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Cryo-assisted resection of primary breast cancer en bloc and tumor cryoablation connected with local drug delivery and targeting of tumor fluids. Experimental and clinical studies

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Objective — to use cryosurgery in combination with simultaneous peritumoral and intratumoral tracer injections of blue dye for further lymphatic mapping in the treatment of primary breast tumors. The effectiveness of intraoperative cryoprobe-assisted injection of blue dye and cytotoxic-tracer mixture for locoregional drug targeting in the VX2 tumor model as well as its translational significance for cryo-assisted breast tumor surgery with blue dye alone were evaluated. Sentinel lymph node mapping, pathological determination of the tumor, and resection margins were achievable.

Materials and methods. Thirty-nine patients with primary breast cancer in stages I to IV, aged 52.4 (±19) years (mean, standard deviation (SD) years), were randomly selected, treated at the Rudolfinerhaus Private Clinic in Vienna, Austria, and included in this preliminary clinical study. Under computed tomography guidance, we injected 2 ml of cytotoxic-tracer mixture in five aliquots into the margins of 16 frozen or normothermic VX2 tumors. We evaluated the intraoperative and post-operative drug targeting and therapeutic efficacy at the tumor-host interface by means of computer tomography, gross examination, and histopathology. In thirty-four T1 to T4 primary breast cancers, we performed an ultrasound-guided cryoprobe-assisted tumor freezing-thawing cycle, blue dye-guided lymphatic mapping, and surgery. We examined an intraoperative and freshly resected specimen and the blue dye distribution pattern in the tumor-host interface, lymph node(s), breast parenchyma, and resection cavity.

Results. 29 of the 38 patients had localized primary breast cancer, which was estimated to be resectable without neoadjuvant chemotherapy. 87 % of patients had one to twelve stained axillary lymph nodes, while 72 % of patients had another quadrant and resection cavity stained. Fluid-impervious frozen VX2 or breast tumors transported drug(s) in an arc-like pattern at the tumor-host interface regardless of freeze dose, number of freeze-thaw cycles, drug dose fractionation, tumor characteristics, or tumor dimensions. During melting, the cytotoxic-tracer mixture spread within 50 % of the VX2 tumor and mirrored that of the tumor-host interface; it was massive in normothermia. In VX2, the CT gap corresponded to 20 % of the focal margin necrosis in pathology. In both studies, blue dye dose-staining spread linearly in the tumor-host interface and tumor.

Conclusions. The study paves the way for intraoperative cryo-assisted cure options for primary breast cancer. We have shown that our cryosurgical technique of repeatedly freezing deep tumors for en bloc resection or for *in situ* ablation of primary breast cancer, facilitated by IOUS monitoring, can be coupled with the simultaneous injection of dye tracers during conventional surgery, which then allows for lymphatic mapping. Intraoperative freezing-assisted drug delivery and targeting techniques during cryoablation of the VX2 tumor translate successfully to locoregional blue dye targeting and lymphatic mapping during cryo-assisted surgery of breast cancer. We explored the ability of our strategy to prevent tumor cell migration, but not that of injected tracers, to the lymphovascular drainage during conventional resection of frozen breast malignancies.

Keywords
Experiment, VX2 tumor, clinical study, primary breast cancer, cryo-assisted tumor resection, cryoablation, intratumoral tracer injection, lymphatic mapping.

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Cryoablation for primary and secondary breast cancer is a well-known procedure [1, 2]. Surgical resection of a frozen tumor has also been previously proposed for primary and secondary breast cancer [3, 4].

Local control of disease and breast cancer–specific survival rates are related; strategies that reduce the rates of local recurrence at 5 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, resection cavity shaving (RCS), partial breast irradiation (PBI), or photodynamic therapy (PDT) are common procedures [4, 5]. During breast conserving surgery, 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 cavity shaving [5] or 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 a selected series of early breast cancer patients. However, PBI and APBI are still controversial in the medical community [9] and are not wildly available in most health-care settings. 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 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] has been used to prevent surgery-induced dissemination or tumor growth. Local washing and multiple CTx platinum agent injections in the resection cavity, breast and axillary region during modified radical mastectomy were shown to be safe and effective at decreasing exfoliated tumor cells and potentially improving the 3-year disease-free survival rate [16].

There is room for intraoperative loco-regional adjuvant therapies during breast conserving surgery 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 reduce the 20 % to 40 % positive margin and reoperation rates after partial mastectomy [17], while still allowing conventional adjuvant RT, CTx, endocrine therapy, or targeted therapy. Such a strategy is based on the cryothermal handling and containment of the target tumor [18, 19] and the simultaneous local injection of cytotoxic drugs 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 interface [22—26] (F-UI) during the tissue freeze-thaw process have considerable potential for the transport of drugs. Indeed, the freezing-extruded tumor fluids contain concentrated tumor metabolic byproducts and debris [24—25]; a «soup» that transiently settles and accumulates in the unfrozen hypothermal region surrounding the frozen mass during a cryosurgical freeze-thaw cycle. Some soup molecules, such as albumin, are natural carriers for drugs [27], including patent blue V (PBV). Thus, we used the unfrozen hypothermal region transport 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. In two recent human studies, [30, 31] we have also evaluated the freezing-induced transport and distribution of blue dye tracers, methylene blue or PBV, which are known for their ability to map the breast lymphatic drainage or bind to albumin [33, 34].

