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RESEARCH ARTICLE

Title

Intraoperative cryosurgical freezing assists with local drug delivery and targeting of tumor fluids in VX2 model, which translates in locoregional drug targeting of tumor fluids during cryoprobe-assisted surgical resection of breast cancer.

Authors

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Abstract

Background

We assess locoregional drug targeting effectiveness of intraoperative (IO) cryoprobe-assisted injection of blue dye (BD) and cytotoxic-tracer mixture (TTM), in VX2 tumor model, and its translational value to cryo-assisted breast tumor surgery with BD alone.

Methods

Under computed tomography (CT) guidance, we injected two ml TTM in five aliquots in the margin of 16 frozen or normothermic VX2 tumors. We evaluated the IO and post-operative drug targeting and therapeutic efficacy in tumor-host interface (T-HI) by CT, gross examination, and histopathology. In twenty-six T1 to T4 primary breast cancer (BRCA) we performed ultrasound-guided (US) cryoprobe-assisted tumor freezing, BD guided lymphatic mapping, and surgery. We evaluated, IO and in freshly resected specimen, BD distribution pattern in T-HI, lymph node(s), breast parenchyma, and resection cavity.

Results

Fluids-impervious frozen VX2 or breast tumor transported drug(s) an arc-like pattern at T-HI regardless of freeze dose, number of freeze-thaw cycles, drug dose fractionation, tumor characteristics or dimensions. During melting, TTM spread within fifty percent VX2 tumor mirrored that of T-HI; it was massive in normothermia. In VX2 twenty percent focal margin necrosis at pathology coincided with CT gap; in both studies, BD dose-staining spread in T-HI and tumor was linear. Eighty-four patients had one to twelve stained axillary lymph nodes; sixty-nine percent and all respectively, had another quadrant and resection cavity stained.

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Conclusion

Intraoperative freezing-assisted drug delivery and targeting techniques during cryoablation of VX2 tumor translate successfully to locoregional BD targeting, lymphatic mapping during cryo-assisted surgery of breast cancer.

Keywords

Targeted drug delivery, VX2 tumor, breast cancer, cryoablation, cryo-assisted injection, cryo-assisted resection, blue dye, epirubicin.

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Introduction

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Breast cancer (BRCA) is the most common cancer and the leading cause of cancer death in women worldwide [1]. Surgery remains the pillar of multimodal therapy. Despite progresses in adjuvant therapies, post-operative locoregional (LR) and/or distant recurrences amount to 18.9%, of which about 7.3% are local [2-4]. Local control of disease and breast cancer–specific survival rates is related; strategies that reduce the rates of local recurrence at 5 years and 10 years translate into an improved breast cancer–specific survival rate at 15 years [5].

Various Intraoperative (IO) techniques aim at detecting and eliminating residual disease, or cell shedding during breast surgery. Margin assessment, sentinel lymph node mapping and biopsy (SLNB), resection cavity shaving (RCS), partial breast irradiation (PBI), or photodynamic therapy (PDT) is common procedure [4, 5]. During breast conserving surgery (BCS), most local recurrences in the conserved breast appear close to the tumorectomy cavity [6-8]; pathologic studies of mastectomy specimens have shown that tumor cells rarely extend 4 cm beyond the index lesion [8]. These clinicopathological facts have spurred the development of techniques that target the tumor bed [9], such as routine circumferential resection cavity shaving [5] or partial breast irradiation (PBI). The latter aim at decreasing the reoperation rates, the side effects of whole breast irradiation [WBI], the treatment duration -accelerated PBI (APBI)- and associated costs. Encouraging results are now available for selected series of early breast cancer patients. However, PBI/APBI is still debatable among the medical community[9] and is not available to most health care facilities. Regarding RCS, most surgeons opt for selective rather than circumferential cavity shaving. Additionally, the determination of an optimal clear margin for invasive cancer is still a challenge, given that even a negative margin does not indicate the absence of residual disease in the breast. Surgery can lead to increased dissemination of epithelial cells[10, 11], and to the local secretion of growth factors[12] that may stimulate tumor cell proliferation, or metastasis formation. Perioperative chemotherapy [CTx] [13, 14], or neoadjuvant local intra- or peritumoral chemotherapy (NLCTx) [15] have been used to prevent surgery-induced dissemination or tumor growth. Local washing and multiple injections of CTx platinum agent in the resection cavity, breast and axillary region during modified radical mastectomy (RM) was demonstrated safe and effective at decreasing the exfoliated tumor cells along with potential improvement of the 3 years disease free survival [DFS] [16].

There is room for intraoperative loco-regional adjuvant therapies during BCS for the prevention of tumor cell shedding and the extension of tumor-free-margins without resecting additional tissue, or doing PBI. The goal is to decrease the 20% to 40% positive margin and reoperation rates following partial mastectomy [17], while allowing conventional adjuvant RT, CTx, endocrine therapy or targeted therapy. Such strategy is based on the cryothermal handling and containment of the target tumor [18, 19] and the simultaneous local injection of cytotoxic targeting tumor margin fluidic pathways. The rationale for this combined technical approach stems from the freezing-mediated tumor cell-fluid entrapment, the dosing advantages of local chemotherapy [15] and its combination with cryosurgery or systemic chemotherapy (CTx). Tumor freezing prevents cells from seeding into circulation [20], or shedding during tumor manipulation and resection [21]. The extrusion and transport of interstitial fluids at the frozen-unfrozen

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interface [22-26] (F-UI) during tissue freeze-thaw process has considerable potential interest for the transport of drugs. Indeed the freezing-extruded tumor fluids contain concentrated tumor metabolic by-products and debris [24-25]; a "soup" that transiently settles and accumulates in the unfrozen hypothermal region (UHR) surrounding the frozen mass during a cryosurgical freeze-thaw cycle. Some molecules of the soup, such as albumin, are natural carrier for drugs [27], like patent blue [PBV]. Thus, we used the UHR transporting potential for a drug locally deposited at the F-UI. We have explored the spatio-temporal aspect of this drug transport at the ice and tumor margin in various in vitro, ex vivo and in vivo experimental tumor model [28, 30] with free drug and/or drug-carrying device systems. We have also evaluated in two recent human studies [30, 31] the freezing-induced transport and distribution of blue dye tracers, methylene blue (MB) or PBV, known for their ability to map the breast lymphatic drainage, or bind to albumin [33, 34].

In this work, we evaluate the translational value of a cryoprobe-assisted drug delivery technique targeting the tumor margin, first in a VX2 tumor model [30], and then in human breast tumor [32]. We describe the distribution and tumor kill pattern of a tracer-and-therapeutic mixture (TTM) deposited under CECT imaging at the frozen edge of a tumor during cryoablation. The procedure simulated the clinical presentation and combined ablation-and-drug therapy of peritumoral residual disease. We tested the clinical translatability of the VX2 procedure to twenty-six T1-T4 resectable primary breast tumors with a special attention to applicability, safety, and efficacy. We injected the blue dye alone under ultrasound (US) guidance at the edge of the frozen breast tumor before surgical resection. Our first goal was to map the lymphatics and assess the intraoperative pattern and distribution of the dye before and after surgical resection. Our secondary endpoint was to evaluate post operatively on the resected specimen, the circumferential distribution of the dye at tumor-host interface. Finally, we compared the experimental and clinical data and we discuss the implications for developing intraoperative fluid-mediated locoregional containment therapies during breast conserving surgery.

Materials and Methods

Overall study design

The first step sought to evaluate the intraoperative (IO) flow and distribution of a tracer therapeutic mixture (TTM) locally injected in a single site of VX2 tumor margin, in normothermic or cryoablated tumor (CA). The conservative cryothermal dosing consisted in maintaining the frozen-unfrozen interface

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(F-UI) on tumor gross margin during five repeat freeze-TTM injection-and-thaw cycles (FIT). Each FIT cycle repeats every three to five minutes. The TTM dose was half the mean tumor volume.

