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Sasa Health Exerts a Protective Effect on Her2/NeuN Mammary Tumorigenesis

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Abstract. *Bamboo grass leaves of different Sasa species have been widely used in food and medicine in Eastern Asia for hundreds of years. Of special interest are Kumazasa (Sasa senanensis rehder) leaves used to prepare an alkaline extract known as Sasa Health. This extract was reported to inhibit both the development and growth of mammary tumors in a mammary tumor strain of virgin SHN mice (1). We found that Sasa Health exerts a significant protective effect on spontaneous mammary tumorigenesis in another mouse model of human breast cancer, the transgenic FVB-Her2/NeuN mouse model. Two cohorts of Her2/NeuN female mice of different age (eleven-week-old and twenty-four-week-old) chronically treated with Sasa Health in drinking water showed both a delay in the development of tumors and reduced tumor multiplicity. Sasa Health also induced inhibition of mammary duct branching and side bud development in association with reduced angiogenesis. Altogether these findings indicate that Sasa Health contains phytochemicals that can effectively retard spontaneous mammary tumorigenesis.*

Chemopreventive phytochemicals are non-nutritive components of edible plants that can block the initiation or reverse the promotion stage of multistep carcinogenesis and halt or retard progression of precancerous cells into malignant ones (2). Approximately one-fifth of seventy-one aqueous botanical extracts used in Asian traditional medicine recently tested *in vitro* revealed some antitumoral efficacy (3). These findings demonstrate a critical need to re-examine plant extracts known to inhibit cancer growth both *in vitro* and *in vivo*.

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Some interesting bio-active extracts are the ones derived from leaves of Sasa species (Gramineae) that are bamboo grasses widely distributed throughout Japan, the Russian part of the Kuril Islands, and the southern part of Sakhalin island (4) *Sasa albomarginata* extract has been described to have anticancer activity (5). Both lignin (6) and polysaccharides from Sasa species were reported to have antitumor activity (7). In particular, two polysaccharide preparations, GK1 and GK2, isolated from *Sasa kurilensis* negatively affected the growth of Sarcoma-180 implanted in mice (8). Antitumoral properties (1) have been documented for an alkaline extract of *Sasa senanensis rehder* known as Sasa Health. Some of the identified phytoproducts in Sasa Health include chlorophyllin and polysaccharides that are known to exert cancer-protective effects (7, 9). The most significant study on Sasa Health showed that, when this extract was administered in drinking water to SHN virgin mice, a significant delay was seen both in mammary tumor formation and growth (1). This effect was tentatively traced to a boost of the host immune response (10). Some reports suggest that Sasa Health contains phytochemicals with anti-inflammatory properties (11). Interestingly, some anti-inflammatory compounds, rather than being immuno-suppressive, seem to increase the immune response (12).

For this reason, we set out to test the effect of Sasa Health in the FVB-Her2/NeuN mouse model of spontaneous mammary tumorigenesis (13). This model is particularly suitable to evaluate the contribution of host-immune response after vaccine and drug treatments by measuring both humoral and cellular response against the Her2/NeuN antigen (14). The FVB-Her2/NeuN mouse is also an important preclinical model because it recapitulates the development of Her2/NeuN-positive human breast cancer, characterized by over-expression of Her2/NeuN antigen and aggressiveness.

Here we show that Sasa Health treatment can retard the onset of Her2/NeuN primary spontaneous mammary tumors and reduce tumor multiplicity when it is administered both to young (11 weeks of age) and older (24 weeks of age) mice. In addition, Sasa Health treatment seems to influence microvessel formation in the developing mammary gland.