In this work, we evaluated 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 described 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 on twenty-six T1-T4 resectable primary breast tumors with 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 the postoperative evaluation of the resected specimen to assess the circumferential distribution of the dye at the tumor-host interface.

Finally, we compared the experimental and clinical data and discussed 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) injected locally in a single site of VX2 tumor margin, in a normothermic or cryoablated tumor (CA). The conservative cryothermal dosing consisted in maintaining the frozen-unfrozen interface (F-UI) on tumor gross margin during five repeat freeze-TTM injection-and-thaw cycles (FIT). Each FIT cycle was repeated 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 in breast cancer patients while adjusting the technical parameters to two IO clinical requirements: map the lymphatics and achieve a conventional resection of a frozen tumor, i.e., cryo-assisted (CR) breast conserving surgery or radical mastectomy, without undue prolongation of general anesthesia. We injected in the F-UI the same blue dye (BD) dose as in the VX2 study, regardless of the tumor volume. The F-UI had to overlap the tumor margin and about 10 mm of normal tissue before injection. The injection needle was always inserted into the 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 the excised specimen, host tissue, and breast lymphatics at gross examination. The image-guided combinatorial local treatment of a peripheral macroscopic residual tumor burden was modelled as part of the VX2 study.

The breast cancer (BRCA) study involved the BD tracer as a surrogate for a small cytotoxic molecule; it assessed intraoperatively (IO) its marginal circumferential and radial spread and pattern as related to the injected dose or cryothermal dose. The VX2 TTM tested the possible therapeutic effect of low-dose epirubicin [29] aliquots in combination with repeat freeze-thaw cycles (CACH) compared to a normothermic tumor (ITCH).

Experimental Study: VX2

The current study, which was previously published in part, [30] can be summarized (Table 1) as follows: bilaterally implanted VX2 tumors develop into 4 milliliter (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 = 28), on the interstitial distribution and marginal targeting of the TTM. Observation data were compared to the injection-alone procedure ITCH (n = 11) in a normothermic tumor. The

Table 1. **Technical and imaging parameters (adapted in part from ref. 32)**

Study	Approach technique	Tumor volume, mm^3	Number of FT cycles	Injection type, volume, BD (dose)	Tumor F dose	Injection timing, frequency	Tracer D imaging
VX2	PC (n = 0) CACH (n = 28) ITCH (n = 11)	4 ± 0.5 (3.6—4.5)	5	TTM, 2 ± 0.2 mL (1.5 mg/mL)	Up to Tm 0 mm	End F, one per FT cycle	IO-CT CECT
BRCA	Open CR (n = 39)	33.5 cm (0.8—158)*	2	BD 2 (10 mg/mL)	Tm positive 0—10 mm	End F, 1st F	IO-US visualization

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/IO-US, intraoperative computed tomography/ultrasonography; CECT, contrast enhanced CT; D, distribution; Vis, visualization

VX2: Five TTM aliquots were injected at a slow flow rate (ca 0.9 mL/min) up to a 2 mL total dose. Each injection (ITCH) or freeze-injection (CACH) sequence was repeated every 3 to 5 minutes. The percutaneous (PC) FT, CT, and CECT-guided procedures contain the frozen margin at tumor margin level. At each time point, two animals were euthanized for tumor specimen gross examination and histopathology.

BRCA: A bolus of 2 mL of the BD dose was 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 10 mm in normal breast tissue; such a 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 average maximal diameter of a freshly resected and bisected tumor was 4 cm.

Table 2. **Patient clinicopathological characteristics (UICC TNM Classification, 8th ed., 2016) (n = 39)**

Characteristic	Number of patience
Stage	
I	8
T_1	8
N_0/N_1	7/1
M_0	8
II	17
T_2	17
$N_0/N_1/N_2$	14/2/1
M_0	17
III	8
T_3	8
$N_2/N_4/N_6$	5/2/1
M_0/M_1	5/3
IV	6
T_4	6
N_{12}/N_{14}	5/1
M_1	6
Pathology	
Invasive ductal carcinoma of no special type	29
Invasive lobular carcinoma	8
Mixed	2
Unifocal	28
Multifocal	7
ulMulticentric	4
Grade	
G1	0
G2	12
G3	27
Mammary location	
Right	28
Left	11
Upper outer quadrant	17
Lower outer quadrant	9
Upper inner quadrant	6
Lower inner quadrant	2
Nipple area	5

methylene blue 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 days 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 also includes methylene blue and ioversol.

Clinical Study: Primary breast cancer

The acute study, which was previously published in part [32], was conducted in a single center, The Rudolfinerhaus Private Clinic, in Vienna, Austria. Thirty-nine patients, aged from 21 to 74 years (mean — 52.4, SD — 19), presenting with primary breast tumors, stages I to III, or *de novo* stage IV, were randomLy selected and treated (Table 2). Tumor size: mean — 4 × 2.7 cm, SD — 3.1 × 2.1 cm. All patients gave oral or written informed consent. All but two patients received chemotherapy before surgery.

