Original Article

Low power ultrasound inhibits cell proliferation and invasion of human cancer cells *in vitro*

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ABSTRACT

Background: Applications of ultrasound in medicine for therapeutic purposes have been accepted, and they have several beneficial uses for many years. However, the outcome of low power ultrasound waves on cell proliferation, especially cell cycle progression and invasion as well as their associated genes on human breast and cervical cancer cells has not been investigated yet. Therefore, we examined the effect of low power ultrasound on BT20, BT20-E6/E7 and HeLa cell lines. **Materials and Methods:** BT20, BT20-E6/E7 and HeLa cell lines were used in this study. On the other hand, cell proliferation, cell cycle, and invasion assays were applied to study the effect of low ultrasound irradiation on these cell lines. Meanwhile, western blot was performed to study the expression patterns of some selected genes associated with this effect. **Results:** We found that low power ultrasound inhibits cell proliferation and provokes G0–G1 cell cycle arrest and reduction of S as well as an increase in the G2-M phase of HeLa cells in comparison with the untreated cells. This is accompanied by a down-regulation of Cdk-6 (cyclin dependent kinase) which is a major control switch for the cell cycle. Moreover, low power ultrasound inhibits cell invasion and consequently down-regulates the expression of Id-1, caveolin, and EGF-R which are widely considered as main regulators of cell invasion and metastasis of human cancer. **Conclusion:** These results suggest that application of low power ultrasound on human breast and cervical cancer could be an effective method to reduce cell proliferation and invasion of these cancers.

Key words: Cancer, cell invasion, cell proliferation, ultrasound

INTRODUCTION

In recent years, bioengineering researchers have expanded their efforts into the area of nonionizing radiation. Nowadays, the great value of ultrasonic methods to biology and medicine is evidently proven. Ultrasound usually interacts with human tissue by generating heat,[1-4] but also through nonthermal effects which are ascribed to cavitation.[5-7] Moreover, a fast development of nonlinear ultrasound took place in the second part of the last century; this was promoted by the emergence of ultrasonic technology and was focused on high intensity ultrasound generation.[8] Meanwhile, investigation of the biophysics of ultrasound interaction with biological samples at a cellular level is evidently complex, due to the small size of a cell. The interaction of ultrasound with biological tissues has led to several clinical therapies such as physiotherapy,[9] transdermal drug delivery,[10] thrombolysis,[11] and cancer treatment.[12] These therapies are generally based on the physical effects of ultrasound on cells and tissues such as controlled disruption of various biological barriers like cell membranes and tissues for drug and gene delivery.[13-15] Several effects of ultrasound have also been studied in isolated cells, cell suspensions and/or cell cultures *in vitro*. It is worth mentioning here that studies of isolated cells or cells in culture provide a means for examining the effect of ultrasound without numerous biological variables operating in the whole organism. Although such simplification reduces the applicability of the experimental

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data in a clinical environment, it nevertheless allows a better understanding of physiological changes due to ultrasound exposure. However, little is known about subtle biological effects such as those which involve up- or down-regulation of specific proteins on cells. The aim of this work was therefore to investigate the effects of low power ultrasound on the cell proliferation and invasion *in vitro* as well as some key genes associated with these events.

MATERIALS AND METHODS

Ultrasound exposure system

The experimental arrangement for the ultrasound exposure of cells is shown in Figure 1. The experiments were conducted using commercially available piezoelectric ceramic transducers Pz27 Disc 5 × 1 mm (FERROPERM Piezoceramics A/S, Denmark). The transducer was glued with a thin conductive epoxy layer at the bottom of the culturing plate, acting as an actuator to generate the ultrasound wave applied to the cells, and was connected to a waveform generator (Agilent 33220A/20MHz function/arbitrary waveform generator, Santa Clara, CA, USA). The signal was a continuous sinusoidal wave with an amplitude of 3 V rms applied to the transducer, giving a thickness variation in service of 10^{-3} μm (initially measured using an accelerometer). The transducer had a bandwidth of 0.7–1.1 MHz, and the frequency was kept at 800 kHz (within the range of therapeutic ultrasound) over an exposure time of 6 h, in order to see the influence of a longer time of ultrasound exposure. The beam profile was not established. The adopted parameters also avoid the cavitation effect, which can be an underlying cause of cell damage.