The second step sought to replicate the VX2 drug delivery technique to breast cancer patients while adjusting technical parameters to two IO clinical requirements: map the lymphatics, and achieve a conventional resection of frozen tumor, i.e. cryo-assisted (CR) BCS or radical mastectomy, without undue prolongation of general anesthesia. We injected in the F-UI the same BD dose as in the VX2 study, regardless of the tumor volume. The F-UI had to overlap tumor margin and about 10mm of normal tissue before injection. Injection needle was always located in tumor margin facing the axilla. We investigated whether and how much tumor volume, and thus frozen zone perimeter would affect BD uptake and its spatio-temporal distribution, compared to VX2.

The third step compared the distribution spread and pattern of the BD tracer in excised specimen, host tissue, and breast lymphatics at gross examination. The VX2 study is modelling the image-guided combinatorial local treatment of a peripheral macroscopic residual tumor burden; the BRCA study uses the blue dye (BD) tracer as a surrogate for a small cytotoxic molecule; it assesses intraoperatively (IO) its marginal circumferential and radial spread, and pattern as related to the injected dose, or cryothermal dose. The VX2 TTM tested a possible therapeutic effect of low dose epirubicin [29] aliquots in combination with repeat freeze-thaw cycles (CACH) compared to normothermic tumor (ITCH).

VX2 Study

This acute study previously published in part [30] sums up (table 1) as follows: bilaterally implanted VX2 tumors develop into 4 milliliters (ml) masses in the paravertebral muscle. Our goal was to assess the safety and kill effect of tumor conservative cryoablation and simultaneous local injection of tracer therapeutic mixture (TTM), the CACH procedure (n=8), on the interstitial distribution and marginal targeting of the TTM. Observation data were compared to the injection alone procedure ITCH (n=8) in normothermic tumor. The methylene blue (MB) concentration in the TTM volume was 1.5mg/ml. The treatment was percutaneous (PC), under computed tomography (CT) -guidance to evaluate the intraoperative TTM flow pattern. Contrast-enhanced CT (CECT) and pathological examination of resected tumors at day 3, 7 and 10, evaluated and compared the contrast agent localization, the dye spatial localization and the marginal kill. Epirubicin (Epi) dissolved in absolute ethanol is the therapeutic component of the TTM, which includes methylene blue (MB) and ioversol (Io).

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Table 1 Technical and Imaging parameters

STUDY	Approach -Technique (n)	Tumor Vol. (range)	FT cycle n	Injection Type, Vol., BD (dose)	Tumor F Dose	Injection Timing, Frequency	Tracer D Imaging
VX2	PC - CACH (8) -ITCH (8)	4 cm3± 0.5 (3.6-4.5)	5	TTM, 2 ml ± 0.2 (1.5mg/ml)	Up to Tm 0mm	End F , one per FT cycle	IO-CT CECT
BRCA	Open -CR (26)	*33.5 cm3 (0.8-158)	2	BD 2 ml (10mg/ml)	Tm positive 0 -10mm	End F, 1st F	IO-US Vis.

Table 1

Description **Technical and Imaging parameters** (adapted in part from ref. 30)

VX2: Five TTM aliquots were injected at slow flow rate (ca 0.9ml/min) up to 2ml total dose. Each injection (ITCH) or freeze-injection (CACH) sequence repeats every 3 to 5 minutes. The percutaneous (PC) FT procedure, CT, and CECT guided, contains the frozen margin at tumor margin level (Tm). At each time point, two animals (n) were euthanized for tumor specimen gross examination and histopathology. **BRCA**: A bolus 2ml BD dose is injected in one minute, in the deep aspect of the frozen tumor margin-breast interface (figure 2), under US guidance. The frozen margin expands about 10mm in normal breast tissue; such positive freeze margin is more harmful to peripheral tumor cells than the VX2 neutral freeze dose. This cryo-assisted resection (CR) of the melting breast mass precedes the axillary exploration. *We assumed a spherical shape for BRCA tumors. The avg. maximal diameter of freshly resected and bisected tumor is 4 cm.

CACH, cryoablation + local chemotherapy (epirubicin); ITCH, intratumor chemotherapy; CR, cryoresection; FT, freeze-thaw; Tm, tumor margin; TTM, tracer and therapeutic mixture; BD (V/V), blue dye dilution. IO-CT, or –US, intraoperative-computed tomography or -Ultrasonography; CECT, contrast enhanced CT; D, distribution; Vis, visualization.

Breast cancer Study

The clinical study, previously published in part [32], was conducted in a single center, The Rudolfinerhaus Private Clinic, Vienna, Austria. Twenty-six patients aged 54 (±14) years (mean, SD), presenting with primary breast tumor, stage I to III or de novo stage IV, were randomly selected and treated (Table 2). All patients gave oral or written informed consent. All but two patients were chemotherapy naïve before surgery.

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Table 2 Patient clinicopathological characteristics				
Age (mean 54.5; range 24-73) years				
Stage (n=26)	Stage I (6)	Stage II (7)	Stage III (7)	Stage IV (6)
T	T1 (6)	T2 (7)	T2 (4); T3 (2); T4(1)	T2 (2); T4 (4)
N	N0 (4); N1 (2)	N0 (4); N1 (2); N2 (1)	N1 (5); N2 (1); Nx (1)	N1 (6)
M	M0	M0	M0	M1 (6)
Tumor Size (mean, SD)	4cm X 2.7 cm ± 3.1 x 2.1			
Pathology	IDC/NST (19); ILC (5); Mixed (2). Unifocal (20) ; Multifocal (4) ; Multicentric (2)			
Grade	G1=0 G2= (9) G3=(17)			
Mammary Location	Right = (15); Left = (11)			
	UOQ (11) ; LOQ (4) ; UIQ (4) ; LIQ (2) ; Nipple area (5)			

Table 2

Description **Patient clinicopathological characteristics** (*UICC TNM Classification (8th ed.). 2016*).

IDC/NST, invasive ductal carcinoma of no special type ; ILC, invasive lobular carcinoma ; UOQ/UIQ, upper outer/inner quadrant ; LOQ, LIQ, lower outer/inner quadrant ;

Following intraoperative tumor freezing with ultrasound guided marginal injection of 2ml BD, and conventional resection of the frozen mass and breast tissue, dubbed cryo-assisted resection en bloc (CR), was conducted [32]. Tumor margin of resected specimen was marked with sutures for spatial orientation. The frozen tumor samples were subjected to tumor characterization and margin evaluation. Twenty-six patients were operated with curative intent, breast conservative surgery in 21, and radical mastectomy in five. The staining pattern and distribution in the resected specimen, the breast parenchyma and the resection cavity was measured and photographed. Lymph node staging, axillary exploration and lymph node clearance were SLNB on 19 patients and 7 ALND of which four were completion ALND following frozen sections examination (Table 4).

Methodological convergences and divergences

The authors had no connection during the conduct of the researches. The VX2 was an acute 10 days study whose results were entirely available before designing the clinical protocol. We tailored the drug delivery technique to the clinical requirements and the preferences of the breast tumor surgeon, Dr. Nikolai N. Korpan (Table 1). Although the growth and invasion patterns of VX2 tumor [35] differ from that of breast tumor [36], the model was considered relevant for translation to human breast tumor. The rationale was that the cooling-injection timing neutralizes the potential influence of tumor

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vascularization, capillary lymphatics, and tumor fluids, thus allowing comparative evaluation of the drug interstitial flow and transport in unfrozen peritumoral tissue.

