, which became a bottleneck in studying the metastasis of lymphoma and antimetastatic therapies<sup>[5]</sup>. We used histologically intact fragments of liver metastasis derived from the surgical specimen

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of a patient with primary gastric lymphoma and continuously passed-on selection in vivo to establish an orthotopic liver metastatic model of human primary gastric lymphoma in nude mice (termed HGBL-0304), based on the model of human primary liver and spleen lymphoma in nude mice<sup>[6,7]</sup>. This model reproduced the natural pathologic processes of human gastric lymphoma and provided an ideal model for basic and clinical research of gastric lymphoma.

## Materials and Methods

### Animals

BALB/C-nu/nu nude mice were from the Chinese Academy of Medical Sciences Cancer Institute. The certificate code was SCXK00-0005. The mice were aged 3–5 weeks, weighed 17–20 g, and housed in a specific pathogen-free (SPF) environment.

### Clinical samples

Fresh tissue was from a male patient of 69 years in the Hepatobiliary Surgery of the People's Liberation Army 202 Hospital, who was pathologically diagnosed as having primary non-Hodgkin's lymphoma (diffuse large B-cell lymphoma) with no history of preoperative radiotherapy and chemotherapy. The surgical resection of the fragment of liver metastasis was cultured in RPMI-1640, nontumor tissue was removed, and the fragment was sheared into pieces of 1 mm × 1 mm × 1 mm for transplantation.

### Orthotopic transplantation and passing-on

The mice were anesthetized by pentobarbital sodium and operated on under a 10 × surgical microscope. A 2-mm long incision was made in the gastric antrum along the lesser curvature side after making an incision in the center of the epigastrium, deep to the submucosa. With dimensions of 1 mm × 1 mm × 1 mm, 2 liver metastatic tumors were fixed on the stomach submucosa using a 10-0 noninvasive suture through the incision, a 7-0 thread for stitching the peritoneum, and a 5-0 thread for sewing skin. In-vivo organ screening method was used. After the tumors formed, the liver metastatic tumors were transplanted into the submucosa of the stomach in the mice. Screening for liver metastasis in the mice was repeated 4 times, then the liver metastases were transplanted into the stomachs of the mice<sup>[8]</sup>.

After the procedure, the mice were housed, freely fed, and observed every 2 weeks. When the tumor-bearing mice were in a dying state, 1 was killed by cervical dislocation surgery and the tumor was removed. One section of the tumor was continuously passed on in the mice as the first generation, and 3–10 mice were used for each passage. The other section of the tumor was stored in liquid nitrogen and used for detection. Other tumor-bearing mice were housed until dying or natural death to observe the growth, invasion, and metastasis of the tumor.

### Observation items

**Anatomy and histology** All killed or naturally dead tumor-bearing mice were monitored by detailed anatomic examinations. The diameter of the tumor was measured and the tumor volume was calculated by  $1/6 \times \pi \times [(A + B)/2]^3$ , where A and B stood for

the longest and the shortest diameter, respectively. The invasion to internal organs was observed and determined tumor metastasis in the neck, the surrounding iliac artery, the arteria iliaca interstitium, mediastinal, mesenteric, pyloric, cardiac, and hepatic lymph nodes, and all internal organs. Tumor tissue, lymph nodes, and internal organs were fixed by 10% neutral formaldehyde, embedded in paraffin, stained by hematoxylin and eosin (H&E), and observed under a light microscope.

**Immunohistochemical staining** The labeled streptavidin biotin (LSAB) method was used. All gastric tumors in the mice or metastatic organs were fixed and embedded. CD3, CD7, CD19, CD20, CD45, and CD79α antibodies were used for immunohistochemical staining (all reagents were from DaKo Company).

**Electron microscope observation** Transplanted tumor tissue was fixed by both 2.5% glutaraldehyde and 1% osmic acid, embedded with Epon 812 in ultrathin sections, stained by uranium-plumbum and observed with a Philips CM-10 transmission electron microscope.

