Focused Ultrasound Therapy for Vulvar Intraepithelial Neoplasia in a Mice Model

Ying Jia, PhD, Jin Wu, MS, Liping Chen, MS, Guangdong Liao, MS, Jiayi Yang, BS, Liangdan Tang, MD, PhD and Chengzhi Li, MD, PhD

Abstract: *Background:* The purpose of this study was to determine the effectiveness and safety of focused ultrasound (FU) for the treatment of vulvar intraepithelial neoplasia (VIN) in a mice model. *Methods:* Estradiol benzoate was subcutaneously injected into the abdomens of eighty 129/J mice. VIN was successfully induced in 56 mice and was divided into the FU group and the control group. Pathologic features and changes in vascular endothelial growth factor expression in the lesions were analyzed before and after treatment. *Results:* Two months after treatment, lesions in 25 of the 56 mice showed restoration of normal skin. Nineteen of the 21 VINI and VINII lesions returned to normal and the other 2 VINII lesions were down graded to VINI, yielding a curative rate of 90.1%. In the control group, all 21 mice had persistent VIN ($P < 0.0001$). In the 14 mice with VINIII lesions, 6 returned to normal skin histology representing a curative rate of 42.9%, 5 were reclassified as VINI and 3 were reclassified as VINII. Thus, the total effectiveness rate was 100%. *Conclusions:* The present study suggests that FU therapy is effective, noninvasive and safe in treating VIN in a mice model.

Key Indexing Terms: VIN; Focused ultrasound therapy; 129/J mice.
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Vulvar intraepithelial neoplasia (VIN) is a general term for a group of vulvar diseases involving precancerous lesions, including the atypical hyperplasia of vulvar epidermis and carcinoma *in situ* of the vulva. The International Society for the Study of Vulvar Diseases divided VIN into differentiated and undifferentiated forms in 2003. These 2 forms are markedly different in morphology, biology and clinical features. The undifferentiated form includes the basal type and the verrucous type and is associated with human papillomavirus (HPV) infection. VINII and VINIII are considered precancerous lesions, whereas the VINI lesions are not. The differentiated form is not related to HPV and usually develops in the elderly. The incidence of VIN is approximately 0.2/100,000. The average age of VIN onset was 50 years in the 1970s and 30 years in the 1990s, a significant trend of earlier disease onset in recent years. Therefore, prompt treatment of these lesions is of paramount importance.

From the Department of Obstetrics and Gynecology (YJ, JW, GL, JY, LT), the First Affiliated Hospital, Chongqing Medical University, Chongqing, China; Department of Breast Surgery (LC), the Third People's Hospital of Chengdu City, Chengdu, China; and Biomedical Engineering College (CL), Chongqing Medical University, Chongqing, China.

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Correspondence: Chengzhi Li, MD, PhD, Biomedical Engineering College, Chongqing Medical University, Box 153, No. 1, Medical College Road, Yu Zhong District, Chongqing 400016, China (E-mail: lichengzhi@cqmu.edu.cn).

Generally speaking, the treatment of VIN includes surgical and nonsurgical treatments. Surgical treatments, such as simple vulvectomy,[1] provide a cure rate of 24%–68%.[2] Surgical scars and deformities may significantly affect quality of life and psychological health.[3–5] Among the many forms of nonsurgical treatments, such as local application of ointment, subcutaneous injection of interferon, freezing and laser treatment, focused ultrasound (FU) appears to be a promising tool for treating dermal lesions. The establishment of animal models of VIN enables researchers to test various therapeutic modalities. Thigpen et al[6] first created an animal model of vulvar squamous cell carcinoma in 129/J mice. OuYang et al[7] also constructed an animal model of vulvar squamous cell carcinoma and induced VIN model at different stages. The aim of this study was to induce a VIN model in 129/J mice and test the efficacy of FU treatment.

METHODS

Experimental Animals

A total of eighty 6-week-old 129/J female mice, weighing 18–20 g, were used in this study. Mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and maintained in specific pathogen-free environments.

Reagents and Equipments

Injectable estradiol benzoate was purchased from Shanghai General Pharmaceutical Co., Ltd. (Shanghai, China). CZF focused ultrasound therapy equipment was manufactured by Chongqing Haifu (HIFU) Technology Co., Ltd. (Chongqing, China). TES-1310 thermometers were purchased from Taiwan TES Electronic Co., Ltd. (Shenzhen, China). Vascular endothelial growth factor (VEGF) antibody and streptavidinbiotin complex reagent kits were purchased from Wuhan Boster Biological Technology Co., Ltd. (Wuhan, China).

Animal Model

Under sterile condition, estradiol benzoate was injected subcutaneously into the mice abdominal skin at the dosage of 20 mg/kg. The injector was withdrawn 2 minutes after injection to avoid leakage. The injection was repeated every 2 days.

Clinical Observation and Pathologic Examination

Every second day after injection, the mice's weight, appetite, vulvar morphology and mental status were observed and recorded. On week 7, each mouse was anesthetized by injection of 0.5% pentobarbital sodium intraperitoneally at a dosage of 30–40 mg/kg and then 1% toluidine blue was applied to the vulva. As the medicine dried naturally, 1% ethylic acid was used to decolorize the blue dye. In the presence of VIN or invasive carcinoma, toluidine blue would combine with DNA in active nuclei to stain the lesion purplish blue and would be decolorized by ethylic acid. Biopsies from the stained areas were performed to verify the successful construction of the model. Once

confirmed, biopsies were taken before treatment, immediately after HIFU treatment and 2 months after HIFU treatment. The mice were exterminated by dislocating spine method after the last biopsies were ended. The tissues were fixed in 10% paraformaldehyde; dehydrated; embedded in paraffin, prepared in serial sections of 4 μm; stained with hematoxylin and eosin (HE) and then examined under a light microscope. A total of 56 mice with VINI, VINII or VINIII were chosen for this study.[8]