Study converging parameters were the drug injection intervening during the end of freezing (Table 1), and the needle positioning in tumor margin. We used a single liquid nitrogen powered probe, a single injection needle, tumor side, and similar 2ml injection volume. The latter matched know values for breast cancer lymphatic mapping [37] and the injection side was facing the axillary region to facilitate the BD migration in this direction.

Study diverging parameters were the approach, the number of FT cycles, and the fractionation of the injected dose, the blue dye concentration, the tumor size (Table 1), and the location of the frozen-margin relative to tumor edge. The 17G (1.47mm) penetrating cryoprobe developed a symmetrical ice ball growth into the VX2 tumor; for the BRCA study, a flat cylindrical cryoprobe – 20mm to 50 mm diameter- contacted the surgically exposed tumor surface, which resulted in an ellipsoidal, asymmetric ice ball growing faster in surface than in depth. Finally, the repeat five FIT including partial thaw sequences (FIT) simulated a “waving” of the TTM dose at the frozen-unfrozen (F-UI) margin interface of VX2 tumor. Given the unchanged tracer distribution pattern from the first to the fifth cycle, only 2 FT cycles were used for the clinical study, a widely recognized clinical technique [38]. The full dose injection of breast tumor took place before completion of the first freeze sequence, when the F-UI reached needle[32]. During the VX2 percutaneous or BRCA surgical approach, we made every effort to minimize the risk of unwanted reflux or drug loss through track of least resistance, such as the probe, the needle tract, or the surgical wound. We injected the deep aspect of the frozen or normothermic lesion margin under CT (VX2) or US guidance for BRCA. For the latter we undermined only the superficial aspect of tumor, where we positioned the contacting probe. The VX2 TTM tested a possible therapeutic effect of low dose epirubicin [30] in combination with freeze-thaw (CACH) compared to normothermic tumor (ITCH). For BRCA, we used blue dye (BD) tracers as surrogate for small cytotoxic drug had. We injected patent blue 2.5% (PBV) in 11 patients, and methylene blue 1% (MB) aqueous solution in 15 patients. All BRCA tumors received a single and similar dose in one tumor side to evaluate the influence of tumor volume on tracer distribution and migration.

Results

The ice zone margin has a directional and patterning effect on the flow and initial distribution of the injectate

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Table 3, figure 1 and 2 show that the injectate, irrespective of the composition, spreads along the frozen tumor mass in an arc-like pattern. The injectate accumulates in the outer edge of the frozen mass, i.e. the frozen tumor-unfrozen tissue interface. During the freezing process, the core of the frozen zone is impervious, and the frozen margin acts as a channel to fluid transport. Indeed, the intraoperative spread of the injectate is remarkably similar in both studies. Upon resection and bisection of fresh sample, tumor margin stains blue over an average 35% to 50% of the perimeter. In order to compare this spread we have averaged volume of BRCA tumor (table 1), and found that tumor staining is a linear function of the BD dose.

Table3 Injectate dosing and distribution in tumor

STUDY (n)	Vi/Vt	mb conc. (mg/ml)	Vd, Tm & Tc %	Vd/Vi	Vmb /Vt (mg/ml)	Tm Kill
Vx2 No F (8)	0.5	1.5	NA, 10% & 80%	0.9	0.8	None
Vx2 F (8)	0.5	1.5	Arc-like, 50% & 80%	0.8	0.8	20%
BRCA F (26)	0.06	10	Arc-like, 35% & 30%	5.5	0.7	NA

Table 3

Description **Injectate dosing and distribution in tumor** (adapted in part from ref. 30)

Tracer distribution, Vd, is described in freshly resected, bisected sample by pattern, and spread in % of target area, on Day 0; n is the number of tumors. Calculation is modelling tumor as a spheroid.

F, freeze; Vi/Vt, injectate to tumor volume ratio; Vd/Vi, tracer distribution in tumor; Tm, tumor margin; Tc, tumor core; mb, methylene blue; Vmb/Vt, estimate of tracer accumulation in tumor margin and core; Kill, marginal necrosis.

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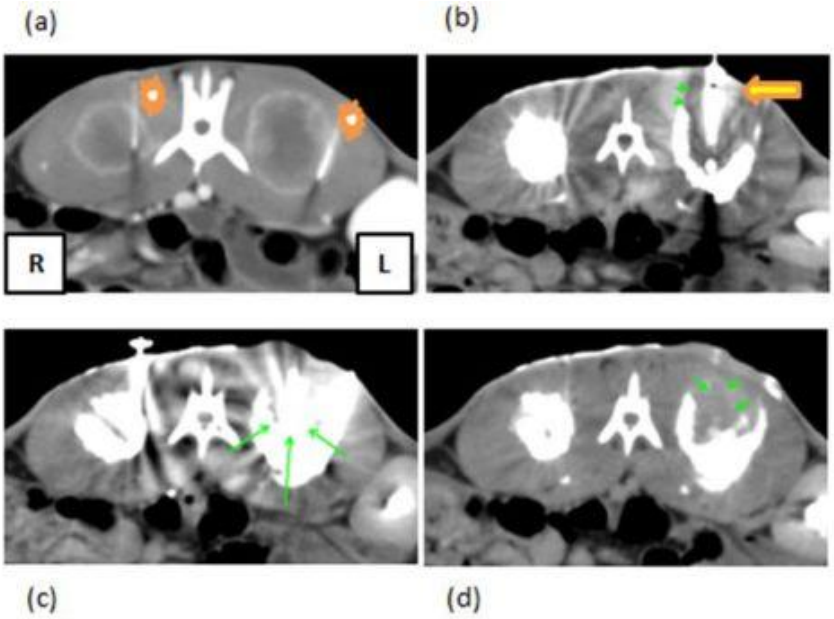

Figure 1

Description **CT guided percutaneous injection, ITCH, (R) or cryoprobe-assisted injection, CACH, (L) of therapeutic mixture in VX2 tumor margin.** (Adapted from Ref. 30) A: CECT guided needle (asterisk) positioning along enhancing tumor rim; B: at end of first 0.4ml injection sequence, the TTM contrast agent tracer (ioversol) does not permeate the frozen core of the left side tumor, penetrated with cryoprobe (yellow arrow) , but permeate the unfrozen tumor of right side; tracer flows along the L ice margin with an arc-like pattern. Reflux through needle tract is minimal C: During the thaw period following each injection, the marginal tracer penetrates the melting ice towards tumor core (green arrows). D: twenty minutes post procedure, a larger amount of fluids and tracer, 50% of the injectate, leaks out of left side tumor core (short green arrows) during probe and needle removal compared to the right side.

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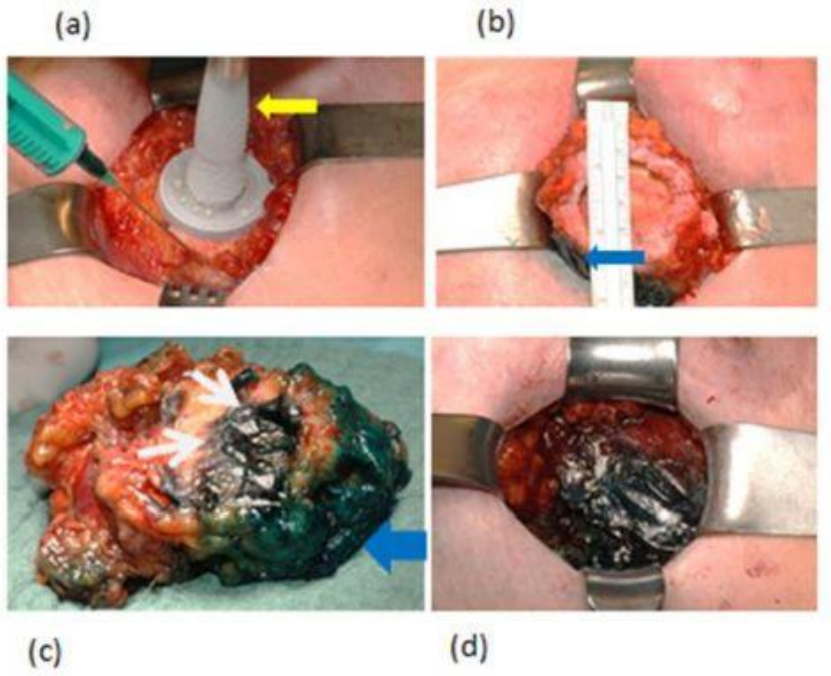

Figure 2.