**Laboratory tests** Serum lactate dehydrogenase (LDH) was measured by the LDH-L method.

**Flow cytometry** An FACS 420 was used. The DNA content of samples of normal gastric mucosa, tumor tissue, and transplanted tumor tissue were measured. Cellfit software was used for statistical analysis according to the literature<sup>[9]</sup>.

**Chromosome examination** Cells were cultured for a short time, harvested without mitogen stimulation, sectioned conventionally, stained by GTG, and observed under the microscope<sup>[7]</sup>.

### Histologic classification of lymphoma

We used the new lymphoma classification adopted by the World Health Organization (WHO) in 2001<sup>[10]</sup>.

### Statistical analysis

Statistical analysis was performed using SPSS 12.0. Data were presented as mean ± standard deviation. Differences between the 2 groups were evaluated by *t* test and a *P* value of < 0.05 was considered statistically significant.

## Results

The liver metastasis model of human primary gastric lymphoma (non-Hodgkin's B cell) constructed using surgical orthotopic transplantation of histologically intact tissue in nude mice was called HGBL-0304.

### The establishment of a liver metastasis model of human primary gastric lymphoma HGBL-0304

Fresh liver metastasis fragments derived from primary gastric lymphoma was successfully transplanted into 5 nude mice. The HGBL-0304 model had been passed on through 45 generations in nude mice. A total 263 nude mice were used for transplantation with a latent period of 7.6 days on average. Among the 28 days of passage, both the growth rate and the resuscitation rate from liquid nitrogen of the transplanted tumors were 100%. The average volume and weight of the transplanted tumors were (2285.00 ± 415.52) mm<sup>3</sup> and (2.18 ± 0.21) g, respectively. After biopsy, all 263 mice bore tumors in the gastric

antrum, the lesser curvature of the stomach, and the pylorus. Some tumors infiltrated the entire stomach of the mouse. Liver metastasis appeared in all tumor-bearing mice (100%), spleen metastasis in 248 mice (94.3%), lymph node metastasis in 164 mice (62.6%), and peritoneal seeding metastasis in 114 mice (43.5%). The longest survival period was 76.5 days and the median survival period was 53 days. Usually, mice died of exhaustion in the 6–7 weeks.

### The growth characteristics of HGBL-0304 orthotopic transplantation

After orthotopically transplanting the liver metastasis of human primary gastric lymphoma, the results from autopsy showed that the tumor grew autonomously and invasively in the submucosal lamina of the stomach in the mice, infiltrated gradually, and invaded the whole layer of the stomach. Irregular multiple ulcers appeared in the lesser curvature of the stomach and the rugae of the gastric mucosa margin became thick. On cross-section, tumors in the ulcer margin were mainly located in the submucosa of the stomach. The tunica muscularis gastris at the bottom were all destroyed. The tumor tissue was gray, homogeneous, and glossy (Figure 1).

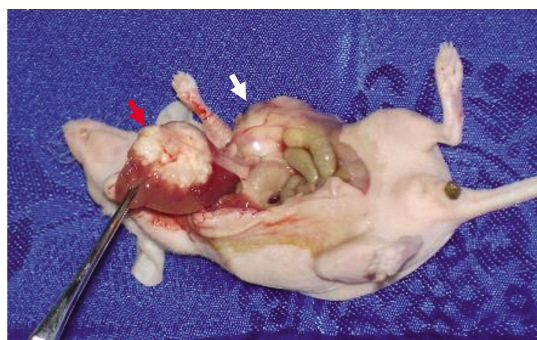


Figure 1 The 45th generation of the HGBL-0304 model (2 weeks after orthotopic transplantation)

The white arrow show the orthotopically implanted tumor, and the red arrow displays the liver metastatic tumor.