The culturing plate was made of polystyrene with a thickness (distance travelled by sound waves before reaching the cells) of 1 mm. Ultrasound waves consist of cycles of compression and expansion exerting a positive and negative pressure. These pressure cycles are known to act on molecules by pulling them together and pushing them away from each other. The ultrasound power was calibrated at <1 mW/cm^2 of radiation sound intensity of the transducer, with the PVDF membrane hydrophone method.

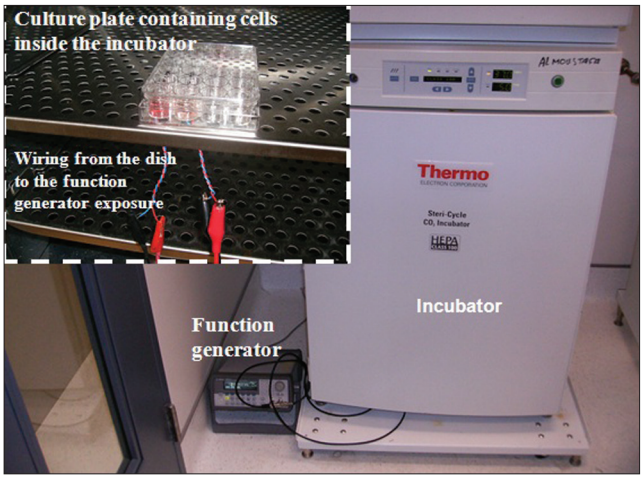

Figure 1: Experimental arrangement for the ultrasound exposure of cancer cells under incubation

Cell lines

Human breast cancer cell lines, BT20 and BT20-E6/E7, as well as human cervical cancer cell line, HeLa, were obtained from the American Type Tissue Culture. HeLa cells were selected for their high invasion ability, while BT20 cells were chosen for their relatively low invasion ability compared to HeLa cells.[16,17] All three cell lines were cultured in RPMI medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin, and 2 mM L-glutamine (Life Technologies, Inc.). Cells were incubated at 37°C in 5% CO_2 atmosphere.

Proliferation assay

The cells were cultured in flat-bottomed 12-well plates (Costar, Cambridge, MA). A concentration of 100 × 10^3 breast cancer cells (BT20 and BT20-E6/E7) were initially plated and incubated 2 h prior to ultrasound exposure. This time is required for the cells to adhere on the substrate before ultrasound excitation. Each cell line was seeded in two wells, one for the control sample (unexposed to ultrasound) and one for the treated sample (to be exposed to ultrasound). Next, ultrasound excitation was turned on for 6 h, and turned off. Cells were left incubated for 56 additional hours before being taken out for counting using a hemocytometer. A triplicate test was performed for each sample, and the average value was considered for our analysis.

Cell cycle analysis

HeLa and BT20 cell lines were exposed to ultrasound under the conditions described above. Next, cells were harvested, washed, fixed and subsequently treated with 50 μg/mL RNase and stained with 50 μg/mL propidium iodide for 30 min. They were then analyzed in a FACS Calibur machine, and data were evaluated with Cell Quest and ModFitLT v3.1 software.

Invasion assay

Cell invasion was performed in 24-well Biocoat Matrigel invasion chambers (8 μm; Becton Dickinson) according to the manufacturer's protocol [Figure 2]. Only BT20 and HeLa cell lines were used.

