The extent of inflammatory infiltration in primary cancer tissues is associated with lymphomagenesis in immunodeficient mice

Lianhai Zhang1*, Yiqiang Liu2*, Xiaohong Wang1*, Zhiyu Li1*, Ying Hu1, Xianglong Zong1, Xiaojiang Wu1, Zhaode Bu1, Aiwen Wu1, Zhiyu Li2, Xiaozheng Huang2, Ling Jia2, Qiang Kang2, Yong Liu3, David Sutton3, Lusong Luo3 & Jiafu Ji1

1Key laboratory of Carcinogenesis and Translational Research (Ministry of Education), Department of Surgery, Peking University Cancer Hospital and Institute, Beijing, China, 2Department of Pathology, Peking University Cancer Hospital and Institute, Beijing, China, 3BeiGene (Beijing) Co. Ltd, No. 30 Science Park Road, Life Science Park, Changping District, Beijing, China.

Xenotransplantation of human cancers into immunodeficient mice is a very useful approach for studying human tumor biology. However, the occasional occurrence of lymphomagenesis in some mice can spoil the model and must be investigated in detail. We found that a high percentage (32.5%, 26/80) of cancer patient-derived xenografts (PDXs) resembled lymphoma in NOD/SCID mice. Of the 26 xenografts, 23 were human-derived expressing human CD45 (hCD45) and proved to be of the B-cell subtype (CD3-/CD20+), and they were all positive for Epstein - Barr virus (EBV). The remaining 3 xenografts proved to be mouse-derived for both hCD45- and negative amplification of a human gene. The most interesting finding is that gastric cancer had much higher rates (24/126, 19.0%) of lymphoma formation in the PDX model than did colorectal cancer (1/43, 2.3%). Statistical analysis revealed that cancer type and inflammation in the parent tumor are significantly associated with lymphomagenesis. Further validation discovered lymphomagenesis by inoculating only gastritis mucosa. Therefore, our findings suggest that it is necessary to take precautions when directly xenografting cancer tissues with remarkable baseline inflammation, such as gastric cancer into immunodeficient NOD/SCID strains. Further, the established xenograft models should be validated by both leukocyte markers and human gene signatures.

Patient-derived tumor xenografts (PDXs), which are established through the xenotransplantation of human cancers into immunodeficient mice, are able to mirror patients' histopathological and genetic profiles1–4 and are very useful for studying human tumor biology. There has been a surge in the use of these experimental models to predict the clinical activity of anti-cancer therapies and discover predictive biomarkers5–7. The most commonly utilized mouse strains, such as the nonobese diabetic severe combined immunodeficiency (NOD/SCID) strains, are deficient in both innate and adaptive immunity and thereby permit the high engraftment rate of human tumor tissues8–10.

The SCID mouse model, however, has several pitfalls. Xenografting primary human solid tumor tissue into immunodeficient mice may fail to induce lymphoma using highly immunodeficient NOD/SCID mice. In the early 1990s, researchers had discovered that B cell lymphomas occurred later in a large percentage of SCID mice that received peripheral blood lymphocytes (PBL) from individuals infected with Epstein-Barr virus (EBV), particularly after the transfer of lymphocytes by intraperitoneal injection11,12. Additionally, in two recent studies, researchers found that after xenografting primary human hepatocellular carcinoma (HCC) and non-small cell lung carcinoma (NSCLC) tumor fragments into NOD/SCID or NSG immunodeficient mice (NOD/SCID/interleukin 2 receptor gamma chain null strains), a high percentage (11 of 21 in HCC, and 19 of 153 in NSCLC) were found to be human B lymphomas13,14.

In this study, through the systematic establishment of a larger panel of xenograft models for pre-clinical drug testing, we discovered a high probability of lymphoma formation when implanting human tumor tissues into immunodeficient NOD/SCID mice, particularly for gastric cancer. The most interesting result is that PDX models...
from gastric cancer (GC) had much higher rates of lymphoma formation than those from colorectal cancer (CRC). We then conducted a comprehensive investigation into the pathogenesis of this phenomenon.

Results
For our initial attempts to generate xenografts from a variety of human cancer tissue specimens, we procured cancer tissues from 170 patients, including 126 gastric cancer (GC), 43 colorectal cancer (CRC) and 1 hepatocellular carcinoma (HCC), from consecutive patients from 2011–2012, which were then xenografted into NOD/SCID mice. Before the implantation, a small portion of the procured specimen was put aside and fixed for pathological evaluation (stage 1, Figure 1a).

