Clinica Chimica Acta xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Investigation of potential breath biomarkers for the early diagnosis of breast cancer using gas chromatography–mass spectrometry

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ARTICLE INFO

Article history:
Received 26 November 2013
Received in revised form 9 April 2014
Accepted 23 April 2014
Available online xxxx

Keywords:
Breast cancer
Early diagnosis
Noninvasive diagnosis
Biomarkers
Breath analysis

ABSTRACT

Background: Breast cancer (BC) remains the most commonly diagnosed malignancy in women. We investigated 4 straight aldehydes in the exhaled breath as potential early BC diagnostic biomarkers.
Methods: End-tailed breath were collected by Bio-VOC® sampler and assayed by gas chromatography–mass spectrometry. Kruskal–Wallis one-way analysis of variance test and binary logistic regression were used for data analysis. The diagnostic accuracies were evaluated by receiver operating characteristic curves. A predictive model/equation was generated using the 4 biomarkers and validated by leave-one-out cross-validation.
Results: All four potential biomarkers demonstrated significant differences in concentrations between BC and healthy controls (HC) ($p < 0.05$). The areas under the curves (AUCs) in HC vs $BC_{I–II}$ model using hexanal, heptanal, octanal, and nonanal were 0.816, 0.809, 0.731, and 0.830, respectively. The AUC for their combined use was 0.934 (sensitivity 91.7%, specificity 95.8%) in the early diagnosis of BC. The predictive model/equation exhibited good sensitivity (72.7%) and specificity (91.7%) in distinguishing between HC and BC (cross-validation: sensitivity 68.2% and specificity 91.7%).
Conclusions: The diagnostic values of 4 exhaled straight aldehydes as early diagnostic biomarkers for BC were successfully verified and the diagnostic accuracy improved in their combined use.

1. Introduction

Breast cancer (BC) remains the most commonly diagnosed malignancy and the second leading cause of cancer-related deaths in women [1]. Early diagnosis is very important for reducing the BC mortality rate. However, the early diagnosis of BC is limited because the disease usually develops asymptomatically, while the conventional diagnostic techniques still have some shortcomings [2]. Therefore, there is still an urgent clinical need to improve or alternate these methods. The combination of molecular screening methods with these conventional methods may help improve this situation [2]. Cancer cells release molecular biomarkers into the blood during their growth, which can provide prognostic symbols that could be useful in early cancer diagnosis through breath, blood, and urine analyses [3–6]. Breath analysis is noninvasive, easy to perform, painless, and present no risks, thus it may allow the development of convenient and safe method that could complement blood and urine tests [7].

Human exhaled breath (EB) mainly comprises N_2, CO_2, O_2, water vapor, inert gases, volatile organic compounds (VOCs) and nonvolatile substances [8,9]. These VOCs are either produced endogenously via metabolism or absorbed exogenously from the environment. Some additional compounds are produced and/or their concentrations are changed in pathological states of the body [10]. Endogenous biomarkers are commonly used for diagnostic purposes.

There is a considerable experimental evidence that the volatile components in EB can be used to detect neoplasms [9,11,12]. The theoretical basis for diagnosing BC based on breath analysis is speculative as follows. BC is accompanied by increased oxidative stress (OS), which can lead to DNA, proteins and lipid damages. The induction of cytochrome P450 enzymes leads to lipid peroxidation of polyunsaturated fatty acids in the cell membranes and resulting in overexpression of volatile alkanes and alkane-derivatives in the breath, and eventually affecting the abundance of VOCs in the EB [13–15]. Besides, free radical-induced oxidation of amino acids and proteins has also been demonstrated to result in the generation of some hydrocarbons along with malonaldehyde [16–18].

Only a limited number of studies have been performed to investigate the feasibility of BC diagnosis based on breath analysis. The published data show that the patterns of VOCs in the EB can distinguish patients with and without BC [19–21]. Unfortunately, the reported potential

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http://dx.doi.org/10.1016/j.cca.2014.04.030
0009-8981/

breath biomarkers for BC differ among studies [22]. Therefore, there is still a need to continue searching breath biomarkers for BC.