Description **Intraoperative ultrasound-guided cryo-assisted blue dye (BD) injection and en bloc resection of breast tumor-T2N0MO**. (Adapted from Ref. 32). Patient D.J; **A**: Aspect of surgical wound following MB injection in frozen tumor margin, during pull out of needle. US transducer has been removed. The cylindrical liquid nitrogen cryoprobe (yellow arrow), 50mm diameter, makes a 89cm3 ellipsoidal ice zone in three minutes that engulfs the 5.45cm3 tumor located in the upper outer quadrant. **B**: Following an 8 minutes thaw and a second freeze, margin of melting tumor evidences BD distribution in an arc-like pattern (blue arrow). **C**: the freshly excised bi-sectioned sample, a 71 cm3 mass, exhibits similar BD intratumor permeation pattern (white arrows), and its diffusion in nearby breast fatty and fibro-glandular tissue (large blue arrow). **D**: blue staining of resection cavity is obvious. Two SLN lymph nodes were removed, non-metastatic at pathology. BD reflux in surgical wound and in needle track is minimal during freezing; we estimated that about 50% of BD migrated to contiguous breast.

BD, blue dye; SLN, sentinel lymph node

The first freezing was sufficient to pattern the concomitant injectate flow, which spread along tumor margin regardless of the conservative (VX2) or curative (BRCA) freezing procedure.

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From the first to the fifth freezing sequence, the concurrent repeated injection of aliquots contrast agent and BD tracer resulted in the same arc-like distribution pattern about frozen-unfrozen interface of VX2 tumor margin(Fig. 1b).. During the first freezing, the conservative cryoablative procedure propagated the frozen margin at VX2 tumor margin level and the following freeze cycles were adjusted in cooling intensity and duration to keep the ice margin steady. Thus, tumor margin was sequentially freezing and melting, which resulted in transient co-accumulation of tumor extruded fluids and tracers during the repeat intensity modulated freeze-thaw cycles. This observation holds true during the first freeze of BRCA tumor whose frozen margin was impervious to the BD, but could engulf the tracer during its planned progression in normal breast tissue; an arc-like marginal staining pattern that did not change during the re-freeze sequence (Fig.2b, 2c) .

The freeze-thaw cycle affected tumor margin and tumor host interface permeation to TTM or BD.

Whether steady (VX2) or advancing (BRCA), the frozen rim kept the co-injected drug-tracers from permeating frozen tumor mass. This effect was constant and independent of the frozen rim dimension, and tumor characteristics. Remarkably, the arc-like pattern of the drug-tracers mirrored the frozen rim shape. An effect that lasted during the freezing period provided that the injection needle tip is located in the unfrozen region and before the ice margin. During tumor freezing, co-injected PB or MB tracer do not permeate the frozen core, regardless of their concentration. However, BD deposited before the advancing ice margin, as exemplified for BRCA, accumulates in the wider positive ice margin, a slushy mixture of fluids and ice crystals. The blue staining of the unfrozen rabbit muscle facing the steady ice margin of figure 3L is narrow in comparison with the large coloration of breast parenchyma surrounding tumor (Figure 2c). During the thaw period, regardless of its duration, or that of the preceding freezing time, 20 minutes for VX2 or seven minutes for BRCA, melting tumor margin becomes permeant to dye penetration towards tumor core. Comparing the injection of frozen and unfrozen VX2 tumor, the injectate penetration into tumor was immediate in the latter and delayed in the former. Although qualitative, the tracer uptake evaluation within tumor looked similar for both groups when the melting process was complete. This observation holds true for frozen BRCA tumor; although blue dye was injected before the end of the freezing process, it penetrated tumor core during the thawing sequence.

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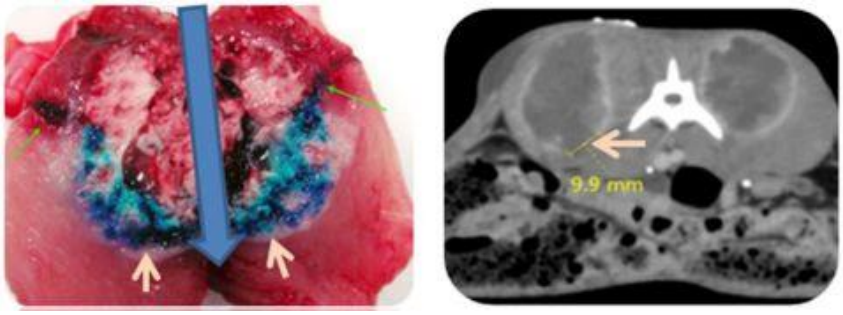

Figure 3

Description **VX2 tumor margin drug targeting and focal kill** (Adapted from Ref. 30) Left: One hour post CACH procedure, transected right side tumor along probe tract (blue arrow) evidences predominant BD staining of tumor margin-muscle interface (orange arrows). The injection needle track (green arrows) stains blue from BD reflux during injection. Right: CECT imaging on day 10 shows a gap of contrast agent (orange arrow) in the enhanced marginal rim, compared to an integral rim in the injection only left side. Histopathological focal margin necrosis coincides with the gap

Tracer migrated in host tissue and breast lymphatic vessels.

In both studies, we observed a rapid migration of the tracer, either the contrast agent and/or blue dye, in the normal tissue-surrounding tumor. From the frozen margin of breast tumor, about 50% of BD dose transported to contiguous breast parenchyma and drained into lymphatics. A little amount tended to reflux along needle tract and superficial wound. The injectate reflux ratio along needle track in the VX2 study ranged from 6% to 17%, with a frequency of 13% to 50% for CACH vs. ITCH group. Following probe pull out nearly 50% of the injectate was lost through probe track, along with tumor necrotic debris and fluids (Figure 1d). We did not compensate for this intraoperative reflux in either study. For sixty-nine percent of BRCA patients BD injectate transported to a single contiguous quadrant in 18/26 patients, and stained the resection cavity in all cases. Twenty-two patients -84.6%- had 1 to 12 stained axillary lymph nodes as seen in Table 4. The time lost during the freeze-thaw injection protocol average 20 minutes for BRCA, which did not prolong much the anesthesia and had no consequence on the patient recovery or wound healing. As expected starting during the intervention, patient urine stained blue. No allergy or durable skin staining were noted. According to preoperative plan, intraoperative lymphatic mapping, SLN and tumor margin evaluation, surgery had been uneventful.

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Drug dose distribution and effect

Tracer distribution volume is evaluated on freshly resected tumor from localization and percentage coverage of target area. We assume spheroid tumor geometry. In Table 3 we show that the injectate (I) to tumor volume ratio (Vi/Vt) is one order of magnitude lesser in BRCA (mean volume) compared to VX2 tumor. After deduction of the estimated dose lost -~50%- (Vd/(Vi-50%) to migration in breast or through probe tract of VX2 target, a quantifiable quite similar estimate of tracer accumulation (Vmb/Vt) in tumor margin (Tm) and tumor core (Tc) ranges respectively from 0.7mg/ml to 0.8mg/ml. The tracer spread pattern is also similar in Tm and Tc.

