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Letter to the editor   **Open Access**

Generation of a tree shrew breast cancer model using lentivirus expressing *PIK3CA-H1047R*

DEAR EDITOR,

Breast cancer is the most common malignant tumor in women, posing a serious threat to health. Tree shrews (*Tupaia belangeri chinensis*) are evolutionarily closer to humans than rodents and are emerging as an attractive experimental animal model for breast cancer. The *PIK3CA* gene is frequently mutated in both human and tree shrew breast tumors. Herein, we effectively overexpressed *PIK3CA-H1047R* in the mammary epithelial cells of tree shrews using a lentivirus to induce breast tumors. The tumor incubation period was approximately 3 weeks, and the incidence rate was 50% within 10 weeks. The pathological characteristics of the tumors were intraductal papilloma (IP) and invasive ductal carcinoma (IDC). In addition, upon transplantation into non-obese diabetic-severe combined immunodeficient (NOD-SCID) mice, these tumors were sensitive to the *PI3Kα* inhibitor alpelisib. This study provides a new animal model of breast cancer that mimics human breast cancer with the *PIK3CA-H1047R* mutation, thereby facilitating the study of breast cancer mechanisms and the evaluation of novel drugs.

The PI3K-AKT pathway plays an important role in breast cell proliferation, survival, migration, and drug resistance. In Chinese breast cancer patients, *PIK3CA* is the most frequently mutated gene in the PI3K-AKT pathway, with *H1047R* mutation also common (Chen et al., 2018). We previously identified frequent *PIK3CA* gene mutations in spontaneous and 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast tumors in tree shrews, with a mutational spectrum similar to that in humans (Xia et al., 2014).

RAS belongs to the family of small G proteins. Mutant *RAS* is a common oncogene associated with cellular transformation and aggressive tumor phenotypes. Approximately 19% of human cancers harbor *RAS* mutations, including *H-RAS*, *K-RAS,* and *N-RAS*. G12, G13, and Q61 are *RAS* mutational

hotspots, which maintain *RAS* in the guanosine triphosphate (GTP)-bound active form and promote downstream transduction of proliferative signals, such as RAS-RAF-MEK-ERK and RAS-PI3K-AKT-mTORC (Chen et al., 2021).

The tree shrew is a promising animal model for human disease, which is more closely related to primates than to rodents in terms of anatomy, behavior, genome, and evolution (Ye et al., 2021). We previously found that tree shrew mammary glands are morphologically and structurally similar to that in humans, thus providing a basis for the generation of breast cancer models (Xia et al., 2012). Tree shrews have become an increasing attractive animal model for human breast cancer research (Ge et al., 2016; Xia et al., 2014). By injecting a *PyMT* lentivirus into the nipple, we successfully generated tree shrew breast tumors within a short incubation period (3–7 weeks) with high efficiency (100%) (Ge et al., 2016). However, the *PyMT* gene does not exist in the human genome, and almost all *PyMT*-induced tumors are papillary carcinomas, inconsistent with humans (Ge et al., 2016).

In the present study, we successfully generated a tree shrew breast cancer model by intraductal injection of a lentivirus overexpressing *PIK3CA-H1047R*, which triggered malignant transformation of cells. The *PIK3CA-H1047R* lentivirus induced breast tumor formation in tree shrews within 3 to 5 weeks, with a total incidence rate of 50% (12/24) within 10 weeks. In addition, a mouse xenograft tumor model derived from tree shrew breast tumors was successfully established and responded well to the *PI3Kα* inhibitor alpelisib. These results suggest that the *PIK3CA-H1047R*-induced tree shrew breast cancer model is a suitable model for fundamental breast cancer research and anticancer drug assessment.

The MCF-10A cell line was chosen as a cell model to test the infection efficiency and function of the *PI3KCA-H1047R*,

H-RAS-Q61L, and *PyMT* lentivirus. At the same titer (4×10^6 transducing units (TU)/mL), the three lentiviruses infected cells with different multiplicities of infection (MOIs) (0, 0.004, 0.04, and 0.4) for 36 h. Immunofluorescence staining showed that green fluorescent protein (GFP)-positive cells increased with increasing MOIs of the three lentiviruses (Supplementary Figure S1A). In order to evaluate the activation of downstream proteins, the three lentiviruses were used to infect MCF-10A cells with different MOIs (*PyMT:* 0, 0.004, 0.04, and 0.4; H-RAS-Q61L: 0, 0.184, 1.84, and 18.4. *PI3KCA-H1047R:* 0, 0.056 0.56, and 5.6) for 36 h. Western blotting showed that the *PyMT* and *PIK3CA-H1047R* lentiviruses increased p-AKT levels at low MOI (~0.4); however, *H-RAS-Q61L* only activated AKT at high MOI (18.4) (Supplementary Figure S1B).

