Supplemental Methods

Cell lines

MM3MG, a murine premalignant mammary epithelial cell line, was transduced with the human HER2 oncogene by retroviral vectors and polybrene to express HER2 (referred to as MM3MG-HER2 cells) in our laboratory (27). MM3MG-HER2 cells were maintained in DMEM supplemented with 10% FBS (Atlanta Biologicals) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Rockford, IL). JC cells, a murine breast cancer cell line, were transduced with human HER3 gene (referred to as JC-HER3) in our laboratory (28). JC-HER3 cells were cultured in RPMI1640 supplemented with 10% FBS and 1% penicillin-streptomycin. 4T1-HER2 cells were obtained from Dr. Michael Kershaw (Peter MacCallum Cancer Center, Victoria, Australia) (29). Cell lines were screened for rodent pathogens at Duke Division of Laboratory Animal Resources (DLAR) Veterinary Diagnostic Laboratory prior to use in animal experiments.

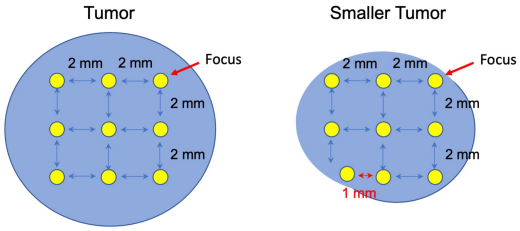
HIFU procedure

The VIFU 2000 system (Alpinion Medical Systems, Bothell, WA) was used for HIFU treatment. Cells or tumors were treated using a 1.5 MHz HIFU transducer under two different protocols (50% duty cycle, 1 Hz pulse repetition frequency, 20W, 10 seconds or 2% duty cycle, 5 Hz pulse repetition frequency, 200W, 20 seconds) to produce either thermal necrosis or mechanical lysis of the tumor cells. The former was defined as thermal HIFU (T-HIFU), and the later was defined as mechanical HIFU (M-HIFU) (30). T-HIFU increased the temperature inside tumor tissues to > 60°C in a few seconds, while the temperature inside tumor tissue was < 42°C during M-HIFU. M-HIFU is similar to boiling histotripsy (31), which produces cavitation activities in vivo that may

damage tumor tissue and cells through shear stresses generated by the complex bubble oscillation and bubble-bubble-tissue-cell interactions (32).

The -6 dB focal dimension of the HIFU transducer of VIFU 2000 system was measured at a low power level of 10W when the effect of nonlinear wave propagation was insignificant by Alpinion Medical System. It was measured to be 0.72 mm x 7.22 mm in the lateral and axial directions, respectively. Under the high-power settings, such as 200W (M-HIFU), the nonlinear effect will be strong, making it difficult to measure the pressure waveforms near the transducer focus and thus determining the -6 dB beam size. Therefore, a numerical simulation test was performed, and -6 dB focal dimensions for T-HIFU (20 W) and M-HIFU (200W) were estimated to be 0.74 mm x 6.50 mm and 0.60 mm x 5.69 mm, respectively.

Concerning focus for the treatment, both X and Y axes intervals were 2 mm, and a total of 9 points were selected in both *in vitro* and *in vivo* studies as shown in below. A shorter interval of 1 mm was used only when tumors did not have enough size to put 2 mm for all intervals. The same spacing strategy was used for both M-HIFU and T-HIFU.



To avoid skin or bone damage by HIFU treatment, tumor tissue just beneath the skin or close to thighbone was spared from exposure of focused ultrasound, thus approximately 20-40% of tumor tissues were ablated by HIFU treatments based on macroscopic assessment.

Mice

5 to 8-week-old female wild-type BALB/c mice or SCID-beige mice, originally purchased from Jackson Labs (Bar Harbor, ME), were bred and maintained in the Duke Cancer Center Isolation Facility (CCIF) under pathogen-free conditions according to NIH guidelines. Human HER2-transgenic mice were a kind gifted by Dr. Wei-Zen Wei, Wayne State University, Detroit, MI (31). F1 hybrid HER2 transgenic mice were established by crossing with BALB/c mice. Human HER3-transgenic mice (MMTV–neu/MMTV-hHER3) with FVB background were a kind gift from Dr. Stan Gerson at Case Western Reserve University (6). FVB mice homozygous for the hHER3 gene were established at Duke University and then crossed with BALB/c mice for establishment of BALB/c homozygous for the hHER3 gene. In Duke CCIF, species-specific heat and humidity are maintained within the parameters outlined in The Guide for the Care and Use of Laboratory Animals. Mice are fed a standard laboratory diet, and are housed in micro-isolated caging on enrich-o-corn cob bedding that is changed every two weeks. Mice are maintained as group housing of up to 5 mice per cage. Environmental enrichment for singly housed mice were provided for mice in survival analysis.