Cryothermal dose affected cryoablation and cryoresection

The BRCA cryo-assisted resection case of figure 2 illustrates the large frozen mass, or freeze dose that engulfs tumor and normal breast tissue. The latter is 15 times grander than tumor volume. It is a positive freeze margin, whose contours cover tumor margins along with normal tissue. The tumor freeze dose, estimated in percentage of frozen tumor and host tissue of table 2, is 100% for VX2 and >100% for BRCA. Assuming an ellipsoidal geometry for the frozen tissue, we have calculated a freeze dose, median 43.6 ml, range 24-177 ml, which means that all targeted BRCA tumors were properly frozen. The resection line locates in normal breast parenchyma, 3cm to 4cm off the palpable and visible frozen contours. The intraoperative selective re-resection rate (Table 4) for close, <2mm, or positive margin is 15%. As expected, freshly excised VX2 tumor after conservative cryoablation exhibited viable tumor clusters in pathological samples of frozen margin [29].

Table 4 Blue dye migration and surgical option in breast cancer patients

BRCA (n)	**BD breast Q (n %)**	**SLN IR (n %)**	**LN meta**	**Selective RCS (n %)**	**BCS**	**RM**	**SLNB**	**ALND**
26	2(69%), 3(12%), 4(19%)	22(84.6%)	17	4 (15%)	21	5	19	7

Table 4

Description **Blue dye migration and surgical option in breast cancer patients**

BD migrated to one or two contiguous quadrants in 81%, and in SLN in 84.6%. The intraoperative selective RCS ratio for positive or close margin (<2mm) was 15%.

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Q, quadrant; SLN, sentinel node; IR, identification rate; RCS, resection cavity shaving; BCS, breast conserving surgery; RM, radical mastectomy; SLNB, sentinel lymph node biopsy; ALND, axillary lymph node dissection.

Discussion

The lack of an effective, translatable strategy for the local delivery of cytotoxic drugs to breast tumor during surgery, has limited the clinical potential of intraoperative local chemo-immunotherapies [15, 39-41]. The goal is to improve local control of disease without the side effects of resection cavity shaving or APBI. Strategies that reduce the rates of local recurrence at 5 years and 10 years translate into an improved breast cancer–specific survival rate at 15 years [5]. Even a modest 10% reduction in the re-excision rate would prevent reoperation in 10,000 to 20,000 of the 180,000 American women who undergo lumpectomy annually in the United States [42]. We propose a cryoprobe-assisted local drug injection-and-resection strategy of solid tumor to minimize tumor fluids leak and maximize drug targeting of tumor-host margin interface during breast conserving surgery.

We have previously demonstrated that solution of small molecular tracer alone or coformulated with cytotoxic drug injected in tumor margin migrate in tumor fluid dissemination pathways. We have shown that concurrent tumor freezing modulates the direction and spread of this migration [30, 32]. However, there is a lack of demonstration that the VX2 cryoablation tumor model is translatable to cyoprobe-assisted surgery, i.e. cryoresection of human breast tumor. There is no known study linking cryosurgical ablation and cryosurgical resection combined with local adjuvant therapy. In the present study, we expand on our previous findings that freeze-thaw assisted local injection of active drug and ablation procedure of VX2 animal tumor is applicable to freeze-thaw assisted local tracer injection and breast conserving surgery in human patient.

The rationale for the translatability of this intraoperative cryothermal and drug-mediated therapy adjuvant to two seemingly opposite local curative procedure, i.e. ablation versus resection, stems from the initiating common event; subzero cooling of living tissues immobilizes all fluids and fluid communication pathways within frozen mass. There is an interruption of blood, lymphatic and interstitial fluid flow in and out of tumor[38]. We hypothesized that, during freezing, the local transport of a co-injected drug along the frozen mass would be similar for a highly vascularized, aggressive tumor like VX2 or for a breast tumor, regardless of their pathological characteristics. A potential advantage of this combinatorial approach is the tumor cell-fluid entrapment, and the lower dosing of local chemotherapy [15] that can be associated with systemic chemotherapy (CTx). Tumor freezing would prevent cells from seeding into circulation [21], or shedding during tumor manipulation and resection [21]. The extrusion and

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transport of tumor interstitial fluids at the frozen-unfrozen interface [22-25] during the freeze-thaw process has considerable potential interest for the transport of drugs. Indeed the freezing-extruded tumor fluids contain tumor metabolic by-products and debris [25, 26], i.e. a "soup" that transiently settle and accumulate in the frozen-unfrozen interface region (F-UI) surrounding the frozen mass during a freeze-thaw cycle. Some molecules of the soup, such as albumin, are natural carrier for drugs [27], like patent blue [PBV]. Thus, the F-UI [22] region has considerable interest for the transport of a locally deposited drug.

The value of this targeted cryothermal-mediated drug delivery technique is its translatability from the VX2 model to breast cancer. With regard to the spatio-temporal drug transport in the F-UI, the imageable or visible tracer(s) distribute at the frozen outer rim with similar arc-like pattern (Fig. 1b, 2b, 3L) in both studies. Although the freezing-assisted injection duration is 8 times longer, and the average frozen mass much smaller in VX2 compared to BRCA, the frozen margin regardless of its size is the drug driver; drug transport within unfrozen interstitial fluid pathways follows the pressure gradient created between needle tip and peritumoral environment. Remarkably, the tracer distribution pattern along the VX2 tumor did not change from the first to the fifth injection. We inferred that the first freeze dose, similar in both studies, draws the directional transport of the tracer that will remain unchanged with additional dose of either. Additionally this pattern was predictable from in vitro, ex vivo, and experimental observations of free drug and/or drug-carrying devices, injected and transported along frozen tumor margin [28, 29]. We found that by injecting the F-UI, either located over tumor margin level for VX2 tumor or progressing towards normal breast parenchyma for BRCA, the drug permeated more widely the latter than the former tumor margin. The extent of tracer radial spread could be attributable to a crystallization of the drug aqueous solution caught in the ice –water phase (slushy ice), at the advancing ice rim location [28]. The injection pressure gradient, a function of the injection rate, and tissue compliance, along with the frozen rim likely contributed to bulk flow in tumor margin and environment. As a result, tracer transported outwardly during freezing in both studies as shown in Fig. 1b, 2b. Although, the no-freeze injection, ITCH series, in the VX2 margin demonstrated an initial tracer distribution pattern resembling the freeze-assisted pattern, the ensuing flow was toward tumor core without accumulation in the margin. This finding suggests that density of interstitial fluid paths of least resistance is higher towards tumor necrotic core than at tumor-muscular interface, thus facilitating the inward direction of the convective flow.

Table 3 shows that tumor staining correlates with blue dye dose, not with injectate volume; for similar injection rate and injected fluid volume, an equal stained/unstained ratio was observed on freshly resected samples of VX2 and BRCA tumor, although the averaged BRCA volume, 33.5cm3 was 8 times larger than VX2 tumor (Table 1). Such result was predictable. Indeed, blue dye guided localization of non-palpable breast tumor shows a direct relation between dose and stained tissue [43]. To evaluate the BD dose distributed in tumor target, we assumed that a part migrated in the tumor and margin, and the rest migrated in breast parenchyma, lymphatics, tumor-host interface, blood vessels; additional amount was lost to reflux through the surgical wound, the injection needle track, or the probe track.

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Based on the tumor stained volume, we assumed that 60 % to 70% of the TTM or BD dose transported along and away from the outer rim of the frozen VX2 or BRCA tumor, within open interstitial fluid channels [36]; a flow velocity and intensity related to the injection pressure gradient, the tumor-to-host interface compliance and hydraulic conductivity. Owing to injection technique, and rate (0.6 to 1.2ml/min), the fluid pressure at needle tip is very high, 200mmHg to 500 mmHg (P. Le Pivert, unpublished data). The interstitial fluid pressure (IFP) in experimental or human tumors (20 ± 13mmHg) [44] and in tumor–host interface (0 mmHg or negative pressure) is much lower; the injection results in a high velocity bulk flow. Such pressure gradient disperses the injected solution in fluid paths and spaces of lower resistance, i.e. tumor margin, tumor necrotic spaces, tumor host interface, and further away in host organ. Tumor draining blood and lymphatic vessels wash out the injectate that may also reflux through needle track.