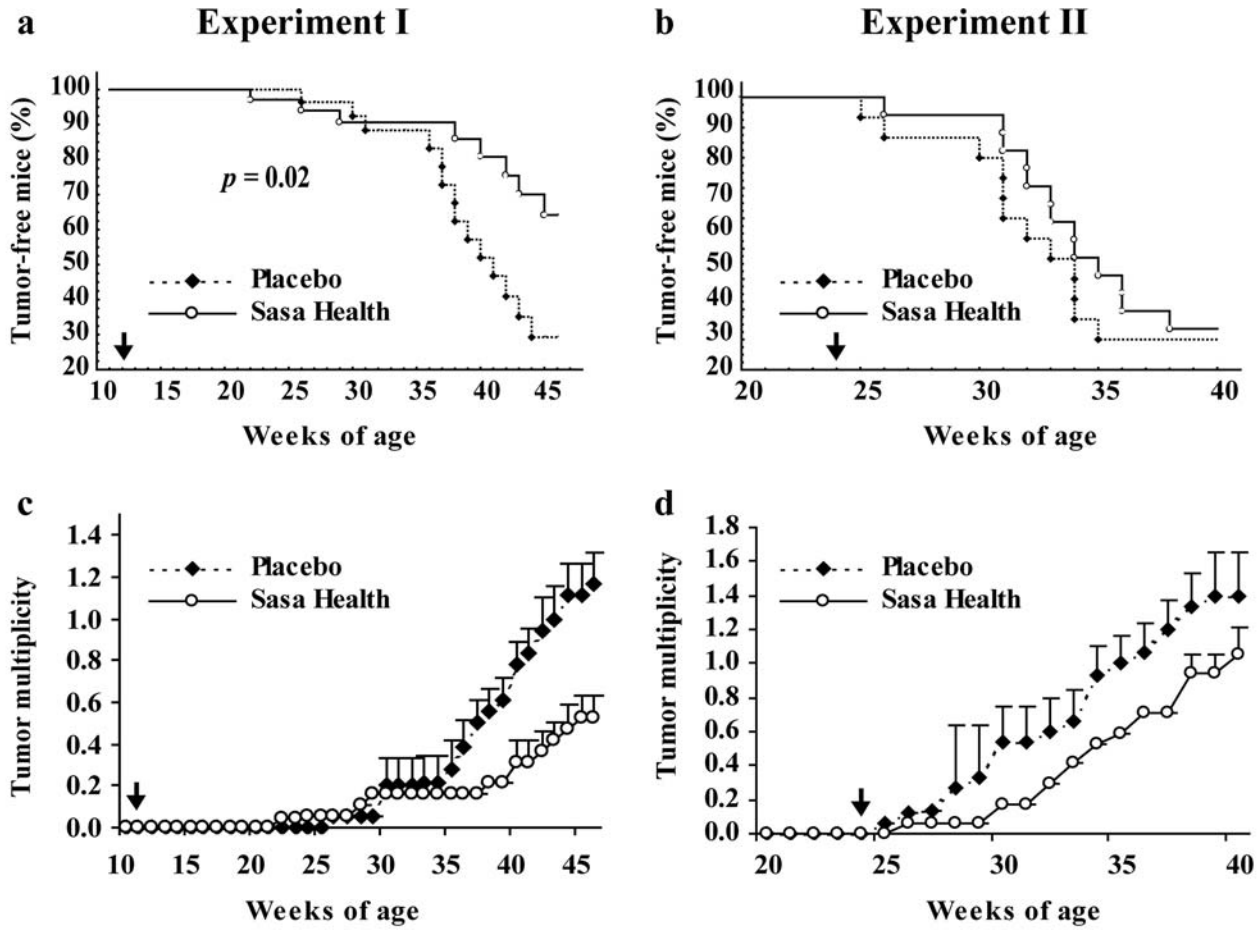

Figure 1. *Effect of Sasa Health on mammary tumorigenesis in Her2/NeuN mice. Kaplan-Meier curves (a and b) and tumor multiplicity (c and d) in two groups of 11-week-old mice (n=21 each for both untreated and treated mice) and 24-week-old mice (n=16 each for both untreated and treated mice). Sasa Health treatment significantly retarded mammary tumorigenesis in the younger group of mice (a) and reduced the tumor multiplicity in both groups (c and d). Tumor multiplicity is calculated as the cumulative number of incident tumors/total number of mice and is shown as mean ± SE (arrows indicate the beginning of treatment).*

Materials and Methods

FVB-Her2/NeuN mice. Transgenic mice bred to homozygosity as described (15) were kept under standard light/dark regimen and fed with laboratory chow *ad libitum*. The Animal Care and Use Committee of Roswell Park Cancer Institute (NY, USA) approved the experiments described in this report.