Following intraoperative tumor freezing with ultrasound-guided marginal injection of 2 mL of BD, conventional resection of the frozen mass and breast tissue, dubbed cryo-assisted resection en bloc (CR), was conducted [32]. The tumor margin of the resected specimen was marked with sutures for spatial orientation. The frozen tumor samples were subjected to tumor characterization and margin evaluation. Thirty-two patients were operated on with curative intent, including radical mastectomy in seven cases. In seven patients, palliative cryoablation was carried out. The staining pattern and distribution in the resected specimen, the breast parenchyma, and the resection cavity were measured and photographed. 28 patients had sentinel lymph node biopsy, and 11 patients underwent axillary lymph node dissection for lymph node staging, axillary exploration and lymph node clearance. In 4 patients, axillary lymph node dissection was followed by the examination of frozen sections (see Table 2).

Methodological convergences and divergences

The authors had no connection during the course of the research. The VX2 was a 10-day acute study whose results were completely available prior to developing the clinical protocol. We tailored the drug delivery technique to the clinical requirements and the preferences of the breast tumor surgeon, Dr. Mykola M. Korpan (see Table 1). Although the growth and invasion patterns of VX2 tumors [35] differ from those of breast tumors [36], the model was considered relevant for translation to human

breast tumors. The rationale was that the cooling-injection timing neutralizes the potential influence of tumor vascularization, capillary lymphatics, and tumor fluids, thus allowing comparative evaluation of the drug's interstitial flow and transport in unfrozen peritumoral tissue.

Study converging parameters were the drug injection intervening during the end of freezing (see Table 1), and needle positioning in the tumor margin. We used a single liquid nitrogen- powered probe, a single injection needle, tumor side, and a similar 2 mL injection volume. The latter matched known values for lymphatic mapping [37] in breast cancer, and the injection side was oriented toward the axillary region to facilitate 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 (see Table 1); and the location of the frozen margin relative to the 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 in diameter- contacted the surgically exposed tumor surface, which resulted in an ellipsoidal, asymmetric ice ball growing faster on the surface than in the depth. Finally, the five repeat FITs including partial thaw sequences (FIT), simulated a «waving» of the TTM dose at the frozen-unfrozen (F-UI) margin interface of the VX2 tumor. Given the unchanged tracer distribution pattern from the first to the fifth cycle, only two 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 paths 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 undercut only the superficial aspect of the 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 a normothermic tumor (ITCH). For BRCA, we used blue dye (BD) tracers as surrogates for small cytotoxic drugs. We injected patent blue 2.5 % (PBV) in 31 patients and methylene blue 1 % aqueous solution in 8 patients. All BRCA tumors received a single, similar dose on 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 dosing.

Table 3, Fig. 1, 2 show that the injectate, irrespective of its composition, spreads along the frozen tumor mass in an arc-like pattern. The injectate accumulates at 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 a fresh sample, the tumor margin stains blue over an average of 35 % to 50 % of the perimeter. In order to compare this spread, we have averaged the volume of BRCA tumors (see Table 1) and found that tumor staining is a linear function of the BD dose.

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

From the first to the fifth freezing sequence, the concurrent repeated injection of aliquots of contrast agent and BD tracer resulted in the same arc-like

Table 3. **Injectate dosing and distribution in tumor (adapted in part from ref. 32)**

Study	Vi/Vt	MB conc., mg/mL	Vd	Tm, %	Tc, %	Vd/Vi	V_{MB}/Vt, mg/mL	Tm Kill
Vx2 No F (n =18)	0.5	1.5	NA	10	80	0.9	0.8	None
Vx2 F (n =21)	0.5	1.5	Arc-like	50	8. 80	0.8	0.8	20 %
BRCA F (n =39)	0.06	10	Arc-like	35	3. 30	5.5	0.7	NA

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.