Over a mean time of 4–6 months, a total of 80 human primary tumor xenograft models were established and serially re-engrafted to maintain tumors in vivo. The clinicopathological features of these patients and matched models are shown in Supplementary Table 1. Routine pathology inspections of the established tumor were conducted. Most xenografts kept the morphology of their parent cancer tissues (Fig. 1b). Unexpectedly, we observed that a very high percentage (26 of 80, 32.5%) of established xenograft models did not resemble carcinoma but instead exhibited the morphological characteristics of lymphoid neoplasms. Immunohistochemistry staining with human CD45 (hCD45) showed that 23 of the 26 lymphomas were positive, suggesting that these lymphomas were derived from the parent tumor tissues of the patients. According to hematoxylin-eosin staining, the morphology of these lymphomas showed characteristics of the B cell type and were composed of a high density of large polymorphic neoplastic cells and scattered small lymphocytes and plasma cells. The pattern of infiltration of the tissues affected is vaguely nodular or diffuse. Geographic necrosis is a prominent feature in all cases. Further CD3/CD20 double staining proved that these tumors are all CD3- and CD20+, consistent with B cell type lymphoma (Fig. 1c). The remaining 3 xenografts were hCD45 negative and were morphologically smaller cells (Fig. 1d) compared with human-derived inflammatory cells. These xenografts were further proven to be mouse-derived by negative amplification of a human gene ALU (Supplementary Table 1) and to be of mouse B-cell origin with CD3- and CD20+ (supplementary Fig. 2). We also analyzed

Figure 1 | Tumor formation in the xenograft NOD/SCID mouse model in stage 1. (a) Illustration of study design. Cancer tissues from 170 patients (including 126 GC, 43 CRC and 1 HCC) were procured and then xenografted into NOD/SCID mice after a small portion was set aside for pathological evaluation. In total, 80 cancer models were established and serially re-engrafted to maintain tumors in vivo. These models were finally shown to consist of 53 patient-derived carcinomas, 23 patient-derived lymphomas and 3 mouse-derived lymphomas. (b–d) Tumor formation in the xenograft NOD/SCID mouse model. (b) the representative morphology of GC and CRC patient-derived carcinomas formed in mice, and (c) 3 representative cases for each cancer type (GC, CRC and HCC) from 23 human-derived lymphoma. All cases were identified with typical B cell type morphology, hCD45 positive, CD3-/CD20+ and were EBER positive. (d) Three morphologically similar cases of lymphoma that are hCD45 negative, finally shown to be mouse-derived lymphoma by negative amplification of human gene ALU (see Supplementary Table 1). Only 1 of the 3 cases were EBER+. Scale bars, 300 μm.
other markers, including the gastric and colorectal tumor markers CD44 and CD133. Immunohistochemistry staining with human CD44 and CD133 antibody showed that all cases were negative (supplementary figure 3), which suggested that the identified lymphomas were not derived from gastric and colorectal tumor cells.

We hypothesized that the observed lymphoid neoplasm was most likely associated with EBV. Because EBV is common in the pathogenesis of lymphoproliferative disorders in immunocompromised humans,[15] we evaluated our xenografts for evidence of EBV infection by in situ hybridization (ISH) for EBV-encoded RNA (EBER) and found that all the human-derived lymphomas were EBER positive. This result suggests that EBV infection is the main cause of immortalization for these inflammatory cells. However, after determining the presence of EBV in the parent tumor specimens by EBER ISH, we found that only one (parent tissue of model BCGA070, Fig. 2a) of the 23 cases was observed to have scattered EBV-positive inflammation cells. A hypothetical pathogenesis of EBV-driven lymphomagenesis in immunodeficient mice can be inferred from observation of several examples: at earlier passages, EBV-infected inflammation cells multiplied and co-existed with tumor cells, as in the model BCCO9116P2 (Fig. 2b), eventually outgrew the carcinoma cells and began to form a lymphoma tumor mass surrounded by mouse inflammatory cells, as in BCGA032P4 (Fig. 2c).

For the mouse-derived lymphoma, EBER was positive in only 1 of the 3 xenografts (Fig. 1d), which suggests that EBV might not play the most critical role in the formation of the mouse-derived lymphoma.

The interesting finding of our study is that the PDX models derived from gastric cancer had much higher rates of lymphoma formation than did those derived from colorectal cancer. In fact, although the take rate for GC was lower than that for CRC, with 25/126 (19.8%) GC tissue inoculation successes vs 29/43 (67.4%) CRC tissue inoculation successes, the lymphoma formation rate was dramatically higher in GC. To be more specific, 24 of the 126 GC cases (19.0%) formed lymphoid neoplasm; among them, 21 were human-derived, and 3 were mouse-derived. In comparison, only 1 of the 43 (2.3%) CRC cases was found to be lymphoma (Fig. 1a).