### Invasion and metastasis of the HGBL-0304 model

**Liver metastasis** Liver metastasis of HGBL-0304 appeared in the second week after transplantation, and was limited to the left lobe of the liver with either single nodular types or diffuse types. Tumors scattered in the left lobe, the right lobe, and even the whole liver with different sizes and numbers of round and elliptical gray tumor nodes and were located in the liver parenchyma (Figure 1). The metastatic tumor was as small as intravenous tumor embolus, or large with the size of 0.7 mm × 0.5 mm–1.0 mm × 1.5 mm. The longest diameter was 2.0 cm × 2.5 cm. Invasive growth of tumor cells were transferred to the center vein and portal area as a metastatic center and infiltrated in the entire liver tissue throughout the hepatic sinusoid (Figure 2). In a serious situation, many liver tissues were replaced by tumor nodules.

**Spleen metastasis** Spleen metastasis of HGBL-0304 appeared in the third week after transplantation with splenomegaly and

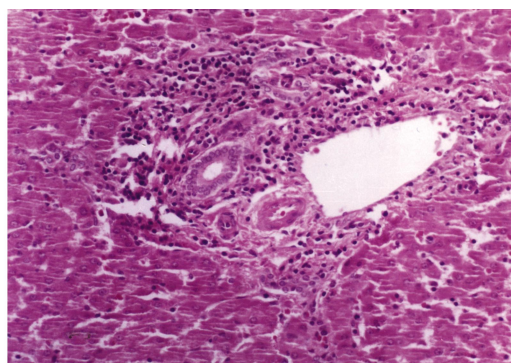


Figure 2 Liver tissue section of the HGBL-0304 model showing liver metastases

Tumor cells diffusely infiltrated the portal areas with disruption of interlobular bile ducts, arteries and veins, and spillover to the surrounding hepatic sinusoid (HE ×150).

congestion. Metastatic tumors were located in the upper or center sections, and the spleen sinus was crushed and broken. The trabecula and the capsula were involved. Cross-sections of the spleen showed single pinkish-gray tumor nodules (Figure 3).

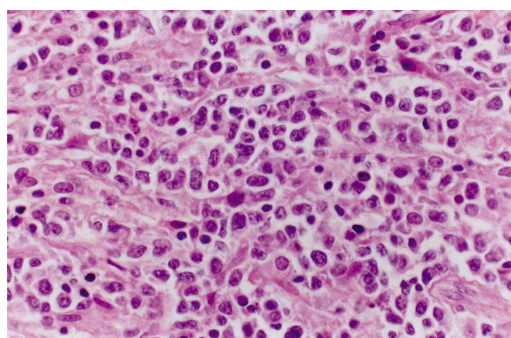


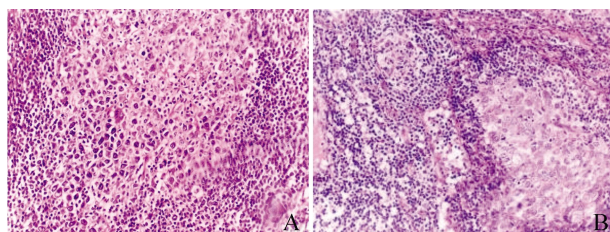
Figure 3 Spleen tissue section of the HGBL-0304 model showing spleen metastases

Tumor cells diffusely infiltrated the red pulp and white pulp with extrusion and disruption of the splenic sinusoid (HE ×200).

**Lymph node metastasis** Lymph node metastasis of HGBL-0304 appeared in the second week after transplantation, and was mainly located in the lesser curvature side of lymph nodes adjacent to the gastric coronary vein and the pyloric lymph nodes. Lymph node metastasis could be divided into 3 phases: (1) the early phase: the structure of the lymph node was still preserved, the lymphatic sinus was amplified, and scattered and small tumors appeared; (2) the metaphase: tumor cells grew invasively into the paracortical area and the medulla; and (3) the anaphase: the structure of the lymph node completely disappeared and replaced by a mass of tumor cells except for a small amount of residual lymphoid tissue. All lymph node structure was destroyed and developed to metastatic tumor nodules under severe conditions (Figure 4).