Drug dose and freeze-mediated injection technique proved efficient at mapping lymphatic drainage of BRCA patients. Indeed, one to twelve nodes of the axillary region stained blue (Table 4) in 22 of 26 cases (84.6%). An identification rate that compares favorably with conventional BD guided lymphatic mapping [37]. Although the VX2 study did not investigate the tracer transport to lymphatic drainage, its dispersion along frozen margin and tumor- muscular interface resulted in its interstitial drainage. Mapping BRCA lymphatic drainage by delivering a bolus dose of BD tracer in a single deep side of frozen tumor margin raises some questions: was the selected injection side facing the axilla optimal for transporting tracer preferentially towards axilla? Was the pressure-mediated bulk flow away from the frozen tumor margin the preeminent factor in the permeation of the lymphatics leading to axilla? The observation that tracer migrated (Table 4) from the deep aspect of tumor margin to a single contiguous quadrant, in 69% of case before reaching the axilla suggests lymphatic drainage directed towards the axilla rather than towards the internal mammary region [45]. Indeed, 15/26 patients had tumor located outside the upper outer quadrant (table 1), in which we did not evidence any internal mammary lymphatic drainage.

Local drug delivery strategies have been investigated [46] over nearly five decades as a means to achieve high concentration of chemotherapeutics (CTx) , augment drug targeting of specific tumor structure, and decrease the side effect of systemic chemotherapy. Local delivery of CTx could sterilize resection margins, and possibly the tumor lymphatic drainage as well [40, 41]. Cryoablation has been combined with adjuvant CTx, systemic or local, to induce cryothermal lethal damages closer to the frozen margin [47], a way of better controlling and predicting the ablative effect. Physicians have designed and optimized cryo-assisted localization (CAL) and cryoablation (CA) to provide better margin clearance and cosmetic result, compared to lumpectomy, in small unifocal breast tumor[48]. Our VX2 preclinical study tests the feasibility and efficacy of intraoperative freeze-thaw assisted drug delivery and targeting of tumor margin, which differs from published combined adjuvant cryoablation-chemotherapies strategies [47]. Our drug delivery strategy consists in using, both tumor margin and ice rim as tunable gel-solid interfaces for accessing and controlling drug distribution over targeted tissue. The working hypothesis, based on

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previous in vitro and pre-clinical studies, is the transient drug entrapment at higher concentration in the peripheral region of the frozen tissue. Frozen VX2 tumor exhibit a solid impervious core and low compliant margin, made of slushy ice-water mixture, partially permeant to injectate that gets distributed along the solid tumor mass , and in the contiguous muscular tissue of Figure 3. The marginal drug targeting that lasts a few hours translates in moderate tissue kill; necrosis is about 20 % of the stained margin on histological samples, and matches with a gap in tumor rim of post-operative CECT imaging of figure 3. Whether this focal necrosis was the result of an additive or synergistic freeze-thaw and drug effect remain an open question. Our previous studies [29] suggest and additive effect; they demonstrated that slow release of low dose cytotoxic from microcapsule deposited in frozen margin of prostate tumor resulted in focal kill subsequent to combined cytotoxic effects of sublethal slush ice and drug injury. Our BRCA study replicated the VX2 protocol with a BD tracer only, to investigate its spread along frozen tumor margin. Remarkably, the peripheral spread was quite similar in both studies, i.e. ranging from 35% and 50% (Table 3). We infer that the nanosized tracers and drug , <10 micrometers, were trapped and released from the frozen-thawed tissues over successive period of time corresponding to the initial peripheral freezing-and-bulk flow sequence and the delayed more central tissue thaw and drug trapping sequence. Due to their small size, the molecules migrate quickly from the point of delivery, which explains the fast rate of staining, < 10 minutes, observed in the draining lymphatics during the operation. Given that about two third of the dose is lost to drainage, reflux or dilution in wound or probe track without affecting the peripheral spread, we think that drug targeting of the entire tumor periphery would be achieved with 0.1 to 0.3ml of 1% solution of MB injected in the deep aspect of three tumor sides. A dosage published by Tang et Al [43] for BD guided cryolocalization of non-palpable breast tumor.

Another implication of this study is in the actualization of local targeted chemotherapy of solid tumor by delivering the drug(s) in tumor margin. In both studies, the finding that the injected solution rapidly permeated tumor core from its marginal location, in the no-freeze ITCH group of VX2 study or, in both studies during the thaw period, suggests the presence of fluid channels bridging inner tumor and exterior tumor environment during tumor growth [36]. Thus, tumor margin seems an optimal region for direct delivery of diagnostic or therapeutic molecules. The transient obstruction of margin fluid channels during freezing, and their re-opening during melting, makes the freeze-thaw process an on-off switch for tumor-host fluid communication pathways, the interstitial fluid channels. In short, the freeze-thaw process is a tunable directional helper of tumor margin permeation to small molecules[31]. The tracer entrapment within tumor core during and after the thaw process as seen in the VX2 study suggests the use of Freeze-thaw assisted injection of active drug not only during the resection but also in the resection cavity margins. The local injection of cytotoxic would help priming residual tumor cells to adjuvant systemic therapies [49, 50], and/or radiotherapy[6].

The clinical validation of our tumor fluid management protocol during breast conserving surgery will require additional steps. Although we could not demonstrate, whether and to which degree, the resection of a frozen breast tumor decreases or prevents cell shedding from tumor manipulation, our

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cryosurgical technique intended to kill as many cells as possible [51-54]. Given that the freeze-thaw process intervening during cryoablation or cryoresection induces tumor necrosis and apoptosis, which may modulate antitumor immunity, our protocol could reinforce the latter with local cytotoxic and/or immuno-modulator [54-57]. Our next research step will investigate the wound fluids biological and cellular composition, and will seek to optimize the cryothermal energy dosing and blue dye delivery to target the whole tumor periphery.

We are aware of some limitations of this study. The short VX2 and BRCA series forbade any quantitative data evaluation. Another limitation may involve the different biomechanical characteristics of the tissue hosting tumor, the back muscle for VX2 and the breast fibro-glandular and fatty tissue for BRCA. The fluid drainage through interstitium may differ for both tissues particularly during TTM bulk flow in VX2 tumor margin in contact with a stiff muscular tissue. We could have implanted VX2 tumor in breast and examined the peritumoral lymphatic capillaries [59]. We thought that our protocol was easier to implement for demonstrating the feasibility of the marginal targeting and its translatability to BRC.

Conclusions

This study confirms that intraoperative blue dye-guided lymphatic mapping with a single deep tracer injection in the margin of frozen breast tumor is feasible. The frozen-thawed tumor-host interface region behaves as an impervious and trapping zone for small molecules. The targeted cryothermal-mediated drug delivery developed during cryoablation of VX2 tumor implant translates in successful margin and lymphatic targeting during cryo-assisted resection of breast tumor. The widespread technique of blue dye-guided lymphatic mapping during breast cancer surgery could benefit from simultaneous tumor margin freezing, and serve as a platform to designing new locoregional breast tumor-containment therapy strategy.

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Author's contribution

PLP conceived the study protocol. PLP, NK, conceived and designed the protocol application to breast cancer study. PLP, XH, and YX conceived and designed the protocol application to VX2 study. XH conducted the VX2 experiments, gathered, and analysed data. NK, OD, conducted the surgery and gathered breast cancer data. PLP compiled and analysed all data, and wrote manuscript. All authors critically revised manuscript, and gave consent for publication.