A concentration of 50 × 10^3 untreated and treated (by exposure to ultrasound) cells were plated in the top chamber. The bottom chamber contained RPMI medium. These cells were also allowed to adhere on the substrate (2 h), and ultrasound was activated for 6 h, then stopped [Figure 2a]. After 16 additional hours of incubation, invasive cells have passed through the Matrigel layer onto the surface of the membrane [Figure 2b]. The noninvasive cells

were removed with a cotton swab. The cells that migrated through the membrane were rinsed, fixed with methanol, and stained with hematoxylin [Figures 2c and d]. For quantification, cells were counted under a microscope in five predetermined fields. A triplicate test was performed for each sample, and the average value was considered in the cell counting. BT20 was used here as a reference because it is a noninvasive cell line. In fact, it has been shown that the invasion and metastatic abilities of BT20 are induced by E6/E7 oncogenes of human papillomavirus (HPV) type 16.[16]

Western blot analysis

This assay was performed as previously described in our works.[17,18] However in our experiment, anti-Cdk-6, Id-1, Caveolin, EGF-R (clone 13) (Bio/Can Scientific), as well as anti-actin (Clone C4, Roche Diagnostics) were used in the assays.

RESULTS AND DISCUSSION

Figure 3a shows the effect of ultrasound exposure on the two breast cancer cell lines proliferation. The cell proliferation rate considerably decreases with ultrasound wave excitation. This effect was already reported in the literature by Watanabe *et al*.,[15] for cancer cells of mouse T lymphoma (EL-4). In their work, Watanabe *et al*. reported that when cancer cells are exposed to ultrasound, hydroxyl radicals are generated and DNA molecules from cancer cells become segmented due to hydroxyl radicals. Apoptosis is then induced and the proliferation of cancer cells is suppressed. Our study complements this observation by using a simpler experimental arrangement and pointing out cell cycle arrest by ultrasound to be the reason in the decrease of the proliferation rate. Moreover, with an ultrasound power far below what was reported by Watanabe *et al*., we succeeded to achieve a 74.6% and 50.2% reductions in the number of BT20 and BT20-E6/E7 cells, respectively, compared to a 90% reduction obtained by Watanabe *et al*.,[15] in 48 h with EL-4 cancer cells. The difference observed in the proliferation reduction of BT20 and BT20-E6/E7 is justified by the E6/E7 genes, which are known to be cell proliferation stimulators.

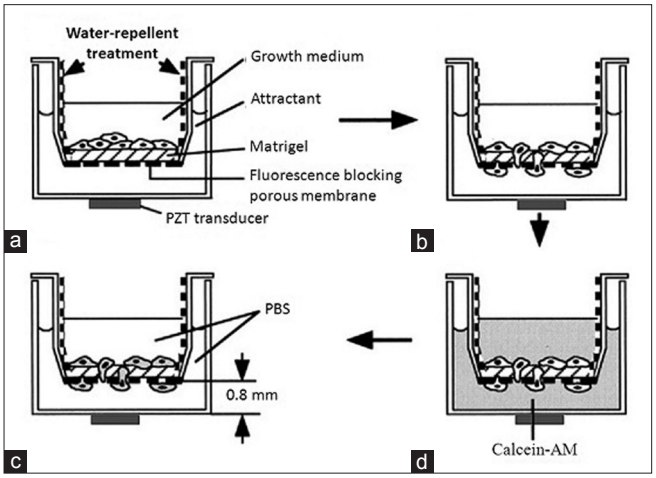

Figure 2: *In vitro* cell invasion protocol

The histogram statistics from the cell cycle data are shown in Table 1 in terms of percentage gated events in each phase of the cell cycle. A total of 9500 and 9953 events were gated out of 12965 and 12395 for the control and the treated HeLa samples, respectively, whereas 9989 and 9810 events were gated out of 12783 and 14019 for the control and the treated BT20, respectively.