Animal studies

All animal studies were performed in accordance with Duke Institutional Animal Care and Use Committee (IACUC)-approved protocols (A274-15-10, A223-18-09) that were prepared and approved before the study.

For all the mouse experiments, mouse number in each group was determined based on our previous experiences using similar animal models with the same cell lines, and considering statistical power.

Mice implanted with tumor cells were included in the experiments. However, in rare cases, mice without palpable tumors at the initiation of treatments for unknown reason were excluded from the experiments, regardless of group allocation, because of difficulties to perform local treatments. Mice were euthanized when the tumor volume reach humane endpoints (>2,000 mm^3).

Mice were randomized into the number of groups scheduled for each experiment using Excel software, randomize function. Tumor sizes were measured using a caliper every 3-4 days. Measurement was done for all the mice for all data points with no exclusion. Tumor volume was calculated as described above. When tumor volume reached humane endpoints (>2,000 mm^3) or tumor ulceration was confirmed, mice were soon euthanized and tissue samples/blood were collected. No exclusion of mice happened for other reasons in this study.

To minimize confounders, we used standardizing of the procedures, such as intraperitoneal injections, tumor measurements and tissue/blood collections. Those procedures were done by the same animal technicians during the experiment period.

During the study, one of our animal technicians performed several experimental procedures, including tumor cell implantations and intraperitoneal injection of antibodies. For intraperitoneal injection of antibodies, information about the antibodies was blinded to them who performed injection. Other investigators performed HIFU procedures to mice, and thus knew which mice received HIFU treatment, but they were not involved in data acquisition. Another animal technician who was blinded for the treatments, including HIFU and antibody injection, monitored tumor sizes or mouse survival and collected tissue/blood samples at the end of in vivo experiments. Data of tumor growth and mouse survival were summarized for individual groups by a different investigator who did not know specific treatments for each group. Finally, data analysis was done by another investigator who knows specific treatments the group of the mice received.

Outcome, such as tumor volume and mouse survival, were measured as scheduled (every 3-4 days) until euthanasia of the mice. Details about outcome measurements for each experiment are described in Results section, Figure Legends or Supplementary Figure Legends.

Statistical methods are described in Method section, and also in each Figure legends when necessary. P-values are shown in Figures and Figure Legends.

For the HIFU procedures, mice were given general anesthesia with analgesics to control pain caused by the tumor swelling after the procedures. Analgesic, buprenorphine (0.1 mg/kg BW), was given by subcutaneous injection every 12 h for 2 days to control pain an reduce stress of mice. Detailed procedures are described in Methods section, Figure Legends, and Supplementary Figure Legends.

The following shows the detailed description of mouse experiments conducted in this study.

To test the immunogenicity of HIFU-treated tumor cells, female BALB/c mice (7-8 week old) received intradermal injections of *in vitro* T-HIFU or M-HIFU treated MM3MG-HER2 cells (1×10^6 cells) into the back on days -14 and -7. On day 0, some mice were euthanized and spleen, draining lymph nodes and blood were collected for *in vitro* assays; IFN-γ ELISpot, flow cytometry, and cell-based ELISA. On day 0, the mice (10 mice/group) were inoculated with 1×10^6 MM3MG-HER2 cells into the left leg. Tumor size was measured serially and tumor volumes were calculated using the formula: long axis × $(\text{short axis})^2 \times 0.5$.

For the therapeutic models using MM3MG-HER2 tumors, MM3MG-HER2 cells were subcutaneously inoculated into the left leg (1×10^6 cells) of the female BALB/c mice on day 0. In the bilateral tumor model, 1 x 10^5 cells (experiments with HIFU monotherapies) or 5×10^5 cells (experiments with combination therapy) were inoculated into the right flank as well as 1×10^6 cells into the left leg on day 0. Established leg tumors were treated with T- or M-HIFU on day 7. Mouse were monitored for tumor growth every 3-4 days until humane endpoints (tumor volume >2,000mm^3) and survival of mouse was assessed. For immune assays to determine the treatment effect on systemic antitumor immune responses, mice (n=4 for each group) were implanted with MM3MG-HER2 cells and treated in the same schedule. On day 11, mice were euthanized and spleen and blood were collected from mice to perform IFN-γ ELISpot assay and cell-based ELISA. For flow cytometry analysis of tumor-infiltrating leukocytes, MM3MG-HER2 tumors were treated with HIFU in the same schedule, and mice were euthanized on day 10 (3 days after HIFU) or day 14 (7 days after HIFU), and tumors were collected, enzymatically digested for flow cytometry analysis (n=4 mice per group for each day). For immunohistochemical analysis, tumors were collected on day 20 (13 days after HIFU treatment), fixed with formalin and stained with anti-mouse CD4 and CD8 monoclonal antibodies (n=3 mice per group).