Sasa Health treatment. Sasa Health was obtained from Daiwa Biological Research Institute Co, Ltd, Kawasaki, Japan and kept at 4˚C. Sasa Health extract is known to contain polysaccharides, chlorophyllin, lignin and flavonoids. Placebo was prepared by mixing yellow and green food colors (McCormick), as per the suggestion of Johns Hopkins Hospital IDS Pharmacy (Baltimore, MD, USA) to match the color of Sasa Health. Virgin female FVB-Her2/NeuN mice of different age (forty-two 11-week-old, and thirty-two 24-week-old mice, respectively) were randomly divided into two groups, the Sasa Health and Placebo groups. The Placebo group received placebo (1:9) in sterile drinking water while the Sasa Health group received the extract (1:2) in sterile drinking water at a final concentration of 0.088% Fe-Chlorophyllin-Na used as a reference compound. Mice were inspected once a week for body weight and appearance of palpable tumors. Water intake was recorded. Tumor growth was measured with a digital caliper and tumor volume was calculated as length x width x height x 0.5236 (16) and reported as mean ± SD.

Assessment of distant metastasis. Magnetic resonance imaging (MRI) was used to assess the development of distant metastases in two groups of five mice each in the Placebo and Sasa Health groups of the younger cohort, 35 weeks after the beginning of treatment. These mice presented palpable tumors of less than 20 mm^3 and continued to receive either placebo or Sasa Health for an additional 12 weeks. The mice were inspected at time intervals by MRI for appearance of metastases in the lungs, liver and kidneys. In preparation for imaging, the mice were anesthetized by administration of ketamine/xylazine. High resolution MRI scans were performed using a General Electric (GE) CSI 4.7T/33 cm horizontal bore magnet (GE NMR Instruments, Fremont, CA, USA) with upgraded radio-frequency and computer systems

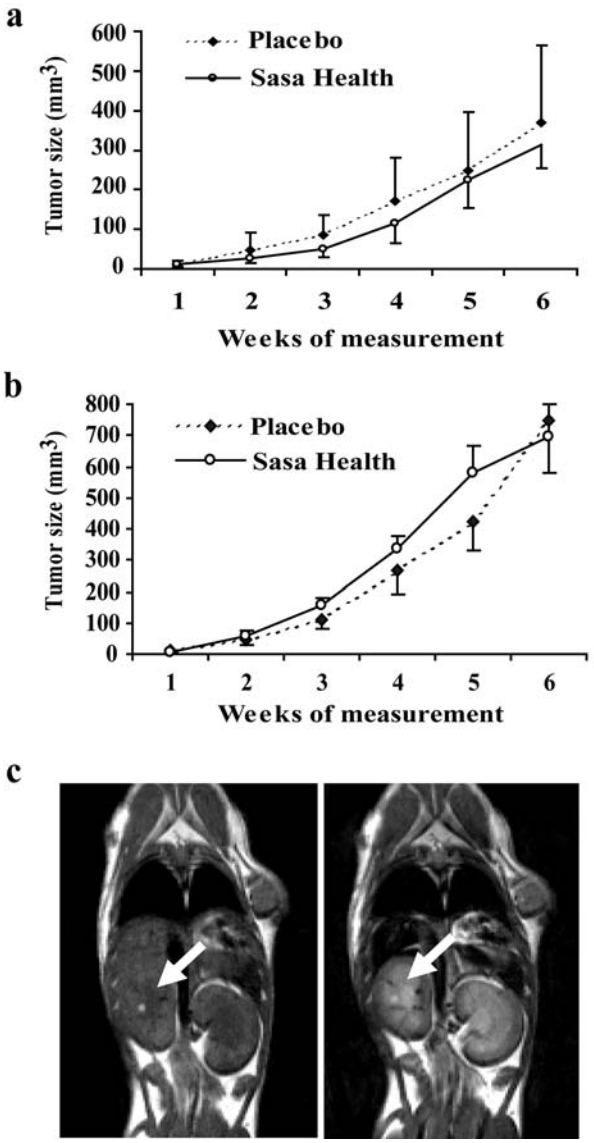

Figure 2. *Sasa Health treatment does not affect tumor growth or metastasis. Tumor growth in a) 11-week-old and b) 24-week-old mice, respectively. c) Representative MR images (T1-weighted, left; T2-weighted, right) of lungs, kidneys and liver showing a mass (arrows) in a kidney of a mouse in the Placebo group.*