Table 1 shows that when exposed to a low power ultrasound, the G2M phase of the cell cycle is significantly affected in HeLa cells. In fact, 30.78% G2 in cells exposed to ultrasound *versus* 15.05% of the "control" population shows that ultrasound significantly induces cell cycle arrest. Moreover, we show that ultrasound exposure of the breast cancer cells studied provokes a loss of cell cycle controllers leading to deregulated cell proliferation. In fact, the cell cycle progression is regulated by the activities of cyclin-dependent kinases and their subunits known as cyclins.[19] When these key genes are deregulated in human neoplasia, they often result in over-/down-expression of CDKs and cyclins, as well as loss of natural inhibitors of CDKs, and consequently hyper-activation of CDKs. In this study, we report for the first time that low power ultrasound partially inhibits Cdk-6, Cyclin D2, and Cyclin D3 in human breast and cervical cancer cells as shown in Figure 3b. Therefore, our data suggest that low power ultrasound, when applied in the conditions described here, inhibits cancer cells proliferation *in vitro*. These results complement those obtained by Hrazdira *et al*.[20] who showed that exposed to a 0.8 MHz low intensity ultrasound (100 mW/cm^2) for 10 min, HeLa cells exhibited partial inhibition of proliferation. Their study showed that cells were most sensitive when undergoing M- and S-phases of the cell cycle.

Figure 4a shows the effect of ultrasound on cell invasion ability with the procedure described in Figure 2. From a total of 50 × 10^3 cells initially incubated, 10% of HeLa cells and 0.32% of BT20 cells not exposed to ultrasound (control samples) have passed the matrigel after 24 h of incubation. For cells exposed to ultrasound, only 4.19% of HeLa and 0.23% of BT20 have crossed the matrigel layer, giving a

Table 1: Cell cycle histogram statistics for HeLa and BT20 cell lines in terms of % gated events.

Cell lines		Go/G1	S	G2M
HeLa	Control	68.05	17.22	15.05
	Treated	57.91	10.46	30.78
BT20	Control	57.25	22.61	18.03
	Treated	54.02	24.25	21.91

Each value represents the average of a triplicate measurement ($P < 0.05$)

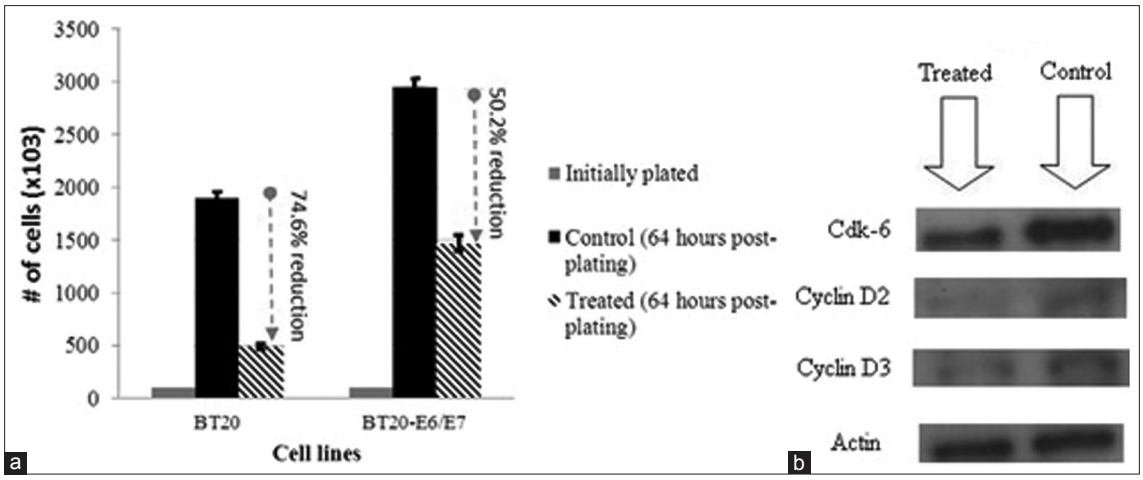

Figure 3: (a) Effect of ultrasound exposure on the proliferation rate of two breast cancer cell lines (BT20 and BT20-E6/E7). Each value represents the average, while error bars are the standard deviation of a triplicate measurement on each sample. (b) Western blot analysis of Cdk-6, cyclin D2, and cyclin D3 expression in BT20-untreated and treated cells