**Peritoneal seeding metastasis** Peritoneal seeding metastasis of HGBL-0304 appeared in the third week after transplantation. Tumor cells grew invasively, penetrated the serous membrane





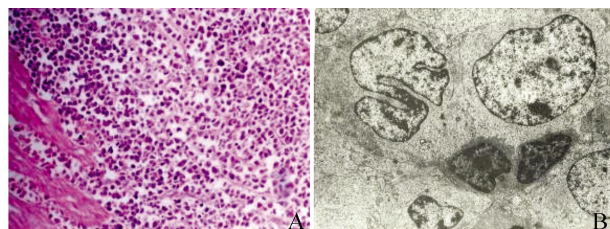
**Figure 4** Gastric lymph node metastases of the HGBL-0304 model

A, sections of gastric lymph nodes showing that tumor cells grew within the sinus of the lymph nodes (HE stain  $\times 200$ ). B, sections of gastric lymph nodes showing that tumor cells diffusely infiltrated lymph nodes with the destruction of the original normal structures and a small quantity of residual lymphatic tissue was present (HE  $\times 150$ ).

and migrated to peritoneum. Different sizes and numbers of gray tumor nodes were shown in the lumen and the parietal layers of the peritoneum, omenta, mesentery, and the parietal peritoneum.

### Histopathologic and electron microscope detection of the HGBL-0304 model

Under a light microscope, the HGBL-0304 cells were mainly large, with a polymorphic nucleus, common nuclear cracking, uneven nuclear chromatin, occasional nucleolus, little cytoplasm, more mesenchymal cells and diffused arrangements, and infiltrated all layers of the stomach. Under the electron microscope, tumor cells had irregular nuclei, deep fissures in the nucleus, bar-shaped heterochromatin along the nuclear membrane, different sizes of nucleoli, close to the nuclear membrane, little cytoplasm, and a small number of swollen and vacuolated mitochondria (Figure 5).



**Figure 5** Histopathologic examination of the HGBL-0304 model

A, sections of transplanted tumor tissues showing that the majority of tumor cells were large, and nuclei were large and obviously polymorphic with cleavage and a scarcity of cytoplasm. Tumor cells were arranged diffusely and infiltrated all layers of the gastric wall (HE  $\times 200$ ). B, ultrastructure of transplanted tumors showing that the nuclei of tumor cells were irregular in shape with a characteristic deep groove ( $\times 5000$ ).

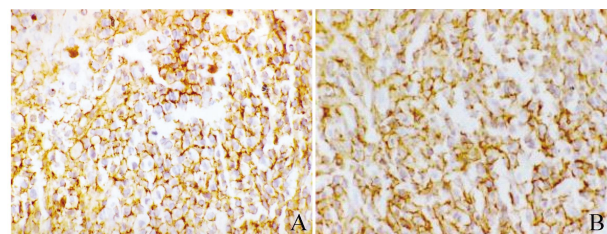
### Laboratory tests

Serum LDH of different passages of the HGBL-0304 transplantation was measured (normal: 313–618 U/L) and results showed that LDH increased and fluctuated between ( $1010.56 \pm 200.85$ ) U/L, which indicated that the transplanted tumors retained the characteristics of self-secretion in human tumors.

### Immunohistochemical staining of the HGBL-0304 model

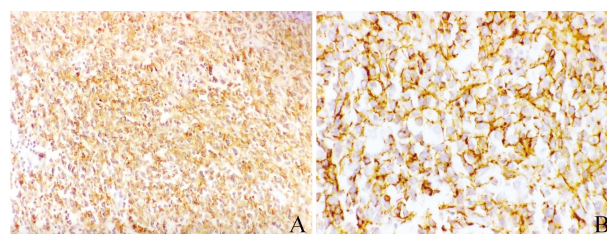
CD19, CD20, CD22, CD45, and CD79 $\alpha$  were positively

expressed in the transplanted cells, all viscera, and lymph nodes, while CD3 and CD7 were negatively expressed (Figures 6 and 7).



