Innovative Surgical System for Breast Cancer Based on Real-time Excising of Hypoxic Lesions Significantly Reduced the Positive Cavity Side Margin

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Research

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Abstract

Background: For most people, the first step in treatment is to take out the tumor (surgery), so precise and fast diagnosis of any sign of high-risk cells in surgical cavity margins is significant. The frozen pathology method is the conventional standard of intraoperative diagnosis, but the low number of slides prepared from non-fixed tissues prevents us to achieve a perfect diagnosis. Although many improvements in intraoperative margin detection were achieved, still no clinically approved intra-operative technique has been reported for the detection of surgical margins with pathologically approved classification in breast cancer.

Methods: From November 2018 to April 2020, 227 patients were registered and 213 with different types of breast tumors (IDC: n=151 (70.9%), ILC: n=6 (2.8%), DCIS: n=38 (17.8%), Atypia: n=8 (3.8%), Benign tumors: n=10 (4.7%)) were randomly assigned for CDP clinical trials. Men made up 2 (1%) of the patients; 211 (99%) were women. Functionalized carbon nanotubes grown on the electrode needles lively and selectively determine the \( \text{H}_2\text{O}_2 \) released from cancer/atypical cells, through reverse Warburg effect and hypoxia assisted glycolysis pathways, in a quantitative electrochemical manner. The study is registered at Iran National Committee for Ethics in Biomedical Research (IR.TUMS.VCR.REC.1397.355).

Result: A real-time electrotechnical system, named cancer diagnostic probe (CDP) (Patent Pub. No.: US 2018/0299401A1), has been developed and clinically approved for breast cancer surgery (National Certificate ID:14006918495) to find the presence of pre-neoplastic/neoplastic cells in vivo. Here, Functionalized carbon nanotubes grown on the electrode needles lively and selectively determine the \( \text{H}_2\text{O}_2 \) released from cancer or atypical cells, through reverse Warburg effect and hypoxia assisted glycolysis pathways, in quantitative electrochemical manner. A matched clinical diagnostic categorization between the pathological results and response peaks of CDP was proposed based on pathological classifications of WHO.

Conclusion: The clinical ability of CDP was verified on more than 1300 human in vivo breast samples with sensitivity and specificity of 93%, and 97%, respectively. After passing many trials and standard examinations, the system received production and clinical use certifications as a surgeon assistant system for usage in the operating room.

Background

Due to the impact of intraoperative margin detection, scientists attempted to develop new methods which resulted in many interesting technologies such as Mass spec pen [1] (based on mass spectroscopy from the lipids/proteins of margin surface), Margin Probe [2] (used radiofrequency for stimulating and sensing the margins), and confocal laser endomicroscopy (CLE) for margin detection of brain tumors [3]. Some of the reported advantages and limitations of these techniques were demonstrated in Supplementary Tables1&2. Although many improvements were achieved, still no intra-operative technique has been reported for the detection of surgical margins with pathologically approved classification in breast cancer (as one of the most important onco-surgeries required to accurate margin detection).

In this paper, a new system named CDP was introduced as a potential tool for live intraoperative clean/involved cavity side margin detection based on an electrochemical tracing of hypoxic functions in suspicious margin cells.

The mechanism of such pathologically classified diagnosis is based on recording the current peaks of \( \text{H}_2\text{O}_2 \) released during three important pathways activated in epithelial cells; First: oncogenic stimulation of normal cells followed by DNA damage and oncogene activation as the signs of tumor initiation [4]. Second: the reverse Warburg effect in which released \( \text{H}_2\text{O}_2 \) from pre-invasive/neoplastic cells in the microenvironment, changes the function of tumor-associated fibroblast (TAFs) from aerobic to glycolysis metabolisms [5],[6]. Third: hypoxia assisted glycolysis of tumor cells, as their distinct metabolism respect to normal cells [7],[8]. It has been shown that \( \text{H}_2\text{O}_2 \) produced and released by oncogenic stimulated normal cells would result in their transformation to atypical/pre-invasive phenotypes [9]. Also, approved reports indicated the strong correlation between activation of hypoxia assisted glycolysis and neoplastic transformation of breast cells [7],[8].

CDP was investigated on wide ranges of human cell lines followed by mice models. Then clinical trials were carried out to approve its efficiency for human models. Also, it is the first time that such an electrical diagnoser would be applied as an intra-operative margin detection approach in clinics. Here, after recording and calibrating hypoxia based margin diagnosis on 258 fresh breast samples had been selected from 74 patients, quantified diagnostic scorings of CDP response peaks were defined in correlation with their permanent histopathology results based on world health organization (WHO) classification of breast tumors [10], [11], [12], [13] that proposed for CDP scores. This diagnostic approach was verified by clinical trials on 188 patients undergone BCS/mastectomy surgeries. Both
external (tumor side) and internal (body side) margins of breast cancer patients underwent were scored by CDP, verified by permanent pathology and compared with frozen results. Next, the system was clinically approved (Certificate ID: 14006918495) and a clinical practice procedure was designed for CDP. It showed more than 97% specificity which shed new light for its application as a surgeon assistant for BCS in this step.

**Results**

CDP consists of an integrated automatic electrochemical readout board and a sensing disposable head probe as the main diagnostic part of the system (Patent Pub. No.: US 2018/0299401A1). The sensing head probe was fabricated by the growth of MWCNTs on the tip of steel needles in the conformation of three electrodes, named Working (WE), Counter (CE), and Reference (RE) with a triangular distance of 3 mm from each other (Fig. 1a). The Head probe was sterilized under plasma sterilizer protocol (standard No: ISO/NP 22441) which didn’t induce any perturbations on the morphology and function of the nanostructures (Supplementary Fig. 1a). The head probe was injected for about 4 times into the bare biological sponge to release any weakly bonded CNTs before sterilizing (Supplementary Fig. 1b-d).

It is well known that the release of H₂O₂ molecules is one of the sequential evidence during tumor initiation, reverse Warburg effect, and hypoxia assisted glycolysis of cancer cells (Fig. 1b-e) [5], [6], [7]. The detection mechanism of CDP has been based on the real-time breaking of released H₂O₂ molecules and selective releasing of 2 electrons on CNT covered sensing needles which resulted in peak current recorded by the readout system (Fig. 1b). As a result, a correlation between the pathologic states of the cells, the concentration of the generated H₂O₂ in the tissue microenvironment, and the level of the electrochemical peak current of the CDP would be observed which can be analyzed and calibrated as a diagnostic profile. CDP tests on various cell lines followed by the investigation of RT-PCR, FTIR analysis, lactate-based assays, and ROS analysis investigated by N-Acetyl cysteine (NAC) (see methods) confirmed the specific hypoxia-related responses of CDP (Fig. 1f,g, Supplementary Sect. 1.1-3, Supplementary Fig. 2,5, and Supplementary Table 3).