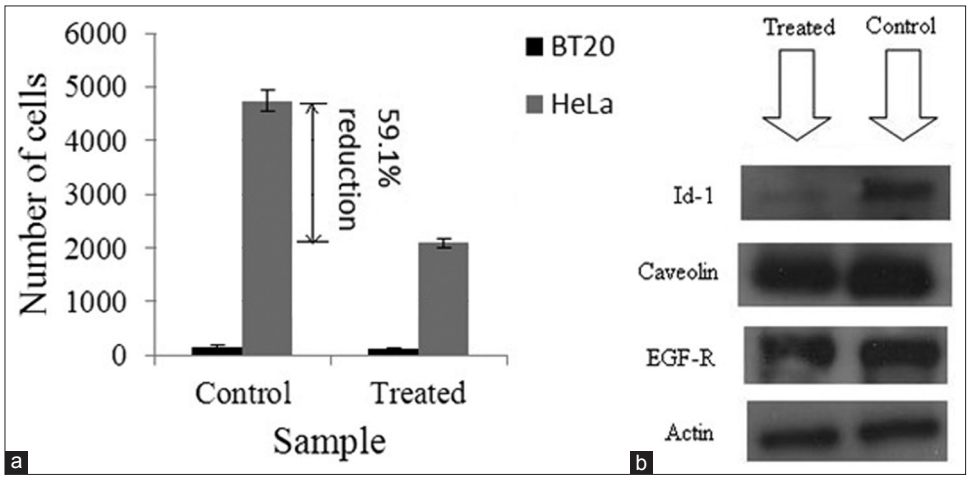

Figure 4: (a) Number of cells crossing the matrigel membrane from the procedure described in Figure 2. Each value represents the average, while error bars are the standard deviation of a triplicate measurement on each sample. (b) Confirmation of down-regulation of Id-1, caveolin, and EGF-R by western blot analysis of HeLa cells

reduction due to ultrasound waves of 59.1% for HeLa cells and 28.1% for BT20 cells.

Western blot analysis was further performed. The results in Figure 4b confirmed the down-regulation of Id-1, Caveolin, and EGF-R genes which are widely considered main regulators of cell invasion and metastasis of human cervical cancer cells.[21-25] Therefore, we report for the first time here that low power ultrasound inhibits cell invasion of human breast and cervical cancer cells through Id-1, Caveolin, and EGF-R down-expression.

From the above results at low acoustic power, nonthermal mechanisms for biological change may be predominant. In general, as the pressure increases, cavitational and thermal effects become more important until, at a higher level, heating effects mask all others.[26] We have chosen to avoid this in our experiment by the use of low acoustic pressure amplitude with a sound intensity far below the intensity necessary for stable production of bubble in mammalian tissue using a pulse echo technique (8×10^{-2} W/ cm^2), as reported by Ter Haar.[27] This is also far below the ultrasound intensity of 0.5–3.0 W/cm^2 used for therapy as reported by Galperin *et al.*,[28] and up to 2750 W/cm^2 as reported by Chapelon *et al.*[29] At such a low ultrasound intensity level, the heat produced is rapidly diffused out, resulting in a negligible change in local temperature [Figure 5]. Moreover, it is generally accepted that many nonthermal effects of ultrasound in biologic systems are attributable to cavitation.[30] Therefore, our study provides an additive information to the scientific community, by illustrating the alteration of cellular proliferation and invasion ability due to sound waves, thus strengthening the use of this type of waves as a potential candidate to stimulate therapeutic effects on cells. This is in line with some other studies reported in the literature.[31,32]

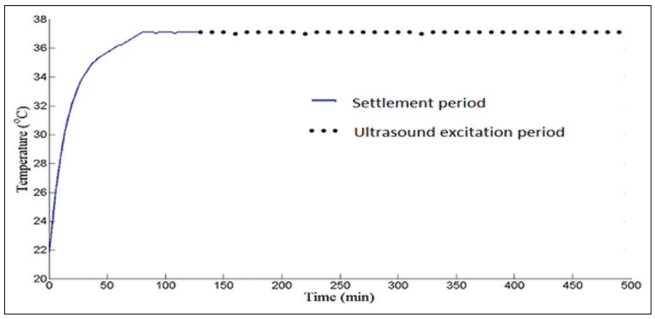

Figure 5: Temperature monitoring on the substrate where cells lie during ultrasound exposure. During the 6 h ultrasound excitation period (dotted line), no meaningful increase in temperature occurs, showing that heating is not the cause of the bioeffects observed on cells