**Figure 6** Immunohistochemical staining of CD20 and CD79 $\alpha$  in the 45th generation of the HGBL-0304 model

Positive expression located in the cytoplasm and the cell membrane is brown. A, sections of transplanted tumors showing that tumor cells were strongly positive for CD20 (LSAB  $\times 150$ ). B, sections of transplanted tumors showing that tumor cells were strongly positive for CD79 $\alpha$  (LSAB  $\times 400$ ).



**Figure 7** Immunohistochemical staining of CD19 and CD22 in the 45th generation of the HGBL-0304 model

Positive expression located in the cytoplasm and the cell membrane is brown. A, sections of transplanted tumors showing that tumor cells were strongly positive for CD19 (LSAB  $\times 100$ ). B, sections of transplanted tumors showing that tumor cells were strongly positive for CD22 (LSAB  $\times 400$ ).

### DNA content analysis of the HGBL-0304 model

As shown by flow cytometry of the tumor samples, HGBL-0304 transplanted tumor cells were all heteroploid (Table 1).

**Table 1** Results of DNA content determined by flow cytometry

Group	DNA index	Proliferation index	Ploidy level
Normal gastric mucosa cells	$1.00 \pm 0.04$	$24.85 \pm 5.40$	Euploid
Original tumor cells	$1.57 \pm 0.59^a$	$33.31 \pm 5.39^b$	Heteroploid
HGBL-0304	$1.45 \pm 0.25^a$	$33.38 \pm 5.19^b$	Heteroploid

Compared with normal gastric mucosa cells, <sup>a</sup> $P < 0.01$ , <sup>b</sup> $P < 0.05$ .

### Karyotype analysis of the HGBL-0304 model

The karyotype of HGBL-0304 was consistent with the human cell karyotype. The chromosomal mode was 75–89 (Figure 8), ranging from hypertriploid to hypotetraploid, and were aneuploid.

### Discussion

Primary gastric lymphoma is the most common extranodal gastrointestinal lymphoma with a dormant onset, unspecific clinical signs, symptoms, and accessory examination, easy to

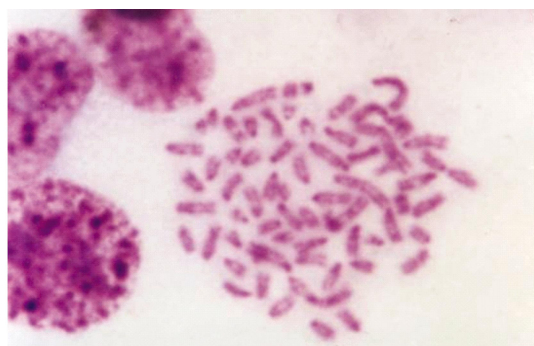


Figure 8 Karyotyping analysis of the 45th generation of the HGBL-0304 model showing human karyotype and heteroploid

confuse with gastric carcinoma and difficult to preoperatively diagnose and stage<sup>[11]</sup>. Biologically, lymphoma originating in the gastrointestinal tract is more susceptible to invasion and metastasis in the liver, which is related to the anatomical characteristics and microenvironment of lymphoma cells<sup>[12]</sup>. However, basic and clinical research about liver invasion and metastasis of lymphoma are limited in both depth and breadth, and few of tumor samples and experimental animal models are available. Therefore, studying the mechanism of tumorigenesis and metastasis of primary gastric lymphoma required the establishment of a high and organ-specific metastatic model of human primary gastric lymphoma by orthotopic transplantation. Previous experimental metastasis models have shown no more than 0.1% of tumor cells survived in blood circulation and less than 0.01% of those cells formed tumors<sup>[13]</sup>. These results indicated that the rate of metastasis was very low in the model of experimental metastasis, and a model of spontaneous metastasis was more difficult to establish. Thus, to establish a good model for spontaneous metastasis is still a problem in the study of human tumor metastasis. In this study, we used a fragment of histologically intact liver metastasis derived from a surgical specimen of a patient with primary gastric lymphoma to orthotopically transplant into the submucosa of the stomach in nude mice, and successfully established an orthotopic liver metastatic model of human primary gastric non-Hodgkin's lymphoma in nude mice (HGBL-0304).