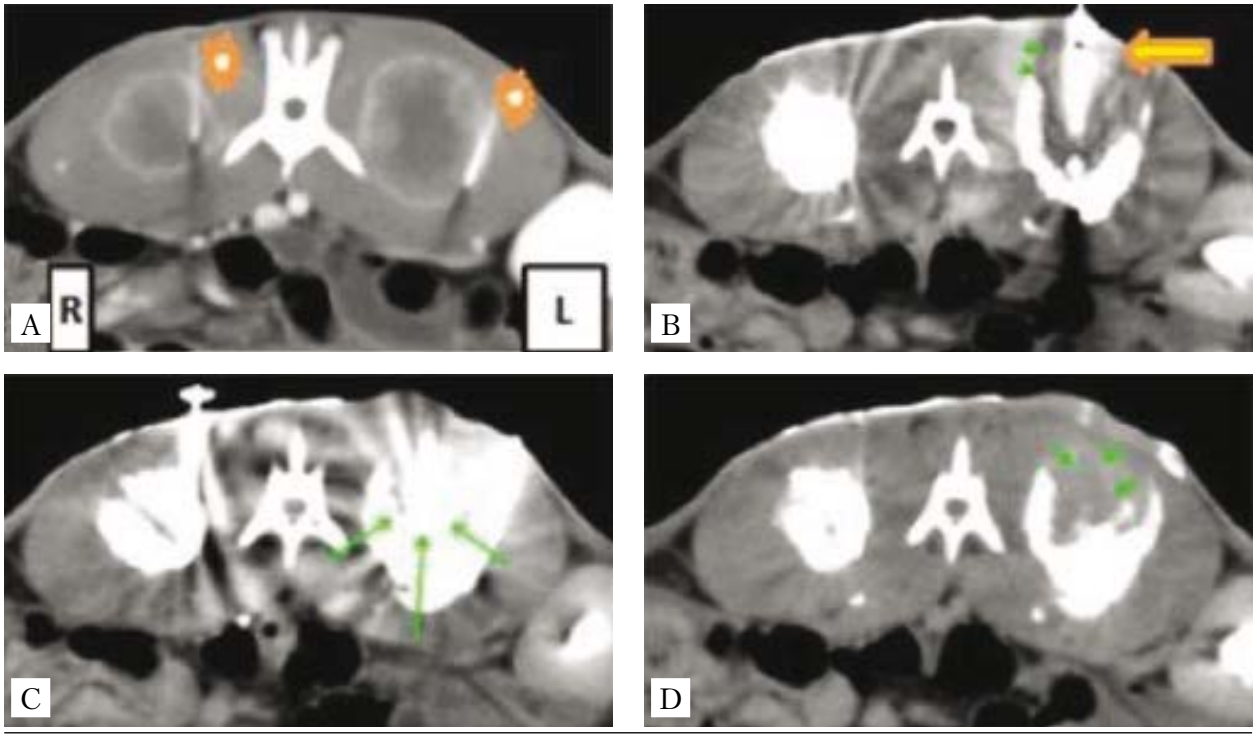

Figure 1. **CT-guided percutaneous injection, ITCH, (R) or cryoprobe-assisted injection, CACH, (L) of therapeutic mixture in the VX2 tumor margin (adapted from ref. 30). A. CECT guided needle (asterisk) positioning along enhancing tumor rim (white arrows); B. at end of first 0.4 mL injection sequence, 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 the 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. 20 minutes after the procedure, a larger amount of fluid and tracer, 50% of the injectate, leaks out of the left side tumor core (short green arrows) during probe and needle removal compared to the right side**

distribution pattern at the frozen-unfrozen interface of the VX2 tumor margin (see Fig. 1*B*). 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, the tumor margin was sequentially freezing and melting, which resulted in the transient co-accumulation of tumor extruded fluids and tracers during the repeat intensity-modulated freeze-thaw cycles. This was observed during the first freeze of a BRCA tumor, whose frozen margin was impervious to the BD but could engulf the tracer during its planned progression in normal breast tissue resulting in an arc-like marginal staining pattern that did not change during the re-freeze sequence (see Fig. 2*B*, 2*C*).

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

Whether steady (VX2) or advancing (BRCA), the frozen rim kept the co-injected drug tracers from permeating the 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 shape of the frozen rim, an effect that lasted throughout the freezing period if the injection needle tip was in the unfrozen region and before the ice margin. During tumor freezing, co-injected PBV or methylene blue tracers did not permeate the frozen core, regardless of their concentration. However, BD deposited before the advancing ice margin, as exemplified for BRCA, accumulated 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 Fig. 3*A* is narrow in comparison with the large coloration of breast parenchyma surrounding the tumor (see Fig. 2*C*). 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 margins became permeable to dye penetration towards the tumor core. Comparing the injection of a frozen and an unfrozen VX2 tumor, the injectate's penetration into the tumor was immediate in the latter