CONCLUSION

We have examined the effect of low power ultrasound in human breast and cervical cancer cell lines, BT20 and HeLa. Its ability to significantly reduce the cell proliferation rate of breast and cervical cancer cells, BT20 and HeLa *in vitro*, was demonstrated. Moreover, it is also shown, for the first time, to consistently reduce cervical cancer cell invasion ability. The data presented suggest that Cdk-6 is one of the most sensitive proteins, among the ones investigated, to low power ultrasound among proteins involved in the cell cycle, whereas Id-1, caveolin, and EGF-R are the most sensitive involved in cell invasion of the cell lines studied. These results are important for medical applications and suggest that low power ultrasound may show a good promise in cancer therapy. This opens the perspective of using a controlled absorption of ultrasound for a therapeutic purpose. However, nonthermal effects of ultrasound such as radiation stress in biological samples still need a better theoretical foundation and physical understanding; therefore, new theories based on numerical and experimental data should be developed in the future.

REFERENCES

1. Dyson M. Mechanisms involved in therapeutic ultrasound. Physiotherapy 1987;73:116-20.
2. Kitchen SS, Partridge CJ. A review of therapeutic ultrasound. Physiotherapy 1990;76:593-600.
3. Draper DO, Schulthies S, Sorvisto P, Hautala AM. Temperature changes in deep muscles of humans during ice and ultrasound therapies: An *in vivo* study. J Orthop Sports Phys Ther 1995;21:153-7.
4. Chan AK, Myrer J, Measom G, Draper DO. Temperature changes in human patellar tendon in response to therapeutic ultrasound. J Athl Train 1998;33:130-5.
5. Loch EG, Fischer AB, Kuwert E. Effect of diagnostic and therapeutic intensities of ultrasonics on normal and malignant human cells *in vitro*. Am J Obstet Gynecol 1971;110:457-60.
6. Pizzarello DJ, Wolsky A, Becker MH, Keegan AF. A new approach to testing the effects of ultrasound on tissue growth and differentiation. Oncology 1975;31:226-32.
7. Webster DF, Pond JB, Dyson M, Harvey W. The role of cavitation in the *in vitro* stimulation of protein synthesis in human fibroblasts by ultrasound. Ultrasound Med Biol 1978;4:343-51.
8. Naugolnykh K. Nonlinear acoustics: From research in physics to application (historical incidents). Acoust Phys 2009;55:335-41.
9. Ter Haar G. Therapeutic and surgical applications - physical principles of medical ultrasonics. Chichester: Ellis Horwood Ltd; 1986. p. 436-61.
10. Mitragptri S, Blankschtein D, Langer R. Ultrasound-mediated transdermal protein delivery. Science 1995;269:850-3.
11. Everbach E, Francis C. Cavitational mechanisms in ultrasound-accelerated thrombolysis at 1 MH. Ultrasound Med Biol 2000;26:1153-60.
12. Hill C, Ter Haar G. Review article: High-intensity focused ultrasound – potential for cancer treatment. Br J Radiol 1995;68:1296-303.
13. Paliwal S, Mitragotri S. Ultrasound-induced cavitation: Applications in drug delivery and gene delivery. Expert Opin Drug Deliv 2006;3:713-26.
14. Mitragotri S. Innovation: Healing sound: The use of ultrasound in drug delivery and other therapeutic applications. Nat Rev Drug Discov 2005;4:255-60.
15. Watanabe A, Nishimura H, Kawashima N, Takeuchi S. Consideration on suppression of cancer cell proliferation by ultrasound exposure using sonochemical and biological measurements. J Phys Conf Ser 2004;1:210-5.