incorporating AVANCE digital electronics (Bruker BioSpec platform with Paravision Version 2.1 Operating System; Bruker Medical, Billerica, MA, USA). MRI data were acquired using a G060 removable gradient coil insert generating maximum field strength of 950 mT/m, a custom-designed 35-mm radio-frequency transceiver coil, standard SE and RARE SE MRI pulse sequences. A typical acquisition consisted of a series of scans including a localizer, T1-weighted (or proton-density-weighted) and T2-weighted RARE SE MR images. At the end of the 12th week all mice had one or more tumors and were euthanized. Pathology of the lungs, kidneys and livers was performed to search for metastasis and to confirm the MR images.

Mammary gland whole-mounts. The fourth and fifth inguinal mammary glands without palpable nodules after 35 weeks of treatment in the younger cohort of mice were surgically removed, immediately spread onto glass slides and processed exactly as described (17).

Cell proliferation and microvessel density. Contralateral mammary glands excised from the same mice used for whole-mounts were used for the evaluation of cell proliferation antigen Ki-67 and microvessel density (MVD). Tissue, fixed either in 10% buffered-formalin or zinc-fixative buffer, respectively, was embedded in paraffin and used to prepare 5-μm sections. Ki-67 antigen was reacted with a rabbit anti-mouse Ki-67 antibody (Abcam Inc., Cambridge, MA, USA) and detected with biotinylated goat anti-rabbit antibody. Proliferating cells were quantified by counting Ki-67-positive cells and the total number of cells arbitrarily selected in 10 fields under a microscope at 400x magnification. The proliferation index (PI) was calculated as P/(P + N) x100, where P indicates the number of Ki-67-positive cells and N indicates the number of Ki-67-negative cells. Microvessel density was evaluated by rat anti-mouse antibody against the platelet endothelial cell adhesion molecule-1 (CD31) (Pharmingen, San Diego, CA, USA) and detected with a goat anti-rat biotinylated secondary antibody. MVD represents the total number of microvessels in 10 random fields of each section visualized at 400x magnification. The data represent the mean ± SE of data collected from two representative sections in the Sasa Health and Placebo groups.

Statistical analysis. Delay in tumor formation was estimated by Kaplan-Meier analysis and log-rank test. The Student's *t*-test was used to estimate the differences in tumor multiplicity, tumor size, body weight, water-intake, PI and MVD. Statistical analysis was performed with the SAS (SAS Institute Inc. Cary, NC, USA).

Results

Sasa Health delays spontaneous mammary tumorigenesis. To test whether Sasa Health administered in drinking water exerts a protective effect on mammary tumorigenesis in the FVB-Her2/NeuN mice, we performed two experiments with mice of different age. We used a group of forty-two 11-week-old transgenic Her2/NeuN mice (Experiment I) and a group of thirty-two 24-week-old mice (Experiment II). Sasa Health treatment did not affect the body weight and water intake in both groups (data not shown). Chronic ingestion of the extract in drinking water induced a significant delay ($p=0.02$) in the appearance of tumors in the cohort of younger mice that were treated for up to 35 weeks (Figure 1a). A two-week-delay in appearance of tumors, which was not significant, was observed in the group of older mice treated for 16 weeks (Figure 1b). However, in both groups of mice the tumor multiplicity was significantly lower after Sasa Health treatment (Figure1 c and d). This became apparent after six weeks of treatment in the group of older mice ($p=0.014$) and after eleven weeks of treatment in the group of younger mice ($p=0.019$). In contrast to observations made in the SHN model (1), Sasa Health

