NMR Metabolomic Analysis on Sh-TINCR of MDA-MB-231 and HS578T Cell Lines: BHMT as a Potential Biomarker in TNBC Patients

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Abstract. The poor outlook for triple-negative breast cancer (TNBC) is due in part to the absence of effective targeted treatments and the variability in clinical response to standard chemotherapy. Previously, we saw positive outcomes in reducing cancer cell survival on TINCR-knocked out cells and in this study TNBC cell lysates and culture medium were subjected to NMR metabolomic analyses. Spermine, Nicotinic acid, Cystathionine and Pyridoxal metabolites were found perturbed by the knocked-out process. This suggests that biosynthesis of cofactors metabolism took place and downstream analysis suggested BHMT protein could have potential for monitoring or prognostic biomarkers in TNBC cancer patients.

Keywords: BHMT, Metabolomics, TINCR, TNBC.

1.0 Introduction

NMR metabolomic analysis has emerged as a powerful tool in cancer research [1]. Recently, researchers analyzed silencing Terminal differentiation-induced non-coding RNA (sh-TINCR) of MDA-MB-231 and HS578T using NMR metabolomics to potential biomarkers in triple-negative breast cancer (TNBC) patients. The study found that BHMT (Betaine- homocysteine S-methyltransferase) could as a potential biomarker for TNBC patients, as its expression was significantly altered in the cells underwent sh-TINCR. These findings could potentially lead to the development of new and therapeutic approaches for TNBC patients, which currently lack targeted therapies. The NMR metabolomic analysis allowed for a comprehensive understanding of the metabolic changes that occur in TNBC patients, providing valuable insights into the disease's pathogenesis and potential treatment options [2].

In this study, the NMR metabolomic analysis was conducted on sh-TINCR of MDA-MB-231 and HS578T cell lines to potential biomarkers for triple-negative breast cancer (TNBC) patients. The cells were cultured in a suitable and treated according to the experimental design, followed by extraction and NMR analysis of the metabolites. The resulting spectra were processed and analyzed using multivariate statistical, and metabolites were using standard reference databases. The identification and

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quantification of metabolites were performed by comparing the spectral with reference spectra. The obtained were analyzed using various statistical methods, including pathway analysis, to identify biomarkers.

2.0 Literature Review

Nuclear magnetic resonance (NMR) metabolomics is a powerful and widely used analytical technique that allows for the identification and quantification of small molecule metabolites in systems [3]. provides a comprehensive snapshot of metabolic activity and can be used to potential biomarkers of disease, including cancer. this technique, samples are analyzed NMR spectroscopy to the unique chemical signatures of metabolites in each sample. This data can be used to build metabolic profiles for different biological systems and identify changes in metabolic pathways associated with disease. Overall, NMR metabolomics is a valuable tool for understanding metabolic regulation and identifying targets for biomarker discovery and drug development.

TNBC accounts for about 10-20% of all cancer cases and is characterized by absence of estrogen receptor (ER), progesterone receptor (PR), and epidermal growth factor receptor 2 (HER2) expression [4]. TNBC is an aggressive breast cancer and is associated with a poorer prognosis compared to other subdued to its high rate of metastasis and increased resistance to and targeted therapies [5]. TNBC is predominantly diagnosed in younger women particularly those of African descent, is more prevalent in developing. Due to the lack of hormonal and HER2 receptors, there limited treatment options available for TN patients, making the identification new biomarkers and therapeutic targets crucial for improving patient outcomes.

Terminal differentiation-induced non-coding RNA (TINCR) is a long non-coding RNA that plays a crucial role in the regulation differentiation and cell fate determination. Recent studies indicate that TINCR expression is altered in TNBC) and may play a role in the progression of this aggressive subtype of breast cancer [5]. In TNBC, TCR promotes the proliferation, survival, and migration of metastatic cancer cells. Additionally, TINCR may function as a mediator of chemoresistance in TNBC. These findings suggest that TINCR could potentially serve as a diagnostic and therapeutic target for the management of TNBC. Investigation is needed to determine the full extent of TINCR role in TNBC and as a therapeutic biomarker.

3.0 Methodology

3.1 Cell Culture and Transduction sh-TINCR Lentivirus

Cell culture techniques are an essential component of this NMR metabolomic analysis. The MDA-MB-231 and Hs578T cell lines were cultured in specific media in a controlled culture medium. Next, these cells were transduced with the sh-TCR lentivirus over a specific long non-coding RNA region which is known as TINCR. The transduction efficiency was confirmed using a fluorescence microscope and qPCR analysis.

3.2 1HNMR Metabolomic Analysis

NMR spectra is acquired on a 600 MHz Bruker Avance III HD/NEO NMR spectrometer equipped with a 5 mm BBI probe. 1D NMR spectra were recorded using a NOESY-presaturation pulse sequence (noesygpprpr1d) with a spectral