16. Yasmeen A, Bismar TA, Kandouz M, Foulkes WD, Desprez PY, Al Moustafa AE. E6/E7 of HPV type 16 promotes cell invasion and matastasis of human breast cancer cells. Cell Cycle 2007;6:2038-42.
17. Yasmeen A, Alachkar A, Dekhil H, Gambacorti-Passerini C, Al Moustafa AE. Locking Src/Abl tyrosine kinase activities regulate cell differentiation and invasion of human cervical cancer cells expressing E6/E7 oncoproteins of high-risk HPV. J Oncol 2010;530130.
18. Al Moustafa AE, Foulkes WD, Benlimame N, Wong A, Yen L, Bergeron J, *et al*. E6/E7 proteins of HPV type 16 and ErbB-2 cooperate to induce neoplastic transformation of primary normal oral epithelial cells. Oncogene 2004;23:350-8.
19. Kong N, Fotouhi N, Wovkulich PM, Roberts J. Cell cycle inhibitors for the treatment of cancer. Drugs Future 2003;28:881-96.
20. Hrazdira I, Skorpikova J, Dolnikova M. Ultrasonically induced alterations of cultured tumour cells. Eur J Ultrasound 1998;8:43-9.
21. Lin CQ, Singh J, Murata K, Itahana Y, Parrinello S, Liang SH, *et al*. A role for Id-1 in the aggressive phenotype and steroid hormone response of human breast cancer cells. Cancer Res 2000;60:1332-40.
22. Fong S, Itahana Y, Sumida T, Singh J, Coppe JP, Liu Y, *et al*. Id-1 as a molecular target in therapy for breast cancer cell invasion and metastasis. Proc Natl Acad Sci U S A 2003;100:13543-8.
23. Koda M, Sulkowski S, Garofalo C, Kanczuga-Koda L, Sulkowska M, Surmacz E. Expression of the insulin-like growth factor-I receptor in primary breast cancer and lymph node metastases: Correlations with estrogen receptors alpha and beta. Horm Metab Res 2003;35:794-801.
24. Nielsen TO, Andrews HN, Cheang M, Kucab JE, Hsu FD, Ragaz J, *et al.* Expression of the insulin-like growth factor I receptor and urokinase plasminogen activator in breast cancer is associated with poor survival: Potential for intervention with 17-allylamino geldanamycin. Cancer Res 2004;64:286-91.
25. Sloan EK, Stanley KL, Anderson RL. Caveolin-1 inhibits breast cancer growth and metastasis. Oncogene 2004;23:7893-7.
26. Ter Haar GR. Biological effects of ultrasound in clinical applications. In: Suslick KS, editor. Ultrasound: Its chemical, physical, and biological effects. New York, NY: VCH Publishers Inc; 1988:305-20.
27. Ter Haar GR, Daniels S. Evidence for ultrasonically induced cavitation *in vivo*. Phys Med Biol 1981;26:1145-9.
28. Galperin H, Kirsch M, Levi S. Fragmentation of purified mammalian DNA molecules by ultrasound below human therapeutic doses. Hum Genet 1975;29:61-6.

29. Chapelon JY, Margonari J, Vernier F, Gorry F, Ecochard R, Gelet A. *In vivo* effects of high-intensity ultrasound on prostatic adenocarcinoma dunning R3327. Cancer Res 1992;52:6353-7.
30. Kondo T, Arai S, Kuwabara M, Yoshii G, Kano E. Damage in DNA irradiated with 1.2 MHz ultrasound and its effect on template activity of DNA for RNA synthesis. Radiat Res 1985;104:284-92.
31. Tsai WC, Chen JY, Pang JH, Hsu CC, Lin MS, Chieh LW. Therapeutic ultrasound stimulation of tendon cell migration. Connect Tissue Res 2008;49:367-73.
32. Mahoney CM, Morgan MR, Harrison A, Humphries MJ, Bass MD. Therapeutic ultrasound bypasses canonical syndecan-4 signaling to activate rac1. J Biol Chem 2009;284:8898-909.

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