To examine the exact factors that may be related to this lymphogenesis, we performed statistical analyses of the association between xenograft phenotypes (human-derived lymphoma or carcinoma) and the clinicopathological features of the corresponding cancer. As shown in Table 1, the cancer type (ORGC vs. CRC = 24.4, 95% CI: 3.1–194.2), preoperative chemotherapy (ORyes vs. no = 4.6, 95% CI: 1.6–13.0) and inflammation in parent cancer (OR+++/+++ vs. –/– = 3.4, 95% CI: 1.2–9.6; the inflammation grade is based on the updated Sydney system for grading of gastritis[16]) were associated with an increased risk of having a human-derived lymphoma in the xenograft. Further, in the multivariate model in which all the listed potential factors were included simultaneously, only the cancer type (adjusted ORGC vs. CRC = 30.0, 95% CI: 2.6–341.3) and inflammation in parent cancer (adjusted OR +++++ vs. –/– = 4.0, 95% CI: 1.1–14.4) were statistically significant.

To validate that the cases of lymphoma formation in GC may be the consequence of the reactivation of latent EBV in remarkable intratumoral gastritis following xenotransplantation, we implanted non-cancerous tissues that were procured adjacent to the GC tumor border from 4 gastric cancer patients into NOD/SCID mice (stage 2, Fig. 3a). The results showed that two of the four mice formed human-derived lymphomas expressing hCD45+ and a B-cell subtype (CD3-/CD20+) and were positive for EBV. Pathological inspection also demonstrated that these two original parent non-cancerous tissues exhibited obvious inflammatory infiltration (Fig. 3b).

**Table 1 | Crude and adjusted odds ratio of the clinicopathological factors of the parent cancer related to the lymphoma formation**

|  | Xenograft Carcinoma | Xenograft Human Lymphoma | Crude OR (95% CI) | Adjusted OR (95% CI) |
|---|---|---|---|---|
| Age | <60 | 26 | 13 | 1.0 |
|  | ≥60 | 28 | 9 | 0.6 (0.2–1.8) 0.5 (0.1–1.7) |
| Sex | Female | 20 | 4 | 1.0 |
|  | Male | 34 | 18 | 2.6 (0.8–8.9) 2.8 (0.5–15.3) |
| Cancer type | CRC | 29 | 1 | 1.0 |
|  | GC | 25 | 21 | 24.4 (3.1–194.2) 30.0 (2.6–341.3) |
| Pre-operative chemotherapy | No | 39 | 8 | 1.0 |
|  | Yes | 15 | 14 | 4.6 (1.6–13.0) 1.0 (0.2–4.6) |
| TNM stage | I/II | 19 | 9 | 1.0 |
|  | III/IV | 35 | 13 | 0.8 (0.3–2.2) 1.0 (0.2–6) |
| Vascular Invasion in parent tumor | – | 36 | 13 | 1.0 |
|  | + | 18 | 9 | 1.4 (0.5–3.8) 0.8 (0.2–4.6) |
| Inflammation in parent tumor | –/– | 33 | 7 | 1.0 |
|  | +/++ | 21 | 15 | 3.4 (1.2–9.6) 4.0 (1.1–14.4) |

**Discussion**

The implications of our results are very important to the establishment of patient-derived xenograft animal models. It is well known that EBV infects over 90% of the human population and remains latent for the lifetime of the host[17]. In the case of xenotransplantation, the small amount of latent EBV might multiply without immunosurveillance in the immunodeficient mice. In clinical settings, patients with HIV/AIDS or those receiving immunosuppressive drugs are at high risk for developing B-cell lymphomas[18]. Without effective immunosurveillance, EBV can efficiently infect B lymphocytes and...
transform them into a proliferative state and eventually form lymphoma. Our experience with the xenotransplantation of human tumor tissues into immunodeficient mice might be a sample of the above-mentioned clinical scenarios. Therefore, the established patient-derived xenograft tumor models in immunodeficient mice should be validated by leukocyte markers such as hCD45 to exclude human-derived lymphoma. In addition, amplification and/or sequencing of specific human genes such as ALU is recommended to exclude the possibility that the tumor is of mouse origin (hCD45-). We also found that NOD/SCID mice were particularly vulnerable because we did not find any lymphoid neoplasms in our previously established 22 BALB/c xenograft nude mouse models, which were generated from 42 gastric cancer and 12 colorectal cancers (data from 2010, not shown). Our studies also showed that once lymphoma was formed in NOD/SCID mouse model, it could survive serial passages in the BALB/c nude mouse model (data not shown). This raises the important and interesting question of why gastric cancer (GC) xenografts had much higher rates of lymphoma formation than did colorectal cancer (CRC). This finding can be explained by two facts. First, a higher extent of baseline inflammation (over 80% with H. pylori related gastritis) is usually present with GC xenografts compared with primary CRC. Second, preoperative chemotherapy often aggravates inflammatory infiltration following its antitumor cytotoxicity. Thus, the extent of inflammatory infiltration in inoculated parent tissue tissues such as gastric cancer is most likely directly associated with the lymphomagenesis observed in NOD/SCID mouse model.