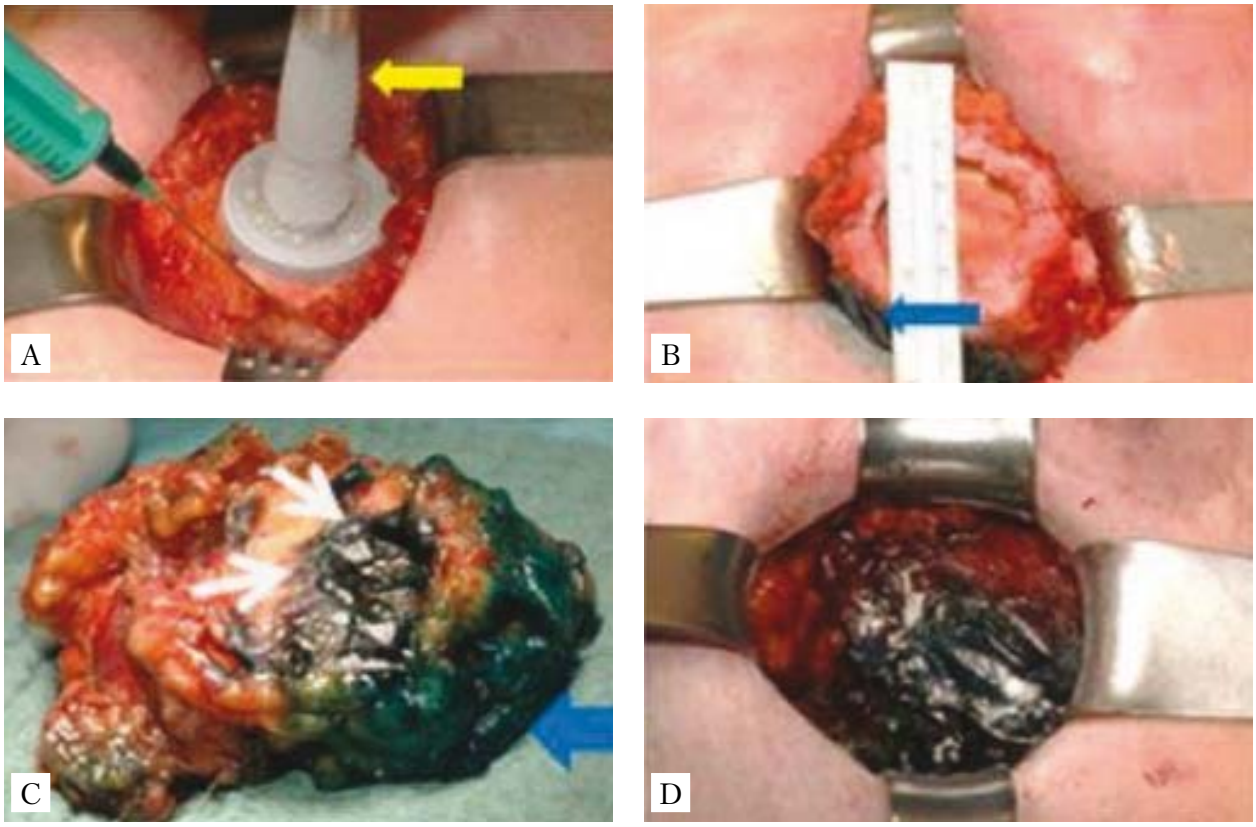

Figure 2. **Intraoperative ultrasound-guided cryo-assisted blue dye (BD) injection and en bloc resection of breast tumor $T_2N_0M_0$ (adapted from ref. 32). Patient D. J.; A. aspect of surgical wound following methylene blue injection in frozen tumor margin, during pull out of needle. The US transducer has been removed. The cylindrical liquid nitrogen cryoprobe (yellow arrow), 50mm diameter, makes a 89 cm^3 ellipsoidal ice zone in three minutes that engulfs the 5.45 cm^3 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 cm^3 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 sentinel lymph nodes were removed, non-metastatic at pathology. BD reflux in surgical wounds and in the needle track is minimal during freezing; we estimated that about 50 % of BD migrated to the contiguous breast**

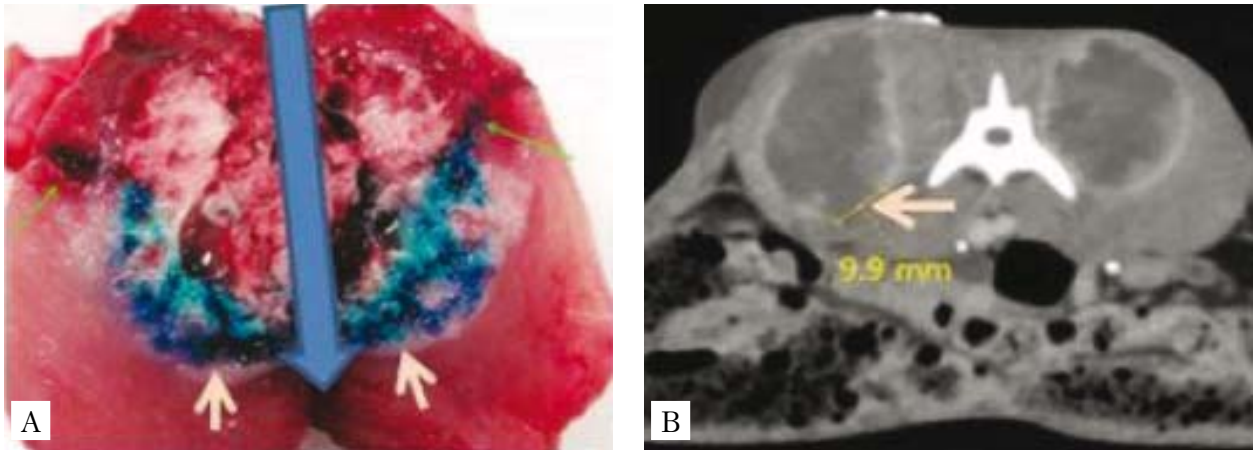

Figure 3. **VX2 tumor margin drug targeting and focal killing (adapted from ref. 32) A. One hour post CACH procedure, transected right side tumor along probe tract (blue arrow) evidences predominant BD staining of tumor margin-muscle interface (orange arrows). The injection needle track (green arrows) 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 on the left side. Histopathological focal margin necrosis coincides with the gap**

and delayed in the former. Although qualitative, the tracer uptake evaluation within the tumor looked similar for both groups when the melting process was complete. This observation holds true for frozen BRCA tumors; although blue dye was injected before the end of the freezing process, it penetrated the tumor core during the thawing sequence.

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 the tumor. From the frozen margin of the breast tumor, about 50 % of the BD dose was transported to the contiguous breast parenchyma and drained into the lymphatics. A little amount tended to reflux along the needle tract and superficial wound. The injectate reflux ratio along the needle track in the VX2 study ranged from 6 % to 17 %, with a frequency of 13 % to 50 % for the CACH vs. ITCH group. Following probe pullout, nearly 50 % of the injectate was lost through the probe track, along with tumor necrotic debris and fluids (see Fig. 1*D*). We did not compensate for this intraoperative reflux in either study. For 69 % of BRCA patients, BD injectate was transported to a single contiguous quadrant in 18 out of 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 averaged 20 minutes for BRCA, which did not prolong the anesthesia much and had no consequence on the patient's recovery or wound healing. As expected, starting during the intervention, the patient's urine stained blue. No allergies or durable skin staining were noted. Surgery was uneventful as determined by the preoperative plan, intraoperative lymphatic mapping, SLN, and tumor margin evaluation.