**Applying CDP in tumor diagnosis of mice models**

To determine the in-vivo efficiency of CDP on scoring both EMs and IMs, the animal model assay is the first step. All mouse studies were performed according to the ministry of health and medical education protocols for use and care of live animals (IR.ACECR.REC). In this regard, 57 female mice were tumorized, and tested by CDP. Before applying CDP on mice models, their tumors had been distinguished from normal regions by sonography. CDP showed high current intensity (> 300 µA vs. <70 µA) just in tumor locations (Supplementary Sect. 2, Supplementary Fig. 6). In the next step, individual tumorized mice were a candidate for surgery and the boundary lesions beneath their tumors were checked by CDP. Pre-surgical sonography declared approximate dimension and location of the tumor (e.g. 8.5 × 6 mm²: Fig. 1h). The center of the tumor (R1), four internal boundary regions beneath the tumor (R2-R5), and a region far from the tumor (R6) were checked by CDP before the surgery (Fig. 1i). Subsequently, the surgery was started (Fig. 1j) and tumor center, as well as the mentioned regions adjacent to the normal stroma, were checked by CDP results showed at least 105 µA differences in current peaks between cancer involved and free lesions (Tabled panel in Fig. 1) confirmed by frozen and permanent H&E (For detail investigation, see Supplementary Sect. 3). As it is shown in ROC analysis and AUC table, for CDP the area under the curve is 0.992 (P-value < 0.0001 and CI99% 0.96-1.00) (Supplementary Fig. 8 & Supplementary Table 10), and this value for Frozen pathology was 0.982 (P-value < 0.0001 and CI99% 0.96-1.00) (Supplementary Fig. 9 & Supplementary Table 11). The result of the ROC test shows that the CDP has better results in comparison with frozen due to the higher area under the curve of CDP (0.992 > 0.982) and it can be used as a diagnostic test of cancerous specimens.

**Pathological classification of CDP responses on in-vitro breast samples**

The main aim of this research was to introduce CDP as a real-time diagnostic tool to find involved body side margins (named IMs) during human cancer surgery. Hence, pathologically confirmed the efficiency of the CDP must be revealed both in-vitro and in-vivo. All methods for the human subjects were also carried out in accordance with relevant guidelines and regulations. They were performed according to the ministry of health and medical education protocols for use of human samples. Moreover, Patients provided consent according to an ethically approved protocol (IR.TUMS.VCR.REC.1397.355) at our breast cancer central clinics and assistant hospitals.

CDP was applied to record the current peaks of 258 *in-vitro* human fresh samples (biopsied/surgically removed) prepared from 74 breast cancer patients. Tissues were tested immediately after dissection from the body (with the non-dehydrated weight of 15–25 mg and size
of up to 1 cm²). All three integrated needle electrodes, assembled on the head probe, were entered into the target tissue at the same time. The whole process includes replacing and connecting a new head probe (~20 sec), entering CDP to the tissue, and recording the data (~15 sec due to synchronized real-time processing) would take place in less than 40 seconds. The permanent pathological diagnostic results of the samples (re-checked by 3 histological slides from each sample) were the gold standards in probable scalability of the CDP responses.

Meaningful results were observed after comparing the experimental categorization of samples through their CDP recorded current peaks with their categorization through their H&E pathological diagnoses presented in Fig. 2 (See detail investigations on CDP calibration method in Supplementary Sect. 4).

Nonmalignant and/or malignant lesion showed hypoxia-related current peaks lower than 200 µA and lesions involved to at least one focus of malignant or two foci of premalignant lesions showed current peaks more than 220 µA. So, an experimentally cut-off was achieved for CDP responses and a pathologically classified table includes negative and suspicious scores as noninvolved margins and positive scores as involved margins which must be re-excised were defined (P-value < 0.0001) (Fig. 2). The sensitivity and specificity of this cut-off definition were 95%, and 92%, respectively (Supplementary Table 13).

After achieved to the calibration of CDP based on pathological classification (DIN, LIN, and FEL), from November 2018 to April 2020, 202 patients were registered and 188 were randomly assigned for CDP clinical trials. Men made up 2 (1%) of the patients; 186 (99%) were women (Fig. 3). All methods for the human subjects were also carried out in accordance with relevant guidelines and regulations.

**Clinical trials**

**Clinical reliability of CDP scoring vs. permanent pathology in BCS/mastectomy**

In the next step, this calibration was applied in the real-time finding of suspicious IMs and EMs during lumpectomy and/or mastectomy of 113 patients with different types of breast tumors in different steps of treatment (Fig. 4a).

897 individual EMs and IMs were intraoperatively scored by CDP and diagnosed by pathology (Supplementary Tables 15&16 for EMs and IMs, respectively).

During the preclinical studies (Completely described in Supplementary Sect. 7), some clinically acceptable diagnostic scores were achieved named CFP+ (marked as CDP/Frozen/Permanent: +/+/+), CP+, C+, and CFP-, and the number of each groups were presented in Fig. 4b,d.

Scoring accuracy of CDP included sensitivity (Correct positive scores on involved margins) and selectivity (Correct positive scores on involved margins and correct negative scores on free margins) on the total number of IMs and EMs were more than 97% and 94%, respectively (Fig. 4c and Supplementary Table 18,19).

The rate of CFP+, CP+, and CFP- as correct diagnoses of CDP were 29%, 36%, and 61%, respectively, with valuable ranges of false negative and false positives (Supplementary Sect. 6.1).

ROC curve analysis has been done to compare CDP and Frozen conventional pathology with the gold standard (Permanent pathology). The result showed that the AUC value for CDP was 0.970 (P-value < 0.0001 and CI99% 0.952–0.987) (Supplementary Fig. 21 & Supplementary Table 20) in comparison to Frozen pathology, 0.881 (P-value < 0.0001 and CI99% 0.844–0.917) (Supplementary Fig. 22 & Supplementary Table 21). So, CDP has better sensitivity, selectivity, and accuracy (Fig. 4f) and it can be used as a diagnostic tool for detection preneoplastic/neoplastic cells during surgery. In addition, the result of the ROC test shows that CDP has better results in comparison with frozen due to the higher area under the curve of CDP (0.970 > 0.881).

* Among 127 patients, 14 cases were excluded due to noisy responses of the system, refused to participate, failed pathological specimens in tissue processing procedures.

IHC evaluation of some C+ lesions which even permanent didn't confirm CDP scores, confirmed the expression of the hypoxia-induced factor (HIF1-alpha) [8][14] markers. this showed promising results in support of CDP scoring but through non-clinical approval of HIF1-alpha (as a diagnostic marker), the scoring values didn't change (Supplementary Sect. 7).
Due to this study (Fig. 4), the efficiency of the hypoxia approach for margin detection in both false negative and positive values was elucidated. To achieve clinical and production certifications for CDP, we designed three clinical trials (one observational and two interventional trials with trial registration ID: IRCT20190904044697N1, IRCT20190904044697N3) to present wide application of this method in helping fast diagnosis of clean and involved margins just by the surgeon through observational and interventional trials. The outcomes of these trials as well as electrical and safety evaluation exams of the system resulted in the achievement of clinical usage certification for CDP by Iran Ministry of Health with the national ID number of:14006918495 and product license number of 23212882 as a surgeon assistant tool in breast cancer surgery.

