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LETTER TO THE EDITOR

rs11046147 mutation in the promoter region of lactate dehydrogenase-B as a potential predictor of prognosis in triple-negative breast cancer

Dear Editor,

Triple-negative breast cancer (TNBC) is a heterogeneous disease characterized by a deficiency in the expression of three well-known biomarkers of breast cancers, namely estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Its treatment has become an important clinical challenge because TNBC is more aggressive than other breast cancer subtypes, and is usually associated with higher tumor recurrence and lower survival rate [1-3]. Breast cancer patients with ER, PR, and/or HER2 positive tumors often respond positively to targeted therapies whereas TNBC patients remain unresponsive to conventional treatment due to lack of specific biomarkers for determining appropriate treatment (e.g. trastuzumab-based therapy). Hence, identifying novel and effective biomarkers for predicting TNBC prognosis and to improve their survival has become an urgent need for developing effective targeted therapy against TNBC.

Currently, the targeting agents of breast cancer generally include epidermal growth factor receptor, vascular endothelial growth factor, and polymerase inhibitors. However, these seem to be ineffective for TNBC [4]. To address this issue, several molecular hallmarks of TNBC have been clarified through genome-wide association studies. One of these studies identified several loci correlated with TNBC risk and confirmed numerous single nucleotide polymorphisms (SNPs) in 25 loci with significant associations with TNBC prognosis [5]. This provides important opportunities and resources to mine effective prognostic markers against TNBC. However, most of these identified SNPs do not show higher frequency in TNBC, as compared to other subtypes. For instance, SNPs in *BRCA1* (a common biomarker of breast cancer) are only present in 7%-18% of TNBC patients [6-8], which cannot be therefore used as a prognostic marker for TNBC. Nonetheless, these results highlight the needs for searching markers from other perspectives, like gene expression shifts or mutations occurring in regulatory regions.

It has been well known that the increased in glycolysis flux is one important hallmark of carcinogenesis [9-11]. TNBC also exhibits very high glycolysis activity [12, 13]. This activity, in turn, gives rise to superfluous lactate production due to increased activity of lactate dehydrogenase (*LDH*), which is encoded by the *LDHA* and *LDHB* genes [13]. Importantly, McCleland *et al*. [14] revealed that *LDHB* expression was significantly up-regulated in TNBC, and *LDHB* knocked-down could significantly decrease TNBC proliferation [14]. Some previous studies further showed that *LDHB* was commonly silenced via promoter hypermethylation in other types of carcinomas, particularly in gastric, pancreatic [15], and prostate cancers [16]. Given the expression level of a gene is highly correlated with its promoter sequences, to which one or more transcription factors can bind [15, 16], this study aimed to address three important questions: (1) whether the up-regulation of *LDHB* is a common feature of TNBC, (2) what is the molecular mechanisms underlying the enhanced expression of *LDHB* in TNBC, and (3) whether there are potential prognostic markers in the promoter region of *LDHB*.

In this study, we sequenced and analyzed the full-length *LDHB* promoter region (160 bp segment: from -3070 bp to -2910 bp) from 90 TNBC and 110 non-TNBC patients (ER, PR and/or HER2 positive), as well as 169 healthy Han Chinese as a test control. All the samples, including TNBC, non-TNBC, and controls, were obtained from participants born and residing in the Guangdong province (China), which guaranteed

Abbreviations: ENCODE, encyclopedia of DNA element; ER, estrogen receptor; FFPE, Formalin-fixed and paraffin-embedded; GO, gene ontology; HER2, human epidermal growth factor receptor 2; LC-MS, liquid chromatography-mass spectrometry; *LDH*, lactate dehydrogenase; PR, progesterone receptor; ROS, reactive oxygen species; SNP, single nucleotide polymorphism; TAF7, TATA-box binding protein associated factor 7; TCA, tricarboxylic acid; TCGA, the Cancer Genome Atlas; TF, transcription factor; TNBC, triple-negative breast cancer.