We then directly infected tree shrews with the *PI3KCA-H1047R* or *H-RAS-Q61L* lentiviruses via intraductal injection to test tumorigenesis *in vivo*. The lentiviruses were injected at the same volume (10 µL per point) but different titer (3×10^7 TU/mL and 1.4×10^6 TU/mL, respectively). Trypan blue was used with the lentiviruses as a tracer (Supplementary Figure S2A). The tumor incubation period for *PI3KCA-H1047R* was 5 weeks (Supplementary Figure S2B), with a tumor incidence of 41.7% within 10 weeks (5/12) (Figure 1A; Supplementary Table S1). In the *H-RAS-Q61L* group, however, no tumors were observed at 10 weeks (Figure 1A). Thus, we again performed *PI3KCA-H1047R* lentivirus infection in tree shrews, only changing the injection volume (5 µL per point). Interestingly, while the incidence was similar (58.3%, 7/12), the incubation period was considerable shortened (3 weeks) (Supplementary Table S1). After the two batches of animals were sacrificed, all breast tumors were surgically removed. Pathological examination showed that intraductal papilloma (IP) was the dominant pathological type, with only one tumor being invasive ductal carcinoma (IDC) (Figure 1B; Supplementary Figure S3). The immunohistochemical (IHC) results for the estrogen receptor-α (ERα), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER-2), and Ki67 biomarkers are summarized in Supplementary Table S2.

We then injected the *PI3KCA-H1047R* or *H-RAS-Q61L* lentiviruses into the fourth pair of mammary glands of FVB background mice, with the *PyMT* lentivirus used as a positive control. The titers and volumes of each lentivirus are provided in Supplementary Table S3. The tumor incubation period for the *H-RAS-Q61L* lentivirus was 5 days compared with 10 days for *PyMT*. At 3 weeks, the tumor incidence rate was 91.7% for *H-RAS-Q61L* (11/12) and 66.7% (4/6) for *PyMT* (Supplementary Figure S4A and Table S3). Interestingly, the *PI3KCA-H1047R* lentivirus failed to induce tumor formation in FVB mice within 3 weeks (Supplementary Figure S4A and Table S3). To further investigate their tumorigenic capacity, the *H-RAS-Q61L* and *PyMT* lentiviruses were injected into a new batch of FVB mice at the same transducing units (4×10^4 TU per point) and volume (10 µL per point). Results showed that the incubation period of the *H-RAS-Q61L* lentivirus was delayed to 16 days compared to 10 days for *PyMT.* On day 16, the tumor formation incidence of the *PyMT* lentivirus was 100% (10/10), while that of *H-RAS-Q61L* was 20% (2/10)

(Supplementary Figure S4B and Table S3). Therefore, we suggest that higher *H-RAS-Q61L* lentiviral titers result in a shorter tumor incubation period and higher tumor incidence, and the carcinogenic ability of *PyMT* is stronger than that *H-RAS-Q61L* under the same viral titer. All mouse breast tumors were surgically removed and subjected to hematoxylin and eosin (H&E) and IHC staining (Supplementary Figure S4C and Table S4).

We orthotopically transplanted *PI3KCA-H1047R*-induced tree shrew breast tumors into immunodeficient (NOD-SCID) mice, then used this xenograft model to evaluate the FDA-approved PI3K inhibitor alpelisib *in vivo*. Different doses of alpelisib were delivered by intragastric gavage when average tumor volume reached 100 mm^3 (Figure 1C). We found both high and low doses of alpelisib significantly inhibited tumor growth and decreased tumor weight (Figure 1D–F). Mouse body weight in the low dose alpelisib treatment group slightly decreased compared to that in the control group (Figure 1G).