Clinical efficacy of CDP based margin detection/cleaning by the surgeon

Three different types of clinical trials were done by CDP. First, an observational trial in which the CDP had no role in the diagnosis and treatment of the patients, and we just recorded the scores of CDP and check their validity by permanent pathology. In this clinical trial, CDP was applied for data recording from IMs of 25 human cases of breast cancer without inducing any perturbation or intervention in the trend of conventional surgery.

This trial was an observational clinical experiment to better realize the impact of CDP in finding involved cavity side margins during breast tumor surgery. In this regard, the surgeon followed the standard guideline-based on frozen pathology, and CDP was applied just as a complementary diagnostic tool to check the IMs after completing the surgery (after checking and removing the involved margins through frozen results of EMs). Hence, when the margins were declared free after one or further sequences of frozen evaluation (frozen might declare some tumor margins as involved EMs and through standard guideline cavity side margins must be re-excised and resend for frozen up to be declared as free EMs by pathologists), the CDP was started to be used by the surgeon in all of the IMs. In this clinical trial, the surgeon checked and scored the IM lesions by CDP and just recorded the results with neither informing the pathologist about the positively scored IMs nor dissection of the positively scored region to prevent from any CDP based intervention. In the next step two days after, the pathologist will check by permanent H&E and IHC (if needed) all of the tumor margins (EMs) and other additional margins re-excised through frozen diagnostics. If a margin (which was declared as free margin in frozen) was positive this means that the frozen missed that margin then, the CDP data would be checked and if CDP positively scored that margin, it means that CDP was capable to detect missed margin of frozen. If CDP negatively scored that margin, it means that CDP was missed the margin similar to frozen. Our gold standard for the pathological states of the margins is permanent H&E/IHC assay (Supplementary Table 23).

In this trial, the precision of CDP was evaluated without any non-pathologically approved dissection or re-excision. Among 25 breast cancer candidates for this trial, 4 of 150 (3%) IMs from 4 of 25 (16%) patients which had been positively scored by CDP was confirmed as involved margins in the permanent evaluation of their reciprocal EMs while they had been reported as free margins in frozen evaluation. 2 of 150 (1%) IMs had been positively scored by CDP, wasn’t confirmed in the permanent evaluation of their EM reciprocal. 144 of 150 (96%) IMs had been negatively scored by CDP (in corroboration to conventional frozen evaluation) while 1 of the reciprocal EMs was declared as involved margin in permanent evaluation (foci of suspicious proliferative UDH with negative CK5/6 and CK14 which was declared as ADH). Totally, in comparison with frozen section (frozen conventional evaluation) as an observational tool, CDP just lost 1 positive margin while truly scored 4 missed margins (e.g. Figure 5b anterior margin of Patient ID 114; invasive ductal carcinoma nuclear grade 2 and Fig. 5c inferior margin of Patient ID 138; DCIS). Also, CDP showed 2 overdiagnosis on free margins.

Also, the sensitivity and specificity of CDP based on permanent evaluation in the first clinical trial were evaluated (Fig. 5a, Supplementary Sect. 8 and Supplementary Tables 24&25).

In the second clinical trial which was an interventional trial and had been registered by the trial registration ID: IRECT20190904044697N1, CDP was applied for data recording from IMs of 25 human cases of breast cancer without inducing any perturbation or bias in the trend of surgery. 25 patients were recruited after the registration. This trial shows the independent role of CDP for margin checking in breast-conserving surgery.

This trial, was a simulation of a real clinical situation by which we can better realize the impact of CDP in breast tumor surgery. In this regard, the main surgeon followed the standard guideline-based on frozen pathology without any information about the CDP results on IMs and a second surgeon applied CDP (just as a complementary diagnostic tool) to check the IMs immediately after tumor dissection (before the declaration of frozen results). In this clinical trial, the second surgeon should only dissect a specimen of the positively scored (by CDP) lesion just in the tested location. These samples were named “CDP Samples”. On the other hand, the results of frozen samples on EMs (named as frozen samples) were the criterion for the main surgeon to continue and complete the surgery. Following such a trend
not only preserved the role of frozen as the main guideline of the intra-operative diagnostics but also presented CDP as an independent complementary system to prevent any false negatives which might be missed by the frozen method and would be helpful for patients in future. The required time for checking all of the IMs by CDP was about 10 minutes (Supplementary Movie1), while at least 50 minutes are required to declare the results of frozen on all of EMs. The main surgeon didn't have any information about the CDP scores of IMs and complete the surgery just by the results of frozen. Finally, permanent pathology was carried out on both frozen and CDP samples. Hence, without any sampling bias, the impact of CDP was evaluated. The gold standard for the pathological states of both EMs (frozen samples) and IMs (CDP samples) is again permanent H&E of EMs dissected through frozen scoring.

In this trial, not only the ability of CDP was evaluated independently, but also the presence of CDP near-frozen in helping the patient to have margins free from suspicious cells was investigated. FPs of CDP would be checked by permanent H&E on samples dissected through CDP scoring and FNs of CDP might be detected by permanent H&E of EMs dissected through frozen scoring.

11 of 150 (7%) samples for 8 of 25 (32%) patients were positively scored by CDP and confirmed by permanent H&E of CDP samples while none of them were diagnosed in frozen sections of their reciprocal EMs (e.g. Figure 5d Lateral margin of Patient ID 143; IDC grade 2/DCIS). 4 of 150 (3%) samples for 2 of 25 (8%) patients were truly scored positive by both CDP and frozen methods. In 133 of 150 (89%) samples, the IMs were negatively scored by CDP which were confirmed in permanent H&E of reciprocal EMs. It is worth noting that on one patient (ID 145), no trace of any high-risk lesion was found neither in frozen nor in permanent of one of the EMs (florid UDH; medial margin) while CDP positively scored its reciprocal IM. Permanent pathological investigations on the scored IM declared the presence of LIN2 (Two foci of LCIS: Fig. 5e). However, CDP showed one LVFN (Superior margin of patient ID 158) which was correctly diagnosed by frozen analysis.

Again, the sensitivity and specificity of CDP and frozen assays in the second clinical trials were evaluated based on permanent results (Fig. 5a, Supplementary Seacyion8 and Supplementary Tables29&30).

A third clinical trial that had been registered by the trial registration ID: IRCT20190904044697N3 was applied to evaluate the role of CDP as a complementary system to cover the misses of frozen pathology. This trial was applied to 25 human cases of breast cancer candidates.