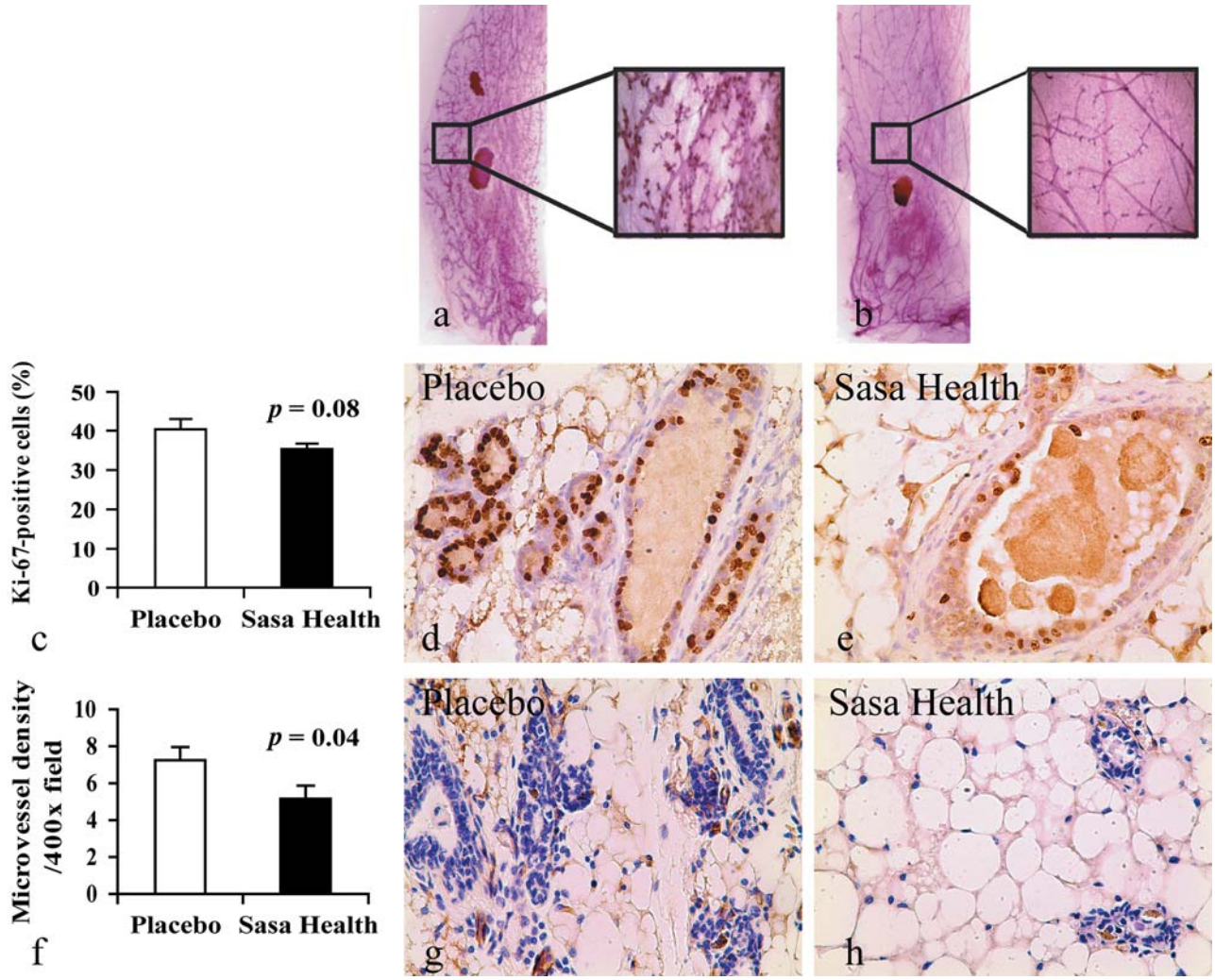

Figure 3. *Sasa Health effect on mammary gland morphology and angiogenesis. Comparison of representative whole mounts preparations (out of five whole mounts each) from a mouse in the Placebo group (a) and a mouse in the Sasa Health group (b) (the nipples are oriented towards the bottom of the images). A dramatic reduction of branching and side-bud development was evident after Sasa Health treatment. Immunocytochemistry showed that cell proliferation was not affected by Sasa Health treatment (c, d, e), in contrast to angiogenesis (f, g, h), which was reduced. Magnification=x 5 (a, b); x 400 (d, e, g, h).*

treatment had no effect on the rate of tumor growth in Her2/NeuN mice (Figure 2).

Altogether these results show that Sasa Health can effectively retard the onset of primary tumors (Experiment I) as well as tumor multiplicity (Experiments I and II) in the Her2/NeuN mouse model.