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Conflict of Interest statement

All authors declare absence of conflict of interest with respect to the research, authorship, and/or publication of this article

Published research connected to the study

This study uses some raw data from two papers originally published in TCRT, http://doi.org/10.1177/1533034615593855, and http://doi.org/10.1177/1533034617746294

References

1. Jemal A, Bray F, Center MM, et al. Global cancer statistics. CA Cancer J Clin. 2011; 61(2):69–90. [PubMed]).

2. Vaidya JS, Wenz F, Bulsara M, et al. Risk-adapted targeted intraoperative radiotherapy versus whole-breast radiotherapy for breast cancer: 5-year results for local control and overall survival from the TARGIT-A randomised trial. *Lancet (London, England)*. 2014;383(9917):603-613. doi:10.1016/S0140-6736(13)61950-9

3. Imkampe A, Bendall S, Bates T. The significance of the site of recurrence to subsequent breast cancer survival. *Eur J Surg Oncol*. 2007;33(4):420-423. doi:10.1016/j.ejso.2006.09.005

4. Aliperti LA, Predina JD, Vachani A, Singhal S. Local and systemic recurrence is the Achilles heel of cancer surgery. *Ann Surg Oncol*. 2011;18(3):603-607. doi:10.1245/s10434-010-1442-0

5. Chagpar AB, Killelea BK, Tsangaris TN, et al. A Randomized, Controlled Trial of Cavity Shave Margins in Breast Cancer. *N Engl J Med*. 2015;373(6):503-510. doi:10.1056/NEJMoa1504473

6. Sabel MS. Locoregional therapy of breast cancer: maximizing control, minimizing morbidity. *Expert Rev Anticancer Ther*. 2006;6(9):1281-1299. doi:10.1586/14737140.6.9.1281

7. Offersen B V., Overgaard M, Kroman N, Overgaard J. Accelerated partial breast irradiation as part of breast conserving therapy of early breast carcinoma: A systematic review. *Radiother Oncol*. 2009;90(1):1-13. doi:10.1016/j.radonc.2008.08.005

8. Holland R, Veling SH, Mravunac M, Hendriks JH. Histologic multifocality of Tis, T1-2 breast carcinomas. Implications for clinical trials of breast-conserving surgery. *Cancer*. 1985;56(5):979-990. http://www.ncbi.nlm.nih.gov/pubmed/2990668.

9. Hickey BE, Lehman M, Francis DP. Partial breast irradiation for early breast cancer (Review) Summary of Findings for the Main Comparison. 2016;(7). doi:10.1002/14651858.CD007077.pub3.www.cochranelibrary.com

10. Pachmann K. Tumor Cell Seeding During Surgery—Possible Contribution to Metastasis Formations. *Cancers (Basel)*. 2011;3(4):2540-2553. doi:10.3390/cancers3022540

11. Choy A, McCulloch P. Induction of tumour cell shedding into effluent venous blood breast cancer surgery. *Br J Cancer*. 1996;73(1):79-82. http://www.ncbi.nlm.nih.gov/pubmed/8554988.

12. Ramolu L, Christ D, Abecassis J, Rodier JF. Stimulation of breast cancer cell lines by post-surgical drainage fluids. *Anticancer Res*. 2014;34(7):3489-3492.

13. Harless WW. Revisiting perioperative chemotherapy: the critical importance of targeting residual cancer prior to wound healing. *BMC Cancer*. 2009;9:118. doi:10.1186/1471-2407-9-118

14. van der Hage JA, van de Velde CJ, Julien JP, et al. Improved survival after one course of perioperative chemotherapy in early breast cancer patients. long-term results from the European Organization for Research and Treatment of Cancer (EORTC) Trial 10854. *Eur J Cancer*. 2001;37(17):2184-2193. http://www.ncbi.nlm.nih.gov/pubmed/11677105.

15. Goldberg EP, Hadba AR, Almond B , Marotta JS. Intratumoral cancer chemotherapy and immunotherapy: opportunities for nonsystemic preoperative drug delivery. *J Pharm Pharmacol*. 2002;54(2):159-180. doi:10.1211/0022357021778268

16. Chen, JX. Pan, M. Li J. Clinical observation of intraoperative local chemotherapy with lobaplatin in breast cancer modified radical mastectomy. Int J Clin Med 2017;10(7):10834-10839

17. McCahill LE, Single RM, Aiello Bowles EJ, et al. Variability in reexcision following breast conservation surgery. *JAMA - J Am Med Assoc*. 2012;307(5):467-475. doi:10.1001/jama.2012.43

18. Le Pivert PJ, Le Petit JC, Fraisse J, Cuiilleret J, Brizard CP. Controlled cryotumorectomy of breast cancers. Technical and theoretical aspects. in “Les colloques de l’INSERM, Cryoimmunologie/Cryoimmunology”. 1976, 62, 267-278.

19. Le Pivert P. Basic considerations of the cryolesion, in: Ablin RJ, ed. Handbook of cryosurgery. New York, NY: Marcel Dekker, 1980: 15-68.

20. Camara O, Kavallaris A, Nöschel H, Rengsberger M, Jörke C, Pachmann K. Seeding of epithelial cells into circulation during surgery for breast cancer: the fate of malignant and benign mobilized cells. *World J Surg Oncol*. 2006;4(1):67. doi:10.1186/1477-7819-4-67

21. Tarkowski R, Rzaca M. Cryosurgery in the treatment of women with breast cancer-a review. *Gland Surg*. 2014;3(2):88-93. doi:10.3978/j.issn.2227-684X.2014.03.04

22. Teo KY, Dutton JC, Han B. Spatiotemporal Measurement of Freezing-Induced Deformation of Engineered Tissues. *J Biomech Eng*. 2010;132(3):031003. doi:10.1115/1.4000875

23. Han B, Miller JD, Jung JK. Freezing-induced fluid-matrix interaction in poroelastic material. *J Biomech Eng*. 2009;131(2):021002. doi:10.1115/1.3005170

24. Bumsoo Han, Ka Yaw Teo. Effects of freezing on intratumoral drug transport. In: *2009 Annual International Conference of the IEEE Engineering in Medicine and Biology Society*. Vol 2009. IEEE; 2009:246-249. doi:10.1109/IEMBS.2009.5333804

25. Wright J, Han B, Chuong C-J. Biphasic Investigation of Tissue Mechanical Response During Freezing Front Propagation. *J Biomech Eng*. 2012;134(6):061005. doi:10.1115/1.4006682

26. Wiig H, Tenstad O, Iversen PO, Kalluri R, Bjerkvig R. Interstitial fluid: the overlooked component of the tumor microenvironment? *Fibrogenesis Tissue Repair*. 2010;3(1):12. doi:10.1186/1755-1536-3-12

27. Hoogenboezem EN, Duvall CL. Harnessing albumin as a carrier for cancer therapies. *Adv Drug Deliv Rev*. 2018;130:73-89. doi:10.1016/j.addr.2018.07.011

28. Le Pivert, PJ. Translational Cryosurgery in Lung Tumor Therapy, Cryoimmunotherapy , Cryochemotherapy , Nanocryosurgery : basics and applications. In "CRYOSURGERY FOR LUNG CANCER" Hongwu Wang, Kecheng Xu, Peter Littrup (Eds); Springer Wien, New York, (2012), Chapter 1.6, 1–38. http://doi.org/10.13140/RG.2.1.5114.2644

29. Le Pivert PJ, Morrison DR, Haddad RS, et al. Percutaneous tumor ablation: microencapsulated echo-guided interstitial chemotherapy combined with cryosurgery increases necrosis in prostate cancer. *Technol Cancer Res Treat*. 2009;8(3):207-216. doi:10.1177/153303460900800305