In this trial, the surgeon followed the standard guideline-based on frozen pathology, and CDP was applied just as a complementary diagnostic tool to check the IMs after completing the surgery (after checking and removing the involved margins through frozen results of EMs). Hence, when the margins were declared free after one or further sequences of frozen evaluation (frozen might declare some tumor margins as involved EMs and through standard guideline cavity side margins must be re-excised and resend for frozen up to be declared as free EMs by pathologists), the CDP was started to be used by the surgeon in all of the IMs. In this clinical trial, the surgeon checked and scored the IM lesions by CDP and informed the pathologist about the IMs which were positively scored by CDP. Then, the pathologist further evaluates all over the last reciprocal EMs of that IMs by slide preparation from much more points on that margin (this EM might be the tumor margin or a re-excised EM). If the pathologist found any suspicious lesions in re-evaluation, he/she informs the surgeon to remove the positively scored margin and if not, the surgeon wouldn’t remove the CDP positive IMs and we just record the data of CDP responses. In the next step, the pathologist will recheck all of the last reciprocal EMs by permanent H&E. Hence, the patient will be recalled to undergone second surgery if any EMs have been missed by frozen and detected in permanent pathology either had been found by CDP or not.

As a result, the impact of CDP in preventing from remaining of the margins missed in frozen pathology would be evaluated.

Again, the gold standard for the pathological states of both EMs and re-excised IMs is a permanent H&E/IHC assay (Supplementary Table 33). In this trial, among 25 breast cancer candidates for this trial, 6 of 150 (4%) IMs from 8 of 25 (32%) patients which had been positively scored by CDP was confirmed as involved margins in the frozen re-evaluation of their reciprocal EMs while they had been reported as free margins in frozen evaluation. 3 of 150 (2%) IMs had been positively scored by CDP, weren’t confirmed in the frozen re-evaluation of their reciprocal EM. Hence, they were re-excised from the surgery site. Permanent pathology not only confirmed all of those 6 of 150 (4%) samples as involved margins but also confirmed the diagnosis of CDP in 2 of 3 (67%) EMs which had been negatively scored by frozen (2 of 3 (67%) those EMs were declared as suspicious to atypia (ADH) in the permanent evaluation and 1 of 3 (34%) was declared as involved to a focus of DCIS, intermediate grade (Fig. 5f,g medial margin of Patient ID 183; DCIS and Fig. 5h,i lateral margin of Patient ID 187; ADH). One of the suspicious ADH samples was rolled out in CK5/6 and CK14 IHC assays). So, the patients with positive margins were recalled for the second surgery. In one sample had been positively scored by CDP and negatively scored by frozen, the CDP score wasn’t confirmed by the permanent pathology of the reciprocal EM.
141 of 150 (94%) IMs had been negatively scored by CDP in corroboration to conventional frozen evaluation. Totally, in comparison with the frozen section (frozen conventional evaluation), CDP gained 8 missed margins without any false negative. Also, CDP showed one overdiagnosis on a free margin.

Similar to previous trials, the sensitivity and specificity of CDP based on permanent evaluation in this clinical trial were evaluated by the confusion matrix method (Fig. 5a, Supplementary Sect. 8).

For three clinical trials, ROC, and AUC for CDP and Frozen conventional pathology were calculated. The area under the curve for CDP in all clinical trials was higher than 0.893 (P-value < 0.003 and CI99% 0.619-1.000) which is higher than this value for Frozen conventional pathology (Lower than 0.656 (P-value > 0.04 and CI99% 0.437–0.876)). These results for Frozen conventional pathology showed that it is not a reliable diagnostic test and it has not a good balance of sensitivity and specificity (Fig. 5j&k, Supplementary Sect. 8).

Discussion

CDP was introduced as a handheld real-time diagnostic tool for intraoperative detection of the IMs involved to atypical, pre-neoplastic and neoplastic breast cells with the capability of pathological classification. The detection has been carried out by electrochemical tracking of the metabolic pathways associated with tumor initiation, progression, and metastasis such as reverse Warburg effects [6], hypoxia assisted glycolysis [5], and field-effect [15]. Quantitative scoring of pre-invasive/invasive lesions in body side margins, in correlation with the standard pathological categorization of breast tumor diseases, would be the distinct role of CDP in cancer surgery. An increase in peak currents of CDP through increased concentration or phenotypic progression of atypical/cancer lesions was meaningful. By in-vitro and in-vivo investigation of more than 1300 tissue samples from about 260 patients (74 in-vitro, 113 in-vivo, and 75 in the clinical trials), the pathological scoring of CDP based on WHO pathological classification of breast tumors, was experimentally proposed and verified. By considering its false negative and positive responses, the CDP correctly scored 473 of 491 (96%) IMs during the surgery of 113 patients in real-time. The whole testing process on one margin sample from preparing CDP to declaring the diagnosis just takes place in less than 40 seconds.

Finally, after passing many clinical trials (on 450 margins) and safety exams is received the clinical certificate as a useful method for margin diagnostics to cover the missed margins might have remained during the BCS or partial mastectomy surgeries. The capabilities of CDP seemed to be comparable with recently developed technologies such as Mass Spec [1], Margin probe [2], and CONVIVO [3] (Supplementary Table 1&2). Pathologically classified responses on finding the involved internal margins, in their live states, ranged from pre-invasive to invasive metastasized lesions (with the sensitivity and specificity of 93% and 97%, respectively). Disposable head probe with the cheap fabrication process and no biological perturbation on the vitality and function of the tested lesions are the strengths of CDP. It also might be able (with a modified head probe) to detect the locations of suspicious regions before core needle biopsy as a guiding method to reduce the number of sampling which was promising in primary tests (Supplementary Fig. 30).

As the CDP received clinical usage certification, it is used under two individual protocols: first: as a complementary diagnostic tool in the presence of frozen assay. Second: as an alternative diagnostic tool in the centers which frozen is not accessible.

The tested lesions by CDP kept their live dynamic function and can be either excised and reanalyzed by pathological methods to assure pathologists or remained in the body without any excision.

We proposed two protocols for using CDP based on our clinical trials. First, in the presence of frozen; the CDP could be applied after treating the margins under frozen guidance. In this regard, all of the IMs would be re-checked by CDP and the frozen pathologist would be informed about the positive results and re-check his/her diagnostic decision by further evaluation or requesting dissection of positively scored specimen for frozen testing.

Second, in the absence of frozen (If frozen is not available for the surgeon); after tumor dissection, the IMs could be checked by CDP and positively scored margins would be dissected through the CDP user guideline (Supplementary Sect. 6, Supplementary Movie1). The test could be repeated for EMs for accurate marginal checking. Finally, in permanent evaluation, the pathologist would check CDP scored specimen near tumor margins. As frozen cannot directly evaluate the IMs, CDP would have a unique role even in the presence of frozen.