Genetically engineered animal models have certain advantages than tumor cell line xenograft models, allowing researchers to: (1) assess the influence of specific gene alterations on tumorigenesis, progression, and/or preclinical therapy; (2) determine the function of genes mutated or modified in human cancer patients *in vivo*, thus facilitating evaluation of novel tumor interventions and chemoprevention strategies; (3) analyze all stages of tumor progression *in vivo*; and (4) clarify the natural tumor environment in an immunocompetent host (Klarenbeek et al., 2013). Although Li et al. (2017) reported that tree shrew spermatogonia stem cells transfected with lentivirus expressing enhanced GFP can successfully generate transgenic offspring, tree shrew genome manipulation remains immature.

In this study, we successfully generated breast tumors in tree shrews by an intraductal injection of a lentivirus overexpressing *PI3KCA-H1047R*, a frequently mutated gene in human breast cancer. The incubation period was 3–5 weeks and tumor incidence rate was 50% (Supplementary Table S1). The present tree shrew model showed lower tumor induction than our previous *PyMT*-induced tree shrew breast cancer model, which was characterized by a short tumor incubation period (3–4 weeks), fast growth, high incidence (100%), and tumor metastasis (lung metastasis) at the later stage (Ge et al., 2016). However, the present tumors were successfully transplanted into NOD-SCID mice, with the xenograft tumors showing a good response to alpelisib. These findings confirmed that these tree shrew breast tumor were indeed triggerd by PI3CA H1047R activated mutation. Pathologically, the *PI3KCA-H1047R*-induced tree shrew breast tumors were primarily IP and atypical hyperplasia (AH), with only one tumor being IDC, similar to that found in humans. Both IP and AH are generally considered as precancerous lesions and can become malignant over time. Interestingly, the tumors in this study were all hormone receptor-positive (HR+) and human epidermal growth receptor-2 negative (HER2−), consistent with patients carrying the *PIK3CA* mutation (Klarenbeek et al., 2013).

Strangely, as a human oncogene, the *H-RAS-Q61L* lentivirus failed to induce mammary tumors in tree shrews, but successfully induced tumors in FVB mice, whereas the
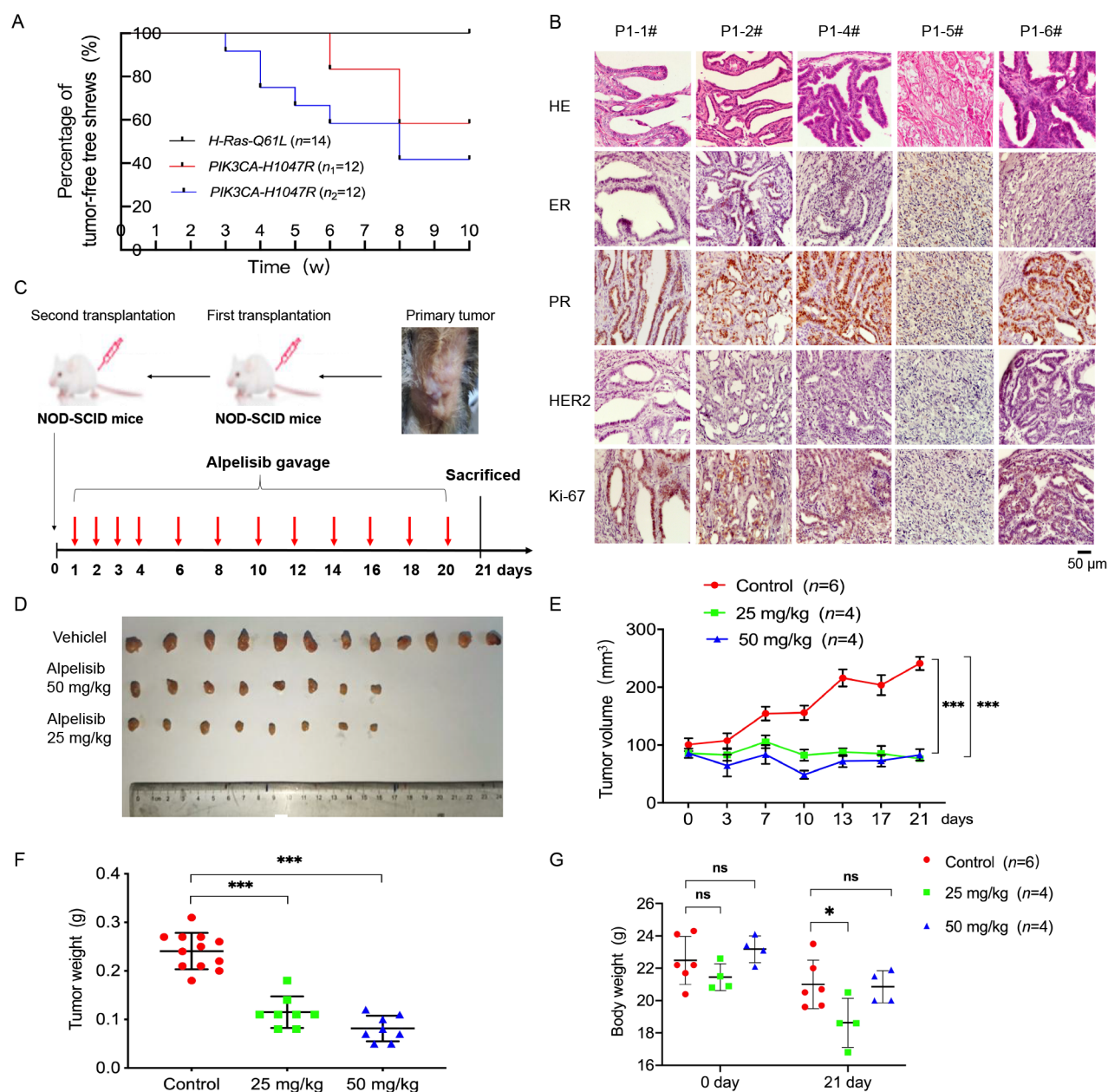
Figure 1 Tree shrew breast tumor model construction and evaluation of alpelisib