For the re-challenge experiment, BALB/c mice cured by M-HIFU treatment received a subcutaneous injection of MM3MG-HER2 cells (1×10^6 cells) into the flank on day 35 (28 days after M-HIFU) and tumor size and mouse survival were monitored. Age-matched naive female BALB/c mice were used as a control group to assess tumor occurrence, growth and survival of mice in the absence of tumor-specific immunity. Mouse were monitored for tumor growth every 3-4 days until humane endpoints (tumor volume >2,000mm^3).

For the combination treatment with anti-PD-L1 antibody, mice received peritoneal injection of 100 μg anti-PD-L1 antibody (clone 10F.9G2, Bio X Cell, West Lebanon, NH) or isotype control IgG (clone LTF-22, Bio X Cell) on days 12, 15 and 18 (5, 8, and 11 days after M-HIFU treatment) in unilateral tumor models, or on days 10, 13 and 16 (3, 6, and 9 days after M-HIFU treatment) in bilateral tumor models. Mouse were monitored for tumor growth every 3-4 days until humane endpoints (tumor volume >2,000mm^3) and survival of mouse was assessed.

To assess the effect of combination therapy on systemic antitumor immunity and tumor microenvironment, MM3MG-HER2 tumor-bearing mice were treated in the same schedule, and 9 days after the initiation of M-HIFU treatment with/without anti-PD-L1 antibody, tumors and spleens were collected for immune assays. Induction of HER2 antigen-specific cellular response was analyzed by IFN-γ ELISpot assay using harvested splenocytes and HER2 peptide mix as a stimulating antigen (n=7 per each group). Collected tumors were enzymatically digested, and tumor infiltrating CD45+ cells were analyzed (n=4 for treatment groups, n=3 for control).

To assess the effector cells and tumor microenvironment in remote tumors after the combination treatment, distant flank tumors (HIFU untreated side) were collected on day 18 and infiltrating immune cells were analyzed by flow cytometry (n=3 for combination group, n=4 for other groups).

As alternative breast tumor models, E0771-OVA cells or JC-HER3 cells (1 x 10^6 cells/injection) were implanted to the leg of female C57BL/6 mice or female HER3 transgenic mice. On day 7 after cell implantation, leg tumors were treated with M-HIFU (n=6) or T-HIFU (n=5), or left untreated (n=5). In JC-HER3 model, M-HIFU treatment and no treatment control were compared

(n=9 mice for each group). Mouse were monitored for tumor growth every 3-4 days and all mice were euthanized when one of these mice reached humane endpoints.

In the bilateral tumor model with JC-HER3 cells, 5×10^5 cells were also implanted to the right flank of female HER3 transgenic mice as well as 1×10^6 cells into the left leg. Established JC-HER3 leg tumors were treated with M-HIFU on day 11 (for M-HIFU monotherapy experiments) or on day 8 (for combination treatment experiments). For the combination treatment, mice received intraperitoneal injection of anti-PD-L1 antibody or isotype control IgG (200 μg/injection) on days 8, 11, 15 in unilateral tumor models, or on days 8, 11, 15 and 18 in bilateral tumor models. Tumor size was measured serially and tumor volumes were calculated as described above.

For depletion of immune cells, mice received peritoneal injection of 250 μg antibody against CD4 (clone GK1.5, Bio X Cell), 250 μg antibody against CD8a (clone 53-6.72, Bio X Cell) or 10 μl antibody against NK cells (anti-Asialo GM1 antibody, FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) 1 day before the first HIFU treatment and 2 days after the first HIFU treatment, followed by injection of the same amount every 5 days throughout experiments. In unilateral MM3MG-HER2 tumor model, tumor size was measured every 3 days until humane endpoint (>2,000 mm^3) or day 40 when the experiment was terminated (n=5 mice for isotype control, n=6 for other groups). Survival of mice was assessed. In MM3MG-HER2 bilateral tumor model, tumor size was measured every 3 days. When one of the mice reached humane endpoint (tumor volume >2,000 mm^3) on day 19, experiments were terminated and all mice were euthanized. In JC-HER3 bilateral tumor model, leg tumors were untreated or treated with M-HIFU on day 8, and mice received intraperitoneal injection of anti-PD-L1 antibody or isotype control IgG (200 μg/injection) on days 8, 11, and 15. Depleting antibody for CD8a or isotype control IgG

was intraperitoneally injected on days 7, 10 and 15. Tumor size was measured every 3-4 days, and when one of the mice reached humane endpoint on day 19, experiments were terminated. n=5 mice per each group.