FU Treatment

Fifty-six mice were divided into FU group and control group with 28 mice each. The FU group included 11 mice with VINI, 10 mice with VINII and 7 mice with VINIII. The mice were anesthetized with intraperitoneal injection of 0.5% pentobarbital sodium and FU treatment was delivered with a power of 4.5 W and frequency of 9.7 MHz. The treatment area included the entire vulvar lesion and the surrounding tissues 0.5 cm outside the lesion. The treatment bar was closely attached to the skin and scanned in uniform linear motion at 3–5 mm/s. The treatment lasted 2–3 minutes until the surface temperature reached 40–43°C, measured with a TES-1310 thermometer. At this point, the treated skin and mucous membrane appeared congested and edematous.

The control group included 11 mice with VINI, 10 mice with VINII and 7 mice with VINIII. Mice with VINI and VINII underwent sham treatment. The treatment procedure was the same as that of the treatment group, but no ultrasound power and frequency were delivered. The mice with VINIII underwent the FU treatment with a power of 4.7 W and frequency of 10.0 MHz.

Electron Microscopic Evaluation

Fresh tissue samples were obtained promptly after treatment. The tissues were fixed in glutaraldehyde (volume fraction as 2.5%) at 4°C for 2 hours and rinsed with phosphate-buffered solution (PBS). The tissues were fixed again in 1% osmiumtetroxide at 4°C for 2 hours, dehydrated with ethanol at different gradients, embedded with epoxy resin and sliced into ultra-thin sections for electron microscopic evaluation.

Immunohistochemistry

Immunohistochemistry staining was performed with streptavidin-biotin complex. Paraffin sections of 4 μm thickness were prepared. The sections were dewaxed with dimethylbenzene and dehydrated with ethanol at different gradients. They were incubated with 3% methanol solution in hydrogen peroxide for 20 minutes at room temperature and rinsed with 0.01 mol/L PBS (pH 7.0). The sections were then heated in a microwave oven until boiling for hot repair of antigen. The treated sections were further incubated with normal goat serum for 20 minutes, and VEGF antibody (ready to use) was added dropwise over a night at room temperature. The samples were then rinsed 3 times with PBS, 5 minutes each. Biotin was added dropwise to mark goat anti-rabbit IgG. The sections were placed in the 37°C incubator for 30 minutes and then rinsed 3 times with PBS. Diaminobenzidine colorization lasted 5–10 minutes and hematoxylin was used for slight restaining.

Determination of Results

Cells with cytoplasm stained tan color were considered positive. Under high power lens, 5 microscopic fields were randomly chosen from each section to count the number of positive cells. The average number of cells per high-power field represented the positive expression of VEGF. Positive standards were detected by the method of Weidner et al.[9]

Statistical Analysis

Statistical analysis was performed with SPSS 11.0. Changes in the vulval epidermis were evaluated using the χ^2 test, and comparisons of positive cell counts between the 2 groups before and after treatment were performed using *t* test.

RESULTS

VIN Model Construction

Gross Observation

In the 4 weeks after FU treatment, congestion and edema were observed on the skin surface of the vulva and bloody secretion was also present. After 6 weeks, some mice showed obvious tumor growth with ulceration and bleeding (Figure 1). During the period of induction of VIN, 5 mice died, all of which developed gross tumors on the vulva. The lesions were harvested in a timely manner and prepared as paraffin sections for HE staining and microscopic evaluation.

Histologic Evaluation

After 7 weeks, all lesions were harvested for HE staining. Fifty-six mice had developed VIN (VINI: 22 cases, VINII: 20 cases and VINIII: 14 cases) and 5 had developed vulvar squamous cell carcinoma, which were excluded from the study. Basal cells and parabasal cells had proliferated upwards to

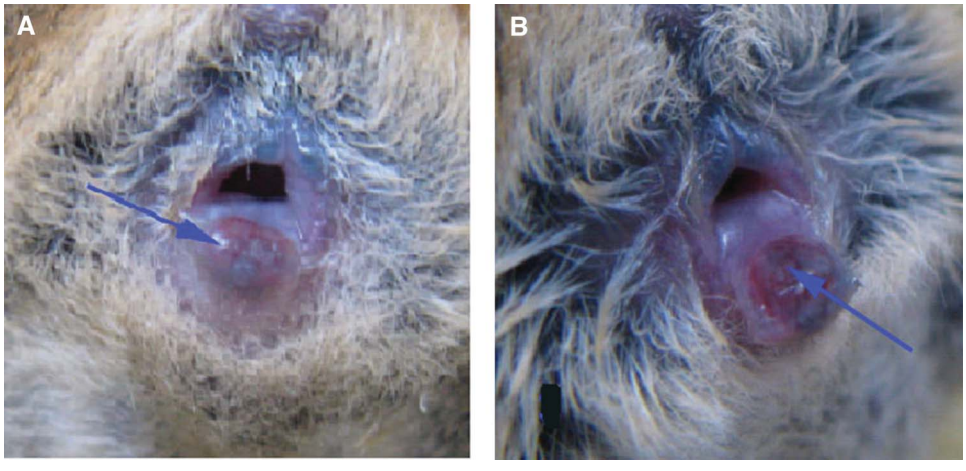

FIGURE 1. Macroscopic vulvar tumors. (A) Vulvar tumor of mice in the sixth week; significant tumor growth on the vulva skin. (B) Vulvar tumor of mice in the seventh week; ulceration and bleeding were present.