In summary, we present here a comprehensive investigation of the EBV-related lymphoma formation in cancer patient-derived xenografts. Our results show that the extent of inflammatory infiltration in inoculated parent cancer tumor tissues is associated with EBV-driven lymphomagenesis in NOD/SCID immunodeficient mice. To better utilize patient-derived xenograft models to evaluate anticancer therapeutics, our findings suggest that the established xenograft tumor model in an immunodeficient mouse model should be validated by both leukocyte markers and human gene signatures, particularly for cancer types with remarkable baseline inflammation, such as gastric cancer. Xenografting purified or sorted tumor cells to excluded potential EBV-infected leukocytes from cancer tissues with baseline inflammation should be considered the method of choice to avoid this problem.

Methods
Patient tumor samples and engraftment in immunocompromised mice. Freshly and surgically removed tumor tissues were obtained from the primary cancer patients in 2011 and 2012 in Peking University Cancer Hospital. This investigation was approved by the Institutional Review Boards of the hospital, and informed consent was obtained from each patient. The cancer type, pre-operative chemotherapy status, histological grade, and vascular invasion were obtained from clinical and histopathological reports. The stage of GC was classified according to the 7th edition of the tumor-node-metastasis (TNM) classification recommended by the International Union Against Cancer. A number of patients were treated by preoperative chemotherapy or radiotherapy following the clinical practice guidelines.

Patient tumor fragments were subcutaneously engrafted into immunocompromised mice. Briefly, after removing and fixing a small portion for further pathological evaluation, the remaining tumor was sliced into 3x3x3 mm3 fragments and inoculated subcutaneously on the flank of mice (NOD/SCID, 6- to 8-week-old female mice, Beijing HFK Bioscience Co., Beijing, China). Tumor growth was monitored weekly using a caliper, and tumor volumes were calculated using the formula: V = 0.5 × (a × b2), in which a and b are the long and short diameters of the tumor, respectively. After established in mice, fragments were frozen in DMEM including...
10% dimethyl sulfoxide and 40% fetal bovine serum. The established tumor models, Amplification of human ALU gene identify human DNA. The successful amplicons were verified to be consistent with melting curves were acquired and analyzed. A Ct cutoff value of 20 was used to was manually set up at the level that reflected the best kinetic PCR parameters, and Master Mix (ABI, Cat#: 4309155). At the end of each reaction, the cycle threshold (Ct) program: 5 minutes of pre-incubation at 95

In situ hybridization for EBER. To detect EBV infection, chromogenic in situ hybridization (CISH) assays were performed on 4-μm-thick sections using a Bond Max
tm autostainer (Leica Microsystems, Germany), according to the manufacturer’s instructions. Cells containing EBER transcripts were evaluated as positive when an intense, brown, predominantly nuclear staining was present in cells.

Amplification of human ALU gene. One piece (approximately 1 × 1 × 1 mm³) of tumor fragment was obtained, to which 200 μl lysis buffer from Invitrogen PuriLink Genomic DNA Kit (Invitrogen, Cat#: K1820-02) was added. Homogenization of tumor tissues was performed in a MP homogenization unit (Fast prep-24) (MP bio, Cat# 6004.2) at a speed setting of 6 for 60 s. The sample was centrifuged at full speed,
the supernatant was transferred to a new sterile tube. The DNA was then reduce the prevalence of precancerous gastric lesions.

Statistical analysis. Odds ratios and 95% confidence intervals were estimated using univariate and multivariate logistic regression to evaluate the association between xenografts (lymphoma or carcinoma) and a series of potential factors including age, gender, cancer type, pre-operative chemotherapy, TNM stage, vascular invasion and inflammation in parent tumor. In the multivariate model, all the listed variables were included at once. Statistical analysis was conducted using the Stata 11.2 software for Windows (College Station, TX: StataCorp LP). All P-values were two-sided, and P < 0.05 was considered statistically significant.

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Author contributions

L.Z. and J.J. conceived and designed the experiments; L.Z., X.W., Z.T., S.L., Y.H., X.Z., X.W., Z.B., A.W., Z.L., Y.L., D.S. and L.W. performed the animal model construction and part of the pathologic experiments; Y.L., Z.L., X.H., L.J. and Q.K. performed the pathological experiments. L.Z., Y.L., X.W. and Z.T. and S.L. analyzed the data and contributed to writing and editing the manuscript; L.Z., L.J. and J.J. supervised the project and wrote the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

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