Drug dose distribution and effect

Tracer distribution volume was evaluated on a freshly resected tumor based on localization and percentage coverage of the target area. We assumed spheroid tumor geometry. In Table 3, we show that the injectate to tumor volume ratio (Vi/Vt) is one order of magnitude lower in BRCA (mean volume) compared to the VX2 tumor. After deduction of the estimated dose lost ~50 % (Vd/(Vi-50 %)) to migration in the breast or through the probe tract of the VX2 target, a quantifiable and quite similar estimate of tracer accumulation (Vmb/Vt) in the tumor margin (Tm) and tumor core (Tc) ranges respectively from 0.7 mg/mL to 0.8 mg/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 Fig. 2 illustrates the large frozen mass, or freeze dose, that engulfs the tumor and normal breast tissue. The latter is 15 times greater than tumor volume. It is a positive freeze margin, whose contours cover tumor margins along with normal tissue. The tumor freeze dose, estimated in percentages of frozen tumor and host tissue in Table 2, is 100 % for VX2 and > 100 % for BRCA. Assuming an ellipsoidal geometry for the frozen tissue, we have calculated a freeze dose of median 43.6 mL and range 24—177 mL, which means that all targeted BRCA tumors were properly frozen. The resection line is located in normal breast parenchyma, 3cm to 4cm off the palpable and visible frozen contours. The intraoperative selective re-resection rate (Table 4) for close, < 2 mm, or positive margin is 15 %. As expected, freshly excised VX2 tumors after conservative cryoablation exhibited viable tumor clusters in pathological samples of frozen margin [29].

Discussion

The lack of an effective, translatable strategy for the local delivery of cytotoxic drugs to breast tumors 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

Table 4. **Blue dye migration and surgical option in breast cancer patients (n = 39)**

Characteristic	Number of patience
Number of BD breast quadrants	
One	12 (69 %)
Two	12 (12 %)
Three	15 (19 %)
SLN identification rate	32 (84.6 %)
LN mts	9
Selective RCS	14 (15 %)
BCS	31
RM	15
Sentinel lymph node biopsy	39
Aaxillary lymph node dissection	18

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 (< 2 mm) was 15 %.
SLN, sentinel node; RCS, resection cavity shaving.

or APBI. Strategies that reduce the rates of local recurrence at 5 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 tumors to minimize tumor fluid leakage and maximize drug targeting of the tumor-host margin interface during breast conserving surgery.

We have previously demonstrated that a solution of a small molecular tracer, alone or co-formulated with a cytotoxic drug, migrates in tumor fluid dissemination pathways after being injected in the tumor margin. 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 cryoprobe-assisted surgery, i.e., cryoresection of a human breast tumor. There is no known study linking cryosurgical ablation and cryosurgical resection combined with local adjuvant therapy. In the present study, we extend our previous findings that freeze-thaw assisted local injection of active drug and ablation procedure of VX2 animal tumor are applicable to freeze-thaw assisted local tracer injection and breast conserving surgery in human patients.

The rationale for the translatability of this intraoperative cryothermal and drug-mediated therapy adjuvant to two seemingly opposite local curative procedures, 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 the tumor [38].

We hypothesized that, during freezing, the local transport of a co-injected drug along the frozen mass would be similar for a higshly 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 transport of tumor interstitial fluids at the frozen-unfrozen interface [22—25] during the freeze-thaw process have considerable potential 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 soup molecules, such as albumin, are natural carriers for drugs [27], including patent blue (PBV). Thus, the F-UI [22] region has considerable potential 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 a similar arc-like pattern (Fig. 1*B*, 2*B*, 3*A*) in both studies. Although the freezing-assisted injection duration is eight times longer and the average frozen mass is 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 the 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, which was similar in both studies, draws the directional transport of the tracer that will remain unchanged with an additional dose of either. Additionally, this pattern was predictable from *in vitro*, *ex vivo*, and experimental observations of free drugs and/or drug-carrying devices injected and transported along frozen tumor margins [28, 29]. We discovered that injecting the F-UI over the tumor margin level for the VX2 tumor or progressing towards normal breast parenchyma for the BRCA tumor resulted in the drug permeating more widely at the latter tumor margin than at the former tumor margin. The extent of tracer radial spread could be attributable to the 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 the tumor margin and environment.

As a result, tracer was transported outwardly during freezing in both studies, as shown in Fig. 1*B* and 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 the tumor core without accumulation in the margin. This finding suggests that the density of interstitial fluid paths of least resistance is higher towards the

tumor necrotic core than at the tumor-muscular interface, thus facilitating the inward direction of the convective flow.

Table 3 shows that tumor staining correlates with blue dye dose, not 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 tumors, although the averaged BRCA volume (33.5 cm^3) was eight times larger than that of the VX2 tumor (see Table 1). Such a result was predictable. Indeed, blue dye-guided localization of nonpalpable breast tumor shows a direct relation between dose and stained tissue [43]. To evaluate the BD dose distributed in the tumor target, we assumed that a part migrated in the tumor and margin, and the rest migrated in the breast parenchyma, lymphatics, tumor-host interface, and blood vessels; an additional amount was lost to reflux through the surgical wound, the injection needle track, or the probe track.