Although CDP would be proposed as a diagnostic tool for both EMs and IMs in breast surgery, the limited number of assayed samples and requirements for experiments on human models with different types of cancer tumors might be its weaknesses ought to be
considered for future developments. Also, further analyses must be done to more elaborate on the reasons for FPs and FNs on CDP scores.

Methods

Fabrication of Cancer Diagnostic Probe for in-vivo assays

First, the sterile needle was rinsed in deionized (DI) water and dried by air. A fixture was designed and fabricated to hold the needles both in E-Beam and DC-PECVD systems to limit the growth of CNTs just in the tips of the needles. Ni catalyst layer for CNT growth with a thickness of 9 nm was coated on Ni needle by E-beam evaporation system (Veeco Co.), at the temperature of 120 °C with depositing rate of 0.1 Angstroms/s. Afterward, Ni-covered samples were located in a direct current plasma enhanced chemical vapor deposition (DC-PECVD) system to grow vertically aligned multi-walled carbon nanotubes (VAMWCNT). The growth has a three-step process named as annealing, graining, and growth. At first, the sample was annealed at 680 °C in an H2 environment with a flow rate of 20 standard cubic centimeters per minutes (sccm) for 30 minutes. During the graining, the surface was plasma hydrogenated for 5 minutes with the intensity of 5.5W.cm-2, which results in the catalyst graining, and formation of Ni Nano-sized islands. In the growth step plasma of C2H2 and H2 mixture with flow rates of 4.5 and 20sccm were introduced to the chamber for 20 minutes. Finally, CNT’s were characterized by FE-SEM. The length and diameter of nanotubes ranged from 2.5 to 5 µm and from 50 to 70 nm, respectively. Figure 1b shows the FESEM image of the CNT biosensor. These CNTs were multi-walled carbon nanotubes of high purity and the presence of nickel on the topside of the CNTs could be related to the tip-growth mechanism. The CNT has been used as the work, counter, and reference electrodes. Then the CNT grown needles were attached to an electrical connector with three pins by conductive paste to form the final probe. Just tips of the needle were extended from the connectors up to 1 cm. The probe was reinforced with a user-friendly homemade holder and connected to the readout system by a noiseless cable which handled all three electrodes.

The electrochemical readout system

The schematic of an integrated portable automatic electrochemical readout board is represented in the Supplementary Fig. 34. In this system, we utilized a low noise, high accuracy, and low power potentiostat. For designing this potentiostat to decrease the loading effect of noise of the environment which their main source the other instruments in the operation room, we used Low Noise Ampliers (LNA). Moreover, to diagnose the current signal, which flows from the Work Electrode (WE) we required a high-speed potentiostat. So, we added Current Buffer Amplifier Classes (CBA)s. The combination of these two classes of amplifiers produces a creative, effective, and appropriate device for high accuracy tests.

On the other hand, to increase the accuracy of the circuit, we used Analog to digital (ADC) and Digital to Analog (DAC) converters with 16 bits. A unit of a processor is installed on the board which receives the data of the converters and transmits them via a Bluetooth module to the user (Supplementary Fig. 34a).

Finally, the software was designed to analyze the data and diagnose whether the figures are cancerous or normal and send the result as the form of an alarm to the surgeon. Not to be neglected that a 3.3V and 800 mA lithium-Ion cell battery is powering all parts of this circuit so it doesn't need to be connected to the power. This ability of the device provides a free and flexible method for the surgeon to utilize the device clinically. For electrochemical measurements, the CV studies were done using DC voltage and no AC frequency was applied. The potential was swept in the range from −0.8 to +0.8 V, using a scan rate of 100 mV s-1 (Supplementary Fig. 34b).

Cell cultures and reagents

Breast cancer cell lines (MCF10A, MCF-7, MDA-MB-231, MDA-MB-468), Colon (COR-L 105, SW-480, HT-29), Hematopoietic (1301, LCL-PI 1), Liver (HEP G2), Lung (QU-DB, MRC-5), Mouth (KB), Neuron (BE (2)-C, LAN-5), Prostate (PC-3, Du-145) were obtained from the standard cell banks of the National cell bank (NCBI) located in the Pasteur institute and they were maintained at 37oC (5% CO2, 95% air) in RPMI medium (Gibco) supplemented with 5% fetal bovine serum (Gibco), and 1% penicillin/streptomycin (Gibco). The fresh medium was replaced every other day. All cell lines were tested and found negative for Mycoplasma contamination. The cells were detached from the plates by trypsin and counted by neobar laam.

ROS assay

ROS generation was analyzed with 5-chloromethyl-2’-7’-dichlorodihydrofluorescein diacetate (CM-H2DCFDA) assay. This probe is changed to 2’-7’-dichlorofluorescein (DCF) with a green fluorescent property by esterase enzymes in the cytosol of the cells. After culturing the fibroblast and MCF-7 cells overnight, the cells are washed with PBS and then 500 µL of the CM-H2DCFDA solution is added
with a concentration of 20 µM. After 30 minutes incubation in room temperature and dark, then cells are again washed and then imaged with a fluorescent microscope. The cells were incubated with 6.5 mM of NAC as a ROS scavenger and then treated with CM-H2DCFDA. Finally, the samples were imaged with a fluorescent microscopy system [16].

**L- Lactate Assay Kit (Colorimetric) Procedure**

The production of lactate in culture was analyzed to determine the rate of hypoxia assisted glycolysis. The intracellular level of lactate was measured by the colorimetric lactate assay kit (Abcam: ab65331, United Kingdom) according to the manufacturer’s protocol. The optical densities were then measured at 450 nm wavelength. The assay was carried out due to the below steps:

- **Reagent preparation:** Solubilize Lactate Substrate Mix and Lactate Enzyme Mix, thaw Lactate Standard, and Lactate Assay Buffer (aliquot if necessary); get the equipment ready.
- **Standard preparation:** Prepare Lactate standard dilution [range 2–10 nmol/well] (according to the manufacturer’s protocol).
- **Sample preparation:** Prepare samples (including deproteinization step) in optimal dilutions so that they fit standard curve readings.

**√ Cell (adherent or suspension) samples:**

- Harvest the number of cells necessary for each assay (initial recommendation = 2 × 10^6 cells).
- Wash cells with cold PBS.
- Resuspend the cell pellet in 500µL of Lactate Assay Buffer.
- Homogenize cells quickly by pipetting up and down a few times.
- Centrifuge 2–5 minutes at 4 °C at top speed in a cold microcentrifuge to remove any insoluble material.
- Collect the supernatant and transfer it to a clean tube.
- Keep on ice.

**Tissue samples:**

- Harvest the necessary amount of tissue necessary for each assay (initial recommendation = 10 mg tissue).
- Wash tissue in cold PBS.
- Re-suspend tissue in 4–6X volumes of Lactate Assay Buffer using a homogenizer sitting on ice, with 10–15 passes.
- Centrifuge samples for 2–5 minutes at top speed at 4 °C in a cold microcentrifuge to remove any insoluble material.
- Collect the supernatant and transfer to a clean tube.
- Keep on ice.