Evaluation of metastasis at distant sites during Sasa Health treatment. One of the goals of cancer therapy is protection against development of distant metastases. For this reason, we tested whether Sasa Health-treatment had any effect in retarding distant metastases in two groups of five mice each (Experiment I) which had evidence of small (less than 20 mm^3) palpable tumors at week 35. These mice were treated for an additional 12 weeks with either Placebo or Sasa Health and were inspected for tumor growth by weekly inspection as well as MRI imaging of the lungs, kidneys and liver. During this period, nine out of ten mice developed two or more primary tumor (data not shown). At MRI, only one mouse in the Placebo group showed a mass in a kidney (Figure 2c). At the end, all mice were sacrificed. Pathology of lungs, kidneys and liver detected a metastasis in the lung of a mouse in the Sasa Health group that was missed by MRI.

Sasa Health treatment affects mammary gland development. To test the effect of Sasa Health treatment on mammary

gland development, we analyzed mammary gland whole-mounts obtained from both Sasa-treated and untreated animals. We examined the 4th and 5th inguinal mammary glands after 35 weeks of treatment from five mice of the Placebo and five mice of the Sasa Health groups of Experiment I. Whole-mount mammary gland analysis showed a remarkable inhibition of mammary duct branching and side bud development in the Sasa Health-treated mice (Figure 3b). Contralateral mammary glands were analyzed both for cell proliferation and angiogenesis. The PI was reduced but not significantly ($p=0.08$, Figure 3c) after Sasa Health treatment (Figure 3e). In contrast, MVD decrease ($p<0.05$) was evident around ducts and duct branches after Sasa Health treatment (Figure 3 f and h).

These data suggest that one of the effects of Sasa Health treatment may be a reduction of mammary epithelial cells at risk of tumor transformation and progression due to reduced angiogenesis.

Discussion

The most important conclusion that can be drawn from our study using the FVB-Her2/NeuN mouse model is that Sasa Health, an extract of *Sasa senanensis rehder*, exerts a protective effect against spontaneous mammary tumorigenesis in a second mouse model of breast cancer. Our data support and extend previous observations on the SHN mouse model that this extract, when administered in drinking water, can delay the appearance and reduce the multiplicity of mammary tumors (1).

It has been reported that the development of Her2/NeuN tumors can be due to hypomethylation of the methylated Her2/NeuN gene in mammary gland tissue (18). Hypomethylation is an epigenetic mechanism that would lead to the expression of the Her2/NeuN antigen, thus triggering tumor growth. Because we could not detect DNA methylation in the Her2/NeuN gene in mammary glands of 11-week-old mice (Ren MQ and Bistulfi G, unpublished data), we exclude that the delay in tumor appearance is consequent to hypomethylation of Her2/NeuN gene induced by Sasa Health.

Instead, similar to what was reported after treatment of Her2/NeuN mice with a combination of tamoxifen and interleukin 12 (19), we observed that Sasa Health significantly affected the morphology of mammary gland. This can be interpreted as a reduction in the number of mammary cells at risk of progression and a reduction in the angiogenic support to mammary development (19). Indeed, we observed a significant reduction in MVD after Sasa Health treatment. It is possible that little delay in tumor formation was seen in the older treated mice because those tumors were present but microscopic at the time treatment was initiated; however, Sasa Health successfully protected against the formation of subsequent tumors in those mice. Thus, this extract may be an excellent chemo-protectant, but is not apparently tumor growth inhibitory.

Our data do not confirm an effect of Sasa Health on tumor growth/progression that was reported in SHN mice (1). It is becoming widely recognized that tumor response to drug treatment is due to the genetic make-up both of the tumor and the host. This is probably also true for the pharmacological response to phytochemicals targeting specific cell signaling pathways (2). This can be an explanation for the different effect on growth in the SHN and Her2/NeuN tumors.

Whether the effects of Sasa Health are due to a boost of the host immune response, as previously suggested (10), needs additional studies. The observations that we gathered in the Her2/NeuN model provide the basis for a rigorous assessment of both humoral and cellular response against the tumor Her2/NeuN antigen (14) in Sasa Health-treated mice.

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