The growing interest in the discovery of breath biomarkers of the OS associated with lung cancer, especially straight aldehydes [23–25], led us to consider whether straight aldehydes are potential effective breath biomarkers for BC. Besides, there have been articles reported that formaldehyde [26] and heptanal [20] may be potential BC breath biomarkers. Aldehydes are secondary breakdown products of lipids oxidation [27,28]. In particular, C5–C10 monofunctional aldehydes (including hexanal, heptanal, octanal, and nonanal) have been identified to be derived from lipid peroxidation [28]. The well-understood biochemical pathways of these aldehydes simplify the evaluation of their diagnostic values. In addition, volatile aldehydes have low solubility in the blood, so they are excreted into the breath within minutes after their formation in tissues [9,29]. Furthermore, hexanal, heptanal, octanal and nonanal were relatively stable than low molecular weight aldehydes and they all could be detected by our method. Moreover, we found that their levels seem to be different between HC and BC in our preliminary experiment results. Therefore, among a variety of straight aldehydes, we choose to study hexanal, heptanal, octanal and nonanal.

Elevated levels of aldehydes imply enhanced OS and they have been proposed as a measure for cancer status diagnosis [30–32]. Moreover, the pattern of aldehydes appeared to be unique to each form of cancer [31]. Hexanal, heptanal, some alkanes, alkane derivatives, and benzene derivatives [33], pentanal, hexanal, octanal, and nonanal [24] were reported to be potential breath biomarkers for lung cancer. Furthermore, formaldehyde [26] and heptanal [20] was also reported as potential breath biomarkers for BC. Therefore, we and other researchers have proposed that exhaled aldehydes are potential diagnostic biomarkers for various human cancers, including BC [20,22,24,26,33,34].

Although Fuchs et al. [24] found out that exhaled aldehydes could be used as lung cancer biomarkers, they only focus on lung cancer. Peng et al. [35] achieved the discrimination between 'healthy' and 'cancerous' breath in a variety of human cancers (including lung, breast, colorectal, and prostate cancers) by a nanosensor array. To the best of our knowledge, no previous studies have evaluated in detail the clinical value of identifying BC using specific volatile straight aldehydes in EB by simple direct gas chromatography–mass spectrometry (GC–MS) analysis without any preconcentration.

Therefore, we designed a systematic study to investigate and identify novel potential breath aldehyde biomarkers for the early diagnosis of BC. Based on literature survey [22–24,31,32] and preliminary experiment results, we choose hexanal, heptanal, octanal and nonanal (which were relatively stable than low molecular weight aldehydes, and were often reported as potential tumor biomarkers, as well as their biochemical pathways were well understood) as our target compounds.

2. Materials and methods

2.1. Chemicals and calibration standards

Hexanal (98%) was obtained from Sigma-Aldrich. Heptanal (97%), octanal (99%), and nonanal (96%) were from Aladdin Chemistry. Absolute ethanol (analytical grade) was purchased from Chengdu Tianhua Chemical Industry. The standard aldehyde solutions were prepared in absolute ethanol.

The standard stock aldehyde solution mixtures (hexanal: 3.98×10^{-2} mol/l, 2.39×10^{-2} mol/l, 1.59×10^{-2} mol/l, 7.96×10^{-3} mol/l, 1.99×10^{-3} mol/l, 9.95×10^{-4} mol/l, 7.96×10^{-5} mol/l; heptanal: 3.61×10^{-2} mol/l, 2.17×10^{-2} mol/l, 1.44×10^{-2} mol/l, 7.22×10^{-3} mol/l, 1.81×10^{-3} mol/l, 9.02×10^{-4} mol/l, 7.22×10^{-5} mol/l; octanal: 3.17×10^{-2} mol/l, 1.90×10^{-2} mol/l, 1.27×10^{-2} mol/l, 6.34×10^{-3} mol/l, 1.59×10^{-3} mol/l, 7.92×10^{-4} mol/l, 6.34×10^{-5} mol/l; nonanal: 2.79×10^{-2} mol/l, 1.67×10^{-2} mol/l, 1.12×10^{-2} mol/l, 5.58×10^{-3} mol/l, 1.40×10^{-3} mol/l, 6.98×10^{-4} mol/l, 5.58×10^{-5} mol/l) were prepared by diluting 50 μl of the respective liquid aldehydes with ethanol and making subsequent dilutions.