30. He X, Xiao Y, Zhang X, et al. Percutaneous Tumor Ablation: Cryoablation Facilitates Targeting of Free Epirubicin-Ethanol-Ioversol Solution Interstitially Coinjected in a Rabbit VX2 Tumor Model. *Technol Cancer Res Treat*. 2016;15(4):597-608. doi:10.1177/1533034615593855

31. Rzechonek A , Blasiak P, et al. The Bi-directional Migration of a Dye Tracer Injected at the Edge of Primary or Secondary Lung Tumors During Surgery. Initial Study on 33 Patients and Clinical Implications. *Inter Collegas*,2017; *4*(3), 106–119. https://inter.knmu.edu.ua/?journal=pub&page=article&op=viewFile&path%5B%5D=177&path%5B%5D=179

32. Korpan NN, Xu K, Schwarzinger P, Watanabe M, Breitenecker G, Le Pivert P. Cryo-Assisted Resection En Bloc, and Cryoablation In Situ, of Primary Breast Cancer Coupled With Intraoperative Ultrasound-Guided Tracer Injection: A Preliminary Clinical Study. *Technol Cancer Res Treat*. 2018;17:1533034617746294. doi:10.1177/1533034617746294

33. Tsopelas C, Sutton R, Bs M. Why Certain Dyes Are Useful for Localizing the Sentinel Lymph Node. *J Nucl Med*. 2002;43:1377-1382. http://jnm.snmjournals.org/content/43/10/1377.

34. Alarcón E, Edwards AM, Aspee A, et al. Photophysics and photochemistry of dyes bound to human serum albumin are determined by the dyelocalization. *Photochem Photobiol Sci*. 2010;9(1):93-102. doi:10.1039/B9PP00091G

35. Liu Y, Ren W, Liu C, et al. Contrast-enhanced ultrasonography of the rabbit VX2 tumor model: Analysis of vascular pathology, Oncology Letters, 2012; 4: 685-690. https://doi.org/10.3892/ol.2012.819

36. Gritsenko PG, Ilina O, Friedl P. Interstitial guidance of cancer invasion. *Journal of Pathology*, 2012;*226*(2): 185–199. http://doi.org/10.1002/path.3031

37. Li J, Chen X, Qi M, Li Y. Sentinel lymph node biopsy mapped with methylene blue dye alone in patients with breast cancer: A systematic review and meta-analysis. Metze K, ed. *PLoS One*. 2018;13(9):e0204364. doi:10.1371/journal.pone.0204364

38. Baust JG, Gage AA, Bjerklund Johansen TE, Baust JM. Mechanisms of cryoablation: clinical consequences on malignant tumors. *Cryobiology*. 2014;68(1):1-11. doi:10.1016/j.cryobiol.2013.11.001

39. Ehrhart N, Dernell WS, Ehrhart EJ, Hutchison JM, Douple EB, Brekke JH, Straw RC and Withrow SJ: Effects of a controlled- release cisplatin delivery system used after resection of mammary carcinoma in mice. Am J Vet Res, 1999; 60(11): 1347-1351.

40. Chen WX, Pan M, Li J, Zhao JH, Zhou JW, Tang JH. Clinical observation of intraoperative local chemotherapy with lobaplatin in breast cancer modified radical mastectomy. *Int J Clin Exp Med*. 2017;10(7):10834-10839.

41. Baitchev G, Gorchev G, Deliisky T, Popovska S, Raitcheva TZ. Perioperative locoregional application of mitoxantrone in patients with early breast carcinoma. *J Chemother*. 2001;13(4):440-443. doi:10.1179/joc.2001.13.4.440

42. Cody HS 3rd, Van Zee KJ. Reexcision—The Other Breast Cancer Epidemic. N Engl J Med 2015; 373: 568–569. pmid:26244311

43. Tang J, Wang X, Wu YP, et al. Significance of methylene blue dye for localization biopsy of nonpalpable breast lesions. *Chin J Cancer*. 2009;28(1):79-81. http://www.cjcsysu.cn/cn/article.asp?id=14395

44. Less JR, Posner MC, Boucher Y, Borochovitz D, Wolmark N, Jain RK. Interstitial hypertension in human breast and colorectal tumors. *Cancer Res*. 1992;52(22):6371-6374. http://www.ncbi.nlm.nih.gov/pubmed/1423283.

45. Kong AL, Tereffe W, Hunt KK, et al. Impact of internal mammary lymph node drainage identified by preoperative lymphoscintigraphy on outcomes in patients with stage I to III breast cancer. *Cancer*. 2012;118(24):6287–6296. doi:10.1002/cncr.27564

46. Wolinsky JB, Colson YL, Grinstaff MW. Local drug delivery strategies for cancer treatment: gels, nanoparticles, polymeric films, rods, and wafers. *J Control Release*. 2012;159(1):14-26. doi:10.1016/j.jconrel.2011.11.031.

47. Goel R, Anderson K, Slaton J, et al. Adjuvant approaches to enhance cryosurgery. *J Biomech Eng*. 2009;131(7):074003. doi:10.1115/1.3156804

48. Ananthakrishnan P, Balci FL, Crowe JP. Optimizing surgical margins in breast conservation. Int J Surg Oncol. 2012;2012:585670. doi:10.1155/2012/585670.

49. Wang J, Lu Z, Gao Y, Wientjes MG, Au JL. Improving delivery and efficacy of nanomedicines in solid tumors: role of tumor priming. Nanomedicine (Lond). 2011;6(9):1605–1620. doi:10.2217/nnm.11.141).

50. Hohenforst-Schmidt W, Zarogoulidis P, Darwiche K, et al. Intratumoral chemotherapy for lung cancer: re-challenge current targeted therapies. Drug Des Devel Ther. 2013;7:571-583. doi:10.2147/DDDT.S46393

51. Korpan NN. Cryosurgery: Ultrastructural Changes in Pancreas Tissue after Low Temperature Exposure. Technol Cancer Res Treat 2007; 6:59-67. PubMed

52. Korpan NN. Cryosurgery: Early Ultrastructural Changes in Liver Tissue in vivo. J Surg Res 2009; 1:54-65. PubMed

53. Korpan NN. Cryoscience and Cryomedicine: Deciphering of the Effect of Deep Low Temperatures on Living Matter. Low Temp Med, 2010; 36(3):76-83

54. Korpan NN, Basics of Cryosurgery. Vienna: Springer, 2001.

55. Haen SP, Pereira PL, Salih HR, Rammensee HG, Gouttefangeas C. More than just tumor destruction: Immunomodulation by thermal ablation of cancer. Clin Dev Immunol. 2011;2011. doi:10.1155/2011/160250 1.

56. Slovak R, Ludwig JM, Gettinger SN, Herbst RS, Kim HS. Immuno-thermal ablations – boosting the anticancer immune response. 2017:1-15. doi:10.1186/s40425-017-0284-8

57. Apetoh L, Ladoire S, Coukos G, Ghiringhelli F. Combining immunotherapy and anticancer agents: The right path to achieve cancer cure? Ann Oncol. 2015;26(9):1813-1823. doi:10.1093/annonc/mdv209

58. Nelson D, Fisher S, Robinson B. The “Trojan Horse” Approach to Tumor Immunotherapy: Targeting the Tumor Microenvironment. J Immunol Res. 2014;2014:1-14. doi:10.1155/2014/789069

59. Li J, Yang X-J, Hu L-N, Sun C-X, Yao C-G. Impacts of steep pulsed electric fields on lymphatic capillaries in VX2 implanted breast cancer in rabbits. *Ai zheng Chinese J cancer*. 2006;25(2):159-162. http://www.ncbi.nlm.nih.gov/pubmed/16480578.