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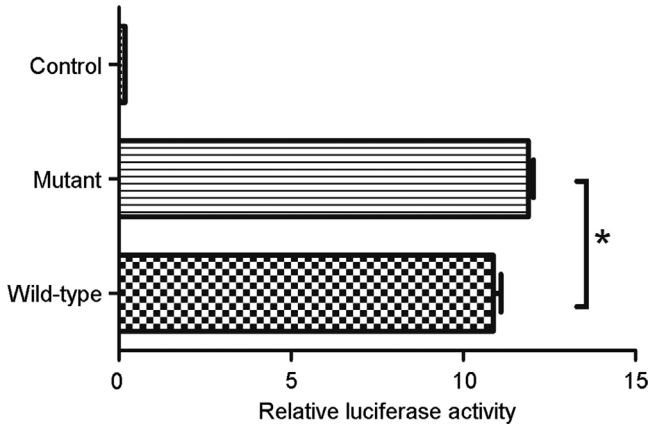

FIGURE 1 Dual-luciferase reporter assays in MDA-MB-468 cells with transfection of luciferase reporter constructs containing either the wide-type LDHB promoter fragment or mutant LDHB promoter fragment. The cells transfected with pGL3-basic vector were used as control. Three independent experiments were done with triplicates each time.*, $P < 0.05$

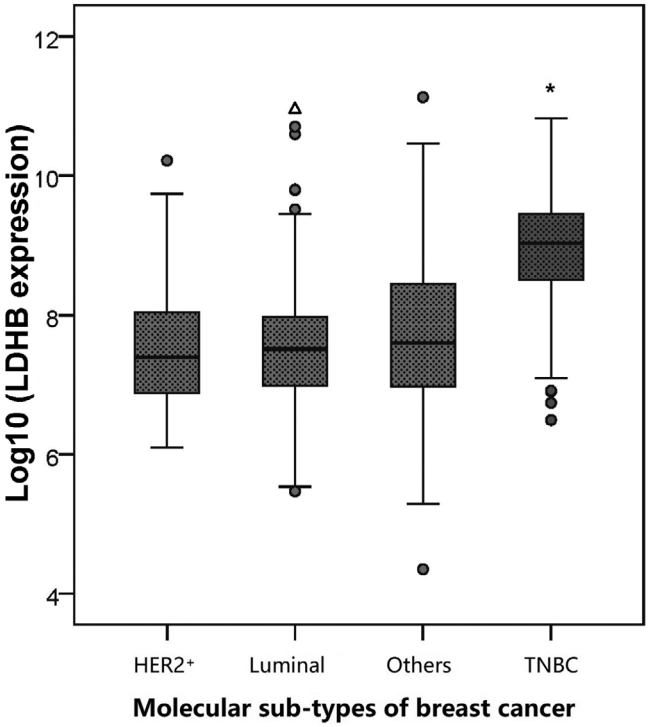

FIGURE 2 The expression levels of *LDHB* in different molecular sub-types of breast cancer. The expression values were obtained from the TCGA database. The box defines the data within its upper quartile and lower quartile. The line within the box defines the median value. Triangle defines a potential "outlier" value of the data. *, $P < 0.05$. Abbreviation: TCGA, the Cancer Genome Atlas; TNBC, triple-negative breast cancer; HER2, human epidermal growth factor receptor 2

high similarity of their genetic background. All the samples were collected as formalin-fixed and paraffin-embedded (FFPE) tissue sections. We sequenced bidirectionally *LDHB* promoter region using BigDye DNA-sequencing kit (Applied Biosystems, Foster City, California, USA) to avoid cases of artificial mutations (details available in Supplementary Information).

We compared the *LDHB* promoter sequences among the three groups of samples and finally identified a prevalent variant G > A (rs11046147, 12:21657712) in the TNBC samples. This variant in *LDHB* promoter demonstrated two-fold higher enrichment in TNBC patients (53/90, 58.9%) as compared to non-TNBC patients (31/110, 28.2%) and healthy controls (27/169, 16.0%). To further evaluate if this variant could alter the promoter activity of *LDHB*, we performed a dual-luciferase reporter assay with a TNBC cell line (MDA-MB-468). Our results showed that the G > A mutation significantly enhanced *LDHB* promoter activity ($P = 0.010$; Figure 1). Next, we compared the expression profiles available in the Cancer Genome Atlas (TCGA) database (https://portal.gdc.cancer.gov/), including 112 and 925 samples for TNBC and non-TNBC patients, respectively. We found that the *LDHB* expression level was significantly higher in TNBC with the median expression level 9.032 (range: 6.494-10.829) versus 7.485 (range: 4.352-11.057) in non-TNBC ($P < 0.05$; Figure 2). Consistent with the study from McClelland *et al*. [14], this result demonstrated that the expression of *LDHB* was up-regulated in TNBC, which could be considered as a common feature of TNBC.

In order to explore the underlying mechanism of rs11046147 G > A mutation affecting the expression level of LDHB in TNBC, we further employed DNA pulldown and liquid chromatography-mass spectrometry (LC-MS) to detect proteins potentially interacting with the rs11046147 G > A mutation. We found that a total of 163 and 325 proteins specifically bonded to the wild-type and mutant probes, respectively (with 388 overlapping proteins binding to both probes; Supplementary Table S1). We further performed gene ontology (GO) enrichment analysis with these protein symbols to better understand their molecular functions. We found that 38.1% of the proteins specifically binding to wild-type probes and 35.3% of the proteins specifically binding to mutant probes, belong to a GO term named "nucleic acid binding proteins".