Based on the tumor-stained volume, we assumed that 60 % to 70 % of the TTM or BD dose was transported along and away from the outer rim of the frozen VX2 or BRCA tumor, within open interstitial fluid channels [36] with a flow velocity and intensity related to the injection pressure gradient, tumor-to-host interface compliance, and hydraulic conductivity. 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, the tumor-host interface, and further away in the host organ. Tumor-draining blood and lymphatic vessels wash out the injectate, which may also reflux through the needle track.

The drug dose and freeze-mediated injection technique proved efficient at mapping lymphatic drainage in BRCA patients. Indeed, one to twelve nodes of the axillary region stained blue (see Table 4) in 22 of 39 cases (84.6 %), a detection rate that is comparable to conventional BD-guided lymphatic mapping [37]. Although the VX2 study did not investigate the tracer's transport to lymphatic drainage, its dispersion along the frozen margin and tumor- muscular interface resulted in its interstitial drainage. Mapping BRCA lymphatic drainage by injecting a bolus dose of BD tracer into a single deep side of frozen tumor margin raises some questions: was the injection side chosen to face the axilla optimal for transporting tracer preferentially towards the 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 fact that the tracer (see Table 4) migrated from the deep aspect of the tumor margin to a single contiguous quadrant in 69 % of cases before reaching the axilla suggests that lymphatic drainage is directed towards the axilla rather than the internal mammary region [45]. Indeed, 25/39 patients had tumors located outside the upper outer quadrant (see Table 1), in which we did not evidence any internal mammary lymphatic drainage.

Local drug delivery strategies have been investigated [46] for nearly five decades as a means to achieve high concentrations of chemotherapeutics (CTx), augment drug targeting of specific tumor structure, and reduce the side effects 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 systemic or local adjuvant CTx to induce cryothermal lethal damages closer to the frozen margin [47], allowing for better control and prediction of the ablative effect. Physicians have designed and optimized cryo-assisted localization (CAL) and cryoablation (CA) to provide better margin clearance and cosmetic results compared to lumpectomy in small unifocal breast tumors [48].

Our VX2 preclinical study investigated the feasibility and efficacy of intraoperative freeze-thaw assisted drug delivery and tumor margin targeting, which differed from previously published combined adjuvant cryoablation-chemotherapy strategies [47]. Our drug delivery strategy consists in using both the tumor margin and the ice rim as tunable gel-solid interfaces for accessing and controlling drug distribution over targeted tissue. The working hypothesis, based on previous *in vitro* and pre-clinical studies, is that transient drug entrapment occurs at higher concentrations in the peripheral region of the frozen tissue. Frozen VX2 tumors exhibit a solid impervious core and low compliant margin, are made of a slushy ice-water mixture, are partially permeable to the 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 into moderate tissue kill; necrosis is about 20 % of the stained margin on histological samples, and matches with a gap in the tumor rim of post-operative CECT imaging (Figure 3). Whether this focal necrosis was the result of an additive or synergistic freeze-thaw and drug effect remains an open question. Our previous studies [29] suggest an additive effect; they demonstrated that the slow release of low-dose cytotoxic from microcapsules deposited in the frozen margin of the prostate tumor resulted in focal killing subsequent to the 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 the frozen tumor margin. Remarkably, the peripheral spread was quite similar in both studies, i.e., ranging from 35 % to 50 % (see Table 3). We infer that the nanosized tracers and drugs (< 10 micrometers) were trapped and released from the frozen-thawed tissues over successive periods 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 thirds of the dose is lost to drainage, reflux, or dilution in the 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 a 1 % solution of methylene blue 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 tumors.

Another implication of this study is the implementation of local targeted chemotherapy for solid tumors by delivering the drug(s) to the tumor margin. The finding that the injected solution rapidly permeated the tumor core from its marginal location, either in the no-freeze ITCH group of VX2 study or in both studies during the thaw period, suggests the presence of fluid channels bridging the inner tumor and exterior tumor environment during tumor growth [36]. Thus, the 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 make the freeze-thaw process an on-off switch for tumor-host fluid communication pathways, the interstitial fluid channels. In short, the freeze-thaw process aids tumor margin permeation to small molecules in a tunable directional manner [31]. The entrapment of a tracer within the tumor core during and after thawing, as observed in the VX2 study, suggests the use of freeze-thaw assisted injection of an active drug not only during resection but also in the resection cavity margins. Local cytotoxic injections would aid in priming residual tumor cells for adjuvant systemic therapies [49, 50], and/or radiotherapy [6].

The clinical validation of our tumor fluid management protocol during breast conservation surgery will require additional steps. Although we could not demonstrate whether and to what extent frozen breast tumor resection reduces or prevents cell shedding from tumor manipulation, our cryosurgical technique was designed to kill as many cells as possible [51—54]. Given that the freeze-thaw process that occurs during cryoablation or cryoresection induces tumor necrosis and apoptosis, both of which may modulate antitumor immunity, our protocol could supplement the latter with local cytotoxic and/or immuno-modulator agents [54—57]. Our next research step will investigate the biological and cellular composition of wound fluids, and seek to optimize the cryothermal energy dosing and blue dye delivery to target the whole tumor periphery.