A: Incidence of tree shrew breast tumors induced by *PIK3CA-H1047R* (n_1=12, n_2=12) and *H-RAS-Q61L* (n=14). n_1, first injection; n_2, second injection. B: Pathological characteristics of breast tumors induced by *PIK3CA-H1047R* for the first time. Statuses of ERα, PR, HER-2, and Ki67 were examined by IHC. Scale bar: 50 μm. C: Schematic of *PI3Kα* inhibitor (alpelisib) treatment of PIK3CA-H1047R-induced tree shrew xenograft tumors in NOD-SCID mice. D: Both high and low doses of alpelisib significantly inhibited growth of *PIK3CA-H1047R*-induced tree shrew xenograft tumors in NOD-SCID mice. After xenograft tumors were established in NOD-SCID mice, the mice were randomly divided into three groups (control group, alpelisib low-dose group: 25 mg/kg, and alpelisib high-dose group: 50 mg/kg). Tumor masses were collected at the end of the experiment. E: Alpelisib significantly inhibited growth of PIK3CA-H1047R-induced tree shrew xenograft tumors in NOD-SCID mice, as measured by tumor size. F: Alpelisib significantly inhibited growth of PIK3CA-H1047R-induced tree shrew xenograft tumors in NOD-SCID mice, as measured by tumor weight. G: Mouse body weight in low dose alpelisib treatment group decreased compared to that in control group. An unpaired two-tailed *t*-test were used. *: *P*<0.05; ***: *P*<0.001; ns: No significance.

PI3KCA-H1047R lentivirus failed to generate breast tumors in mice. As the *H-RasV12-shp53* lentivirus invariably induces malignant glioma in tree shrews (Tong et al., 2017), one plausible explanation is that *H-RAS-Q61L* alone is not sufficient to trigger carcinogenesis in highly evolved species, and *H-RAS-Q61L* mutations may be rare in breast cancer.

Alternatively, *H-RAS-Q61L* may be more oncogenic than *PIK3CA-H1047R* in mice.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

L.Z. and F.B.L. performed the experiments and analyzed the data. H.Y.Z. assisted in writing the manuscript. C.S.C and F.B.L. designed the experiments and supervised the project. C.Y.Y. helped with pathological examination. Z.C., Q.Y.J., and Y. Luo helped perform the experiments. Y. Li provided the plasmids. All authors read and approved the final version of the manuscript.

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