Additionally, the results of LC-MS also showed that there were more proteins specifically binding to the mutant promoter (325 specifically bind to the mutant promoter versus 163 specifically binding to the wild-type promoter), indicating the rs11046147 G > A mutation allowed the mutant promoter to attract more regulatory proteins than the wild-type promoter. Indeed, among the 325 specific interacting proteins of mutant promoter, we identified 42 transcription factors (TFs) that were bounded to the 15-bp mutant *LDHB* promoter region through LC-MS experiment (Supplementary Table S2). This might be the underlying mechanism of the

enhanced expression of *LDHB* in TNBC. To determine the most likely TF affecting the expression of *LDHB*, we further compared our results to the annotation results from the encyclopedia of DNA elements (ENCODE) project (https://www.encodeproject.org/) whose aim is to build a comprehensive parts list of functional elements in the human genome. Based on the ENCODE annotations, there were 28 TFs binding to the rs11046147 loci, all of which have been confirmed by other studies through chromatin immunoprecipitation followed by sequencing (Supplementary Table S3). Of these 28 TFs, only TATA-box binding protein associated factor 7 (TAF7) was identified in our study, indicating that TAF7 was most likely to upregulate LDHB expression via its mutant promoter.

Finally, we wondered whether the rs11046147 G > A mutation could be a potential prognostic marker of TNBC. To undermine this, we should further understand the potential roles of the *LDHB* variant in TNBC tumorigenesis, which could be also essential for the development of therapeutic strategies. It is well known that TNBC expresses both glycolysis and mitochondrial metabolism-related proteins, but its glycolysis flux is much higher than that of non-TNBC [12]. Moreover, the high glycolysis flux glycolytic form is the most common phenotype of basal-like breast cancer (a subtype of TNBC) [12]. This may be related to the high lactic acid production of TNBC which can promote tumor formation and reactive oxygen species (ROS) production. A similar lactate metabolism mechanism has also been observed in human lung cancer [17]. This evidence indicating that superfluous lactate production in cancer cells could be recycled into the cancerous tricarboxylic acid (TCA) cycle, which could further promote oncogenotypes [17]. As a predominant LDH isoform, *LDHB* exhibits lower affinity for pyruvate and greater sensitivity to substrate inhibition by pyruvate than *LDHA* [14]. It also primarily functions in the conversion of lactate to pyruvate. Therefore, the *LDHB* variant, as a potential proxy of the tumors that overexpress LDHB, provides an alternative means of energy generation and increases metabolic flexibility of cells to adapt to the tumor microenvironment [14]. Similar to our *LDHA* expression results (Supplementary Figure S1), a previous study has demonstrated that the *LDHA* expression level had no significant difference between TNBC and non-TNBC patients [14]. However, this study still observed that LDHA played an extremely important role in tumor metabolism and growth of breast cancer. Subsequent studies further showed that *LDHB* may play a vital role in the conversion of lactate to pyruvate in TNBC cells [18, 19].

In conclusion, we identified a *LDHB* variant (rs11046147, 12:21657712) that was prevalent in TNBC patients by comparing large-scale promoter sequences. Additionally, this variant may have crucial functions in TNBC tumorigenesis by enhancing promoter activity via recruiting additional TFs. Our study provides a new genetic hallmark (*LDHB* variant) with higher prevalence than the *BRCA1* variant in TNBC patients. It also provides a potential target to discover efficient predictive factors of TNBC, given that ~60% of TNBC patients carry the *LDHB* promoter mutation. Thus, our findings, to some extent, lend support to the increased oncogenic risk in the presence of high *LDHB* expression. Nonetheless, further investigations are still needed to unravel the pathogenesis and therapeutic approaches of TNBC.

DECLARATIONS

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, and was approved by the Ethics Committee of the Seventh Hospital affiliated to Sun Yat-sen University. Each participant signed an informed consent before participating in this study.

CONSENT FOR PUBLICATION

Consents for publication were obtained from all patients or his/her guardians.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analyzed during this study are included in this published article and its supplementary information files.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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AUTHORS' CONTRIBUTIONS

Q.P.K., Z.Y., and J.L. designed the study. J.L., Y.L., X.Q.C., C.S., and X.L.S. performed the molecular and cytology experiments. J.L. and Y.L. analyzed the data. J.L., Y.L., and Q.P.K. wrote the manuscript. All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

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