We are aware of some of the study's limitations. The short VX2 and BRCA series forbade any quantitative data evaluation. Another limitation may involve different biomechanical characteristics of the tissue hosting the tumor: the back muscle for VX2 and the breast fibro-glandular and fatty tissue for BRCA. The fluid drainage through the interstitium may differ for both tissues, particularly during TTM bulk flow in the VX2 tumor margin in contact with stiff muscular tissue. We could have implanted the VX2 tumor in the breast and examined the peritumoral lymphatic capillaries [59]. We believed that our protocol would be easier to implement in order to demonstrate the feasibility of 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 a 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 VX2 tumor implant ablation translates into 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 for designing a new locoregional breast tumor-containment therapy strategy.

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Declaration of interests

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

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Published research connected to the study

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Authors contributions

M. M. Korpan, Yueyong Xiao, Xiaofeng He, O. I. Dronov created the study protocol; compiled and analyzed all data, wrote and critically revised the manuscript. M. M. Korpan, O. I. Dronov developed the protocol application to the breast cancer study; conducted the surgery and collected breast cancer data. Yueyong Xiao, Xiaofeng He designed the protocol application to the VX2 study. Xiaofeng He carried out the VX2 experiments, collected and analyzed data.

All authors the manuscript and gave consent for publication.

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Кріо-асистована резекція первинного раку молочної залози в один блок та кріоабляція пухлини в супроводі з місцевою доставкою ліків із прицілом на рідинний стан пухлини. Експериментально-клінічні дослідження

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Мета — лікування первинної пухлини молочної залози із застосуванням кріо в поєднанні з одночасним навколо- та внутрішньопухлинним введенням синього барвника для оцінки лімфатичного картування. Було визначено локальну регіонарну ефективність інтраопераційної ін'єкції синього барвника та суміші цитотоксичних індикаторів із застосуванням кріозонду в моделі пухлини VX2, а також її трансляційну цінність у кріохірургії пухлини молочної залози лише з використанням синього барвника. Картування сторожових лімфатичних вузлів, патологічне визначення пухлини та межі резекції були досяжними.

Матеріали та методи. Тридцять дев'ять пацієнтів віком (52,4 ± 19,0) року (середнє значення, стандартне відхилення) з первинним раком молочної залози I—IV стадій були рандомізовано відібрані та проліковані у приватній клініці Rudolfinerhaus у Відні, Австрія. Під контролем комп'ютерної томографії було введено 2 мл суміші цитотоксичних індикаторів у п'яти аліквотах на краю 16 заморожених або нормотермічних пухлин VX2. Було оцінено інтраопераційну та післяопераційну ефективність доставки препарату та його терапевтичну ефективність у первинній пухлині за допомогою комп'ютерної томографії, загального обстеження та патоморфологічного дослідження. Тридцяти чотирьом пацієнтам з первинними формами раку молочної залози від T1 до T4 було виконано цикл заморожування-розморожування пухлини за допомогою кріозонду під контролем ультразвуку, картування лімфи із застосуванням синього барвника та оперативне втручання. Досліджували резектований зразок, поширення синього барвника по поверхні первинної пухлини, лімфатичний(і) вузол(и), паренхіму молочної залози та порожнину резекції.

Результати. Двадцять дев'ять із 38 пацієнтів мали локалізований первинний рак молочної залози, який, за оцінками, був операбельним без необхідності застосування неоад'ювантної хіміотерапії. 87 % мали від одного до дванадцяти забарвлених пахвових лімфатичних вузлів; у 72 % було виявлено поширення барвника на інший квадрант і резекційну порожнину. Непроникні для рідини заморожені VX2 або пухлини молочної залози транспортували препарат(и) за дугоподібною схемою на межі «пухлина-хазяїн» незалежно від дози заморожування, кількості циклів заморожування-розморожування, фракціонування дози препарату, характеристик пухлини або розмірів пухлини. Під час плавлення суміш цитотоксичних індикаторів поширювалася в межах 50 % пухлини VX2 і відображала межу «пухлина — хазяїн»; цей процес був масштабним при нормотермії. Пробіл на знімку КТ відповідав 20 % некрозу фокального краю при патології (VX2). В обох дослідженнях дозове фарбування синім барвником відбувалося лінійно на поверхні межі «пухлина-хазяїн» і в пухлині.

Висновки. Дослідження прокладає шлях для варіантів інтраопераційного кріолікування первинного раку молочної залози. Ми показали, що нашу кріохірургічну техніку багаторазового заморожування глибоких пухлин для резекції en bloc або для абляції первинного раку молочної залози in situ, сприяючи моніторингу IOUS, можна поєднати з одночасним введенням індикаторних барвників під час традиційної хірургії, що потім дозволяє проводити лімфатичне картування. Інтраопераційні методи введення препаратів за допомогою заморожування та таргетування під час кріоабляції пухлини VX2 успішно перетворюються на локорегіональне таргетування синім барвником і лімфатичне картування під час кріоасистентної хірургії раку молочної залози. Ми дослідили здатність нашої стратегії запобігати міграції пухлинних клітин, але не введених індикаторів, до лімфатичного дренажу при стандартній резекції заморожених злоякісних пухлин молочної залози.

Ключові слова: експеримент, пухлина VX2, клінічне дослідження, первинний рак молочної залози, резекція пухлини із застосуванням кріо, кріоабляція, внутрішньопухлинна ін'єкція індикатора, лімфатичне картування.

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