**Calculation:**

Duplicate the test for each standard and sample and extract the average. If the background control of the sample is significant, then deduct it from the sample readings. Decrease the mean absorbance value (Standard #1) from all standard and sample readings.
Corrected absorbance will achieve. Consider the corrected absorbance values for each standard as a function of the final concentration of Lactate.

To form the standard curve, draw the curve through these points. Most plate reader software can outline these values and curves. Based on your standard curve data calculate the trend line equation (use the equation that presents the most accurate fit).

The concentration of L-lactate in the test samples is determined as:

\[
Lactate\ concentration = (La/Sv) \times D
\]

La = amount of Lactic acid in the sample well calculated from a standard curve (nmol)

Sv = volume of sample added into the well (µL)

D = sample dilution factor

Lactic acid molecular weight = 90.08 g/mol

**Tumor formation in mice models**

Female inbred BALB/c mice on 6–8 weeks of age were acquired from Pasteur Institute. They were kept at 22–24 °C with a 12 h light/dark cycle in a designed pathogen-free isolation facility and allowed to adapt for a week before tests. All procedures were approved by the animal ethics committee. In the next step, \( 3 \times 10^6 \) 4T1 cells/200 µl, and in the case of non-malignant tumor injection, a total of \( 3 \times 10^6 \) MC4L2 cells/200 µl were injected into the back of the neck or right side of the mice. Tumor sizes were measured by portable sonography. After 10 days, to assess the superficial tumor regions, individual head probes of CDP (fabricated with the distance of ~1 mm between the needles due to the tumor sizes in the mice) were entered into them from the skin.

**In-vitro sample collection from the patients**

All methods for the human subjects were also carried out in accordance with relevant guidelines and regulations. Patients provided consent according to an ethically approved protocol (IR.TUMS.VCR.REC.1397.355) at our breast cancer central clinics and assistant hospitals. Live slices from CNB or surgically removed samples were cut in similar specimens and directly transferred through micro wells contain RPMI-1640 without any preprocessing (Supplementary Table 38).

**Tissue staining procedure with hematoxylin-eosin (H&E)**

H&E are used to illustrate the nucleus and cytoplasmic inclusions in clinical specimens. Hematoxylin works as a mordant, and stains the nucleus light blue. In the presence of an acid, the dye turns into the red to achieve the differentiation, we should put the tissue in an acid solution. By using eosin, the counterstaining is done, which provides pink color to the cytoplasm. The H&E staining process starts with the deparaffinization of a tissue section, flaming the slide on the burner, and placing it in the xylene. After the Hydration process, the treatment process must be repeated. To hydrate the tissue section, it should pass through a decreasing concentration of alcohol (100%, 90%, 80%, and 70%). Next, for approximately 3 to 5 minutes, the sample should be stained in hematoxylin, followed by washing in the tap water until the sections become blue. In the next step, the sample should be contrasted in 1% acid alcohol (1% HCL in 70% alcohol) for 5 minutes. Afterward, the sample should be washed in running tap water, soaked in an alkaline solution (e.g. ammonia water) until it turns blue, followed by another tap water wash. Moreover, the sample should be stained in 1% Eosin Y for 10 minutes and washed in tap water for 1–5 minutes. Finally, the sample should be soaked in an increasing concentration of alcohol to dehydration and then clear it within the xylene.

**Patients’ samples staining by the immunohistochemical (IHC) procedure**

Discrepancy cases were picked for further immunohistochemical procedures (IHC). The samples were cut and put on poly l-lysine coated slides. Using a microwave oven with heat-induced epitope retrieval antigen, retrieval was conducted. By the clone AE1/AE3 (dilution 1:50), avidin-biotin method (utilizing labeled streptavidin-biotin (LSAB) + kit) with Dako Monoclonal anti-Human PCK, Anti-HIF-1 alpha antibody (ab82832), Anti-Cytokeratin 5 + 6 antibody [D5/16 B4] (ab17133), Anti-Cytokeratin 14 antibody [LL002] (ab7800), and Recombinant Anti-p63 antibody [EPR5701] (ab124762) the IHC were done.

**RNA sequencing of breast cell lines**
RNA samples including MCF10A, MCF-7, MDA-MB 231, and MDA-MB 468, extracted from the breast cell lines, thawed, and incubated for 90 seconds at 70 °C. To produce cDNA, we treated samples with reverse transcription master mix and incubated them on thermocycler at 50 °C for 30 minutes, and 70 °C for 15 minutes. To extract the free primers, we added 1.0 µl of EXOSAP mix to each sample, then, incubated the mixture at 37 °C for 30 minutes, and inactivated it at 80 °C for 25 minutes. afterward, by incubating in the master mix at 37 °C for 15 minutes and inactivated at 70 °C for 10 minutes, a 3’ poly (A) tail was added to the cDNA in each sample. By dividing each sample into 4 and incubating in master mix at 95 °C for 3 minutes, 50 °C for 2 minutes, and 72 °C for 10 minutes, the second layer of cDNA was integrated. PCR increase (95 °C for 3 minutes, and then, 20 cycles of 95 °C for 30 s, 67 °C for 1 minutes, and 72 °C for 6 s) was performed with the master mix.

The four responses of each sample were pooled, filtered applying the Qiagen PCR purification kit (cat. no 28106), and eluted in 50 µl EB buffer. By testing for genes, HIF-1α, c-Myc, HKII, PGAM1, and LDHA the samples were chosen.

Primers and probes (Supplementary Table 43) were produced by AlleleID (Premier Biosoft) and synthesized by BonYakteh Corp. Nucleotide series applied for the probe-primers design were obtained from the NCBI database. The designed probe-primers were aligned by BLAS to approve gene specificity.

Again, each sample was divided into 4, and the second round of PCR amplification (nine cycles of 98 °C for 3 minutes, 67 °C for 1 minutes and 72 °C for 6 minutes 6 s) was made with a master mix. Samples were pooled and washed using Agencourt AMPure XP beads and eluted in 40 µl 1× low-TE buffer (Supplementary Table 39).

In-vivo intra-operative use of CDP and marking the prepared margins for pathology assays

During the lumpectomy or mastectomy surgery, the CDP was tested for checking both IMs and EMs to observe any probable matching between suggested pathological classification of CDP scores and the final diagnostics of the samples declared by pathologists. At first Sterilized CDP (had been stored in ambient exposed to formalin tablet for one day) was turned on and connected to the software. Then the head probe (had been sterilized by plasma standard protocol) was connected to the CDP and the bodyside margins of the patient undergone tumor dissection was checked by CDP though the process presented in Supplementary Movie1. Tested margins were dissected and evaluated through standard frozen and permanent assays. Similar procedures were done for external margins on tumor sides. The protocol of marking the dissected lesion by pathological ink was presented in Supplementary Fig. 14. The boundary of the lesion was inked in parallel to the path of probe entrance. Hence, in all of the prepared slides, we can follow the trace of the ink and also would be ensured from the interaction of the probe needles with the prepared tissue slides. Finally, the resulted data were used for better calibration of CDP pathological categorization.

Finally, Applying CDP near the frozen in future surgeries may prevent any probable false negatives of either CDP or frozen methods (and would be helpful for the patient in a real surgery). The required time for checking all the internal margins by CDP was less than 10 minutes while at least 50 minutes is required to declare the results of frozen on all External margins. The gold standard for the pathological states of both External margins (frozen samples) and internal margins (CDP samples) is again permanent H&E/IHC assay.

Breast surgery

Applying the CDP in detecting both internal and EMs during the lumpectomy or mastectomy of the patients was done under the Ethics committee confirmation. The gold standard in all surgeries was pathological results. In the cases that even permanent pathological results weren't matched by CDP, we experimented with IHC on the samples. When the IHC results were matched by CDP in contrast with permanent H&E, we based the diagnosis on IHC results due to the opinion of our tumor board (surgeon, oncologist, and pathologist). For example, patient ID: 93, were diagnosed as usual ductal hyperplasia by H&E but CDP scored it as cancer involved region and SMMH confirmed the infiltration of neoplastic cells from the stroma.

Statistical analysis

SPSS software (version 26) was used for statistical analysis. To evaluate each of the diagnostic tests (CDP and Frozen conventional pathology), the receiver operating characteristic (ROC) curves and area under the ROC curve (AUC) have been done to assess and compare the detection ability of each group with the gold standard test (Permanent pathology). Also, the sensitivity, selectivity, accuracy, and specificity of each of them were calculated with SPSS software. A P-value of less than 0.01 was considered significant.
Abbreviations

H&E: Hematoxylin and Eosin; IHC: immunohistochemical procedure; DC-PECVD: Direct current plasma enhanced chemical vapor deposition; VAMWCNT: Vertically aligned multi-walled carbon nanotubes; HIF-1α: Hypoxia-inducible factor 1-alpha; HKII: hexokinase II; PGAM1: Phosphoglycerate mutase 1; LDHA: Lactate dehydrogenase A; PDK1: Pyruvate Dehydrogenase Kinase 1; DIN: Ductal intraepithelial neoplasia; LIN: Lobular intraepithelial neoplasia; FEL: Fibro-epithelial lesion; FCC: Proliferative fibrocystic changes; UDH: Usual ductal hyperplasia; SA: Sclerosis adenosis; CCC: Columnar cell changes; ADH: Atypical ductal hyperplasia; DCIS: Low grade ductal carcinoma in-situ; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; ALH: Atypical lobular hyperplasia; LCIS: Lobular carcinoma in-situ; PCK: Pancytokeratine

Declarations

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Authors' contributions

Mohammad Abdolahad designed and coordinated the research, analyzed the data, and wrote the manuscript. Zohreh Sadat Miripour manufactured the head probe sensors for clinical studies, performed most of the experiments, categorized the tabled data and did the statistical analysis. Fereshteh Abbasvandi do most of the surgeries and provided the clinical samples in the manner as was designed for the research by Mohammad Abdolahad. Parisa Aghaei sorted the results and also assisted in the experimental process. Fatemeh Shojaeian assisted in performing experiments and validating statistical data. Mahsa Faramarzpour and Pooneh Mohaghegh assisted in categorized the tabled data, did the statistical analysis and sorting the results. Parisa Hoseinpour did the pathological experiments and declared diagnosis. Naser Namdar and Morteza Hassanpour Amiri design and fabricated the hardware and software of the CDP system. Hadi Ghafar designed and fabricated the body shape of the CDP probe. Sahar NajafiKhosnnoo assisted in the animal experimental process. Hassan Sanati and Mohammad Ali Khayamian assisted in the biological experimental process. Mahna Mapar assisted in the interpretation of the experimental test results. Nastaran Sadeghian did some of the surgeries through the protocol designed for the research by Mohammad Abdolahad. Mohammad Esmaeil Akbari supervised the surgical procedure and the clinical samples. All authors and participants reviewed the paper and approved the final manuscript.

Ethics approval and consent to participate

Patients provided written informed consent according to an ethical approved protocol by the institutional review board of Tehran University of Medical Science (Trial registration: IR.TUMS.VCR.REC.1397.355, IRCT20190904044697N1, IRCT20190904044697N3. Registered at 18 August 2018, http://ethics.research.ac.ir/PortalProposalListEn.php?code=IR.TUMS.VCR.REC.1397.355&title=&name=&isAll=&GlobalBackPage=http%3A%2F%2Fethics.research.ac.ir%2FindexEn.php) at our breast cancer central clinics and assistant hospitals for the use of their samples. All animal studies were performed according to ministry of health and medical education protocols for use and care of live animals (IR.ACECR.REC).

Consent for publication

Not applicable.

Availability of data and materials

The authors declare that all the other data supporting the findings of this study are available within the article and its supplementary information files and from the corresponding author upon reasonable request. Also, the authors declare that all codes supporting the findings of this study are available from the corresponding author upon reasonable request.
Competing Interest

A patent application has been published on the basis of this work. M.Abdolahad is the member of the scientific advisory board of Arya Nano biosensor Manufacturer Co., a company that is commercializing CDP technology. The remaining authors declare that they have no competing interests.

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Figures

Figure 1
a) Image of CDP system with a changeable head probe consists of three needle electrodes coated by multi-wall carbon nanotubes, b) Selective electrochemical reactions of released H2O2 on MWCNTs and production of the cathodic ionic peak. Distribution and abundance of nanotubes make a conformal surface for signal extraction and it presented in FESEM images. Cancer cells release H2O2 due to hypoxia assisted glycolysis, as their distinct metabolism respect to normal cells, c) The reverse Warburg effect also releases H2O2 from pre-invasive/neoplastic cells in the microenvironment and changes the function of TAFs from aerobic to glycolysis metabolisms, d) Intracellular reactive oxygen species (e.g., H2O2) levels were measured using CM-H2DCFDA fluorescent that emits green fluorescence in the presence of H2O2 but it decreased in MCF-7 treated cells (1*106) with 6.5 mM of N-Acetyl-L-cysteine (NAC) as a ROS Inhibitor (ab143032), f) Hypoxia assisted glycolysis related electrochemical cyclic voltammetry cathodic peaks of different phenotypes of breast cell lines (MDA-MB-468, MDA-MB-231, MCF-7, and MCF10A) in comparison with standard culture media (RPMI) and lactate solution (Concentrations of 0.3Mm). The intensity of the peaks is in correlation with the cancerous progression of the cells, g) CV responses of different concentrations of standard unstable H2O2 contained solution (with the base of lactate) as a reference sample, h) Tumorized mouse by 4T1 breast cancer cell lines checked by sonography. The CDP was tested on tumor and suspicious regions before, and during the surgery. k) The tumor contained suspicious regions were removed and sent for frozen pathology and the H&E images taken from the center of the tumor (R1) as well as its, right (R2), left (R3), up (R4) and bottom (R5) margins, with the distance of 1mm from the histologically distinct region, were demannded by the CDP results as presented in the table. Green and red marks in the tables indicated the normal and cancerous regions with different intensities of infiltrated malignant cells through the sharpness of the color. The results of CDP before and during surgery exhibited a great correlation (99% CI, p-value=0.00001). Triangular arrows in pathologial images refer to the cancer cells with hyperchromic and deformed nuclei distributed among the stroma in suspicious regions, l) Schematic of applying CDP in real-time detection of suspicious margins during breast cancer surgery. The assay conducted on a suspicious margin inside the body of the patient (Lateral margin of patient ID: 2) which is the significance of CDP. It also positively scored the margin and the removed specimen showed a negative result for malignancy in frozen analyses meanwhile the permanent H&E showed the papillary lesion with the Atypia region, which must be removed by the surgeon. Inferior IM of the other patient (ID 62) was negatively scored by CDP and confirmed by both frozen and permanent H&E as usual hyperplasia and, m) CDP as a surgeon assisted tool in the surgery room for finding involved IMs to pre-invasive/invasive cells.

![Image of CDP system](https://via.placeholder.com/150)

**Figure 2**

(a) CDP current peak results of 258 in-vitro samples from 74 patients in association with pathological diagnosis based on pathological classification (DIN, LIN, and FEL). Normal breast (e.g. breast fatty tissue), UDH (e.g. FCC lesions), and DIN1a (e.g. FCC with CCC and a small focus of ADH) showed response peak ranges from 0 to 196 µA which were negatively scored by CDP. DIN1b (e.g. two or more foci of ADH), DIN1c (e.g. low grade-DCIS), DIN2 (e.g. intermediate DCIS), DIN3 (e.g. high-grade DCIS) and IDC lesions showed response peaks in the ranges between 203µA to more than 600µA which were positively scored by CDP, (b) Results show meaningful consistency between pathological diagnosis and CDP scoring.
Figure 3

CONSORT diagram for CDP and standard Frozen pathology in the efficacy analysis.
Figure 4

a) Clinical and pathologic characteristics of patients randomly assigned to this study. Investigation of margins in 113 patients with breast cancer during surgery by CDP, Frozen H&E, permanent H&E, and IHC (if required), b) The number of patients ID which all three CDP/Frozen/permanent were positive (CFP+), c) The number of patients ID that CDP and permanent were positive and frozen declared negatives (CP+), d) The number of patient ID which CDP was positive and permanent H&E couldn't declare final diagnosis, therefore, IHC was recommended and confirmed CDP results (C+), e) The number of patients ID which all three CDP/Frozen/permanent declared negative (CFP-). Internal circles in each diagram indicate the number of tested margins for one patient, f) Comparison of the accuracy, sensitivity, specificity, and selectivity parameters for CDP, and conventional Frozen pathology for preclinical trial. * Among 127 patients, 14 cases were excluded due to noisy responses of the system, refused to participate, failed pathological specimens in tissue processing procedures.
| Characteristic or Outcome       | Clinical Trial 1 (Observational) | Clinical Trial 2 (International) | Clinical Trial 3 (International) |
|--------------------------------|----------------------------------|----------------------------------|----------------------------------|
| Age                           | 45.6 (30-65)                     | 35.3 (22-45)                     | 47 (29-71)                       |
| Sex                           |                                  |                                  |                                  |
| Female                        | 25 (100%)                        | 25 (100%)                        | 25 (100%)                        |
| Male                          | 0                                | 0                                | 0                                |
| Race                          | White                            | White                            | White                            |
| Type of surgery               |                                  |                                  |                                  |
| Mastectomy                    | 5 (20%)                          | 1 (4%)                           | 2 (8%)                           |
| Lymphectomy                   | 20 (80%)                         | 24 (96%)                         | 23 (92%)                         |
| Tumor grade                   | IDC                              | ILC                              | IDC                              |
| Grade                          | 22 (88%)                         | 18 (72%)                         | 18 (76%)                         |
| DCIS                           | 2 (8%)                           | 2 (8%)                           | 4 (16%)                          |
| Alveolar                      | 1 (4%)                           | 0                                | 2 (8%)                           |
| Benign                        | 0                                | 0                                | 0                                |
| State of patient              |                                  |                                  |                                  |
| Re-Surgery after Re-currency  | 1 (4%)                           | 0                                | 2 (8%)                           |
| Re-Surgery due to involved margin | 0                                | 1 (4%)                           | 1 (4%)                           |
| Surgery after neoadjuvant     | 24 (96%)                         | 3 (12%)                          | 5 (20%)                          |
| First Surgery                 | 21 (84%)                         | 17 (68%)                         |                                  |

**Diagnoses for the 150 IMs**

|                  | CDP          | Frozen       |
|------------------|--------------|--------------|
| TP               | 4            | 0            |
| TN               | 143          | 133          |
| FP               | 2            | 0            |
| FN               | 1            | 0            |
| Accuracy         | 99%          | 97%          |
| Sensitivity      | 99%          | 97%          |
| Specificity      | 99%          | 97%          |
| Selectivity      | 99%          | 97%          |

**Figure 5**

a) The baseline of three clinical trials characteristics and overall study outcomes, H&E images of IMs positively scored by CDP, b) Anterior margin of Patient ID 114 which was reported as free margin in frozen section but was confirmed as IDC nuclear grade 2 on its reciprocal EMs by permanent pathology, c) Inferior margin of Patient ID 138 while Frozen declared free margin on its reciprocal margin (EM-) but Permanent pathology diagnosed margin involvement to DCIS on the same EM, d) IDC grade 2/DCIS lesions found in an internal margin that positively scored by CDP while Frozen declared free margin on its reciprocal margin (EM-) but Permanent pathology diagnosed margin involvement on the same EM (Patient ID:143), e) LIN2 lesion which CDP score on IM was positive (ID 145), Frozen on reciprocal EM was negative, and Permanent on reciprocal EM was negative, Medial margin of Patient ID 183 positively scored by CDP but Permanent pathology diagnosed margin involvement to ADH lesion on the same EM and confirmed CDP, Receiver operating characteristic (ROC) diagram for j) CDP, and k) Frozen vs. Permanent pathology for total 450 EM and IM margins on 75 patients in the three clinical trials.

**Supplementary Files**

This is a list of supplementary files associated with this preprint. Click to download.

- [CDPUsersmanual.v.En7.0.pdf](#)
- [SupMovie1MarginDetectionprotocolbyCDP.mp4](#)
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