After the orthotopic transplantation of human primary gastric lymphoma in the mice, the ability to maintain the original biologic characteristics is the key point. In the present study, we established the HGBL-0304 model, which passaged 45 generations through 4 years of continuous passage in the mice. Such metastatic tumors formed by the liver metastasis model of the patients with gastric lymphoma was not only identical with human gastric lymphoma in histopathology, tumor-associated antigens, immunohistochemical phenotype, ultrastructure, DNA ploidy level, and karyotype characteristics, but also the rates of growth of the transplanted tumor, of liquid-nitrogen resuscitation, and of spontaneous liver metastasis were all 100%. All the results indicated that the biologic characteristics of the model were stable.

Our data found that the biologic behavior of the HGBL-0304 model and in clinical patients showed striking similarities, such as extensively invasive growth of the transplanted tumors in the stomach of the mice, blood-route metastasis (liver and spleen involvement), lymphatic metastasis (lymph node involvement), and peritoneal seeding metastasis. The results confirmed that HGBL-0304 model completely reproduced the whole 4 stages of the clinical process of human gastric lymphoma according to the Ann Arbor method<sup>[14]</sup>. Therefore, HGBL-0304 was an ideal animal model for elucidating the invasion and metastasis mechanisms of gastric lymphoma.

HGBL-0304, a liver metastasis model of primary gastric lymphoma in nude mice by using orthotopic transplantation, which accurately simulated the clinical process of human gastric lymphoma, not only provided a better animal model for simulating clinical lymphoma patients for drug studies for anti-lymphoma and anti-liver metastasis, but also supplied a large number of biologically stable human primary lymphoma and metastatic tumor tissues for malignant lymphoid tumor biology, metastasis biology, diagnostics, and pharmacogenomic studies. Thus, the overall experimental study of spontaneous metastasis of human malignant lymphoma was realized.

Tumor heterogeneity is the radical reason for tumor metastasis. Tumor cells have to search for a suitable target organ to grow and proliferate. Our results suggested that liver was the organ-specific metastasis site of the HGBL-0304 model. Most tumor cells were of monoclonal origins, however, due to the instability of tumor genetics and the selectivity of the host, tumor cells appeared as a continuously variant group. In this study, we isolated a subgroup cells with the unique advantages of high growth, and invasive and metastatic potential (preferences to migrate, clone, and grow in the liver) through orthotopically transplanting liver metastasis of human gastric lymphoma and continuous selection in vivo, which had great significance in studying the molecular mechanisms of lymphoma invasion and liver metastasis. Jaffe *et al.*<sup>[15]</sup> reported that the rate of liver metastasis for human non-Hodgkin's lymphoma reached up to 40%. Other studies have shown that the rate of liver invasion was 54.8% in 73 patients with non-Hodgkin's lymphoma, and the liver involved rate was up to 66.7% postmortem or puncture pathologic examination after death<sup>[16,17]</sup>. In our study, the rate of liver metastasis was 100% in the HGBL-0304 model of 263 tumor-bearing nude mice. In-vivo selection of liver metastasis tumor cells was helpful for researching the liver metastasis gene, and provided an experimental basis for selecting early diagnostic liver metastatic markers and therapeutic targets.

In summary, the liver metastasis model of primary gastric lymphoma (HGBL-0304), completely reproduced the natural clinical pathology and biologic behavior of primary gastric lymphoma, and supplied an ideal animal model for basic and clinical research of primary gastric lymphoma.

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