Standard aldehyde gaseous mixtures were produced by injecting 10 μl of the stock solution mixtures into 3 L Tedlar bags, which were prefilled with 3 l of high purity nitrogen via septa. Before use, the Tedlar bags were flushed with high purity nitrogen five times and the residual gas was evacuated. Compared to standard Tedlar bag handling procedures [36], we did not heat the Tedlar bag filled with N_2 at 85 °C for 8 h in the bag cleaning process. Because standards and real samples were stored in different Tedlar bags with different sizes in our experiment, and no residual contamination interference was found in our study or other literatures [37], thus, we simplified the clean procedures of Tedlar bag. The gas volumes in the Tedlar bags were measured using a mass flow controller. We found that 20 min at room temperature (approximately 20 °C) was sufficient for the standards to evaporate completely.

Indoor air is an unsuitable sample matrix because the background levels of aldehydes are relatively high and fluctuating. Nevertheless, it has been reported that the composition of the matrix did not affect aldehyde determinations [37]. Therefore, the calibrations were performed using high purity nitrogen.

2.2. Human subjects

Three subject groups were studied: 24 HC, 17 breast benign tumor (BBT) patients, and 22 BC patients (five at stage I, seven at stage II, nine at stage III, and one at stage IV). All of the subjects were Chinese females who had no history of smoking.

The patients were recruited from the Galactophore Department of China West Hospital of Sichuan University. The patients were selected randomly. Subjects were eligible to participate if they were ≥18 years old, and if clinical breast examination or imageological examination exhibited breast neoplasm. All patients' EB samples were collected before surgery, radiotherapy or chemotherapy. The patient diagnoses were all confirmed by the postoperative pathological diagnoses. The HC were healthy volunteers with no medical history of malignancy or clinically significant breast disease. Subjects were eligible to participate if they were aged ≥18 years and had no history of previously diagnosed cancer at any site. Informed consent was obtained from each subject at the time of enrolment. The study was approved by the Ethics Committee of Sichuan University and conducted in accordance with the Declaration of Helsinki.

2.3. Breath collection apparatus and sample storage device

The end-tidal breath samples were collected from the human subjects using a Bio-VOC® sampler (Markes International Ltd.) which is a preproduction device based on a syringe made from an inert plastic and fitted with a one way valve piston [38]. The subject blows through the sampler syringe and the last portion of breath is retained in the sampler, and subsequently the sample is transferred by pushing the piston with a 94% transfer efficiency and no significant loss of analyte or contamination [38]. The Bio-VOC® sampler collects a representative sample of end-tidal air (approximately 150 ml), without contaminating or diluting with breath from the bronchial tubes or mouth. It can even be used to collect breath samples from a small child and elderly or bedridden patients, without causing discomfort. Compared to breath sampling equipment with standard CO_2 trigger valves [39], the Bio-VOC® sampler is more simple, miniature, and do not need any electronic power supply, therefore, it is more flexible. Moreover, its usage is simpler and desired end-tailed breath samples can be obtained without any professional operators.

The breath sampling for the subjects was done in a separate room after participants had rested for 10 min. The sampling room had good ventilation and no obvious chemical pollution source. The subjects were asked to exhale a single slow breath through a disposable

Please cite this article as: Li J, et al, Investigation of potential breath biomarkers for the early diagnosis of breast cancer using gas chromatography–mass spectrometry, Clin Chim Acta (2014), http://dx.doi.org/10.1016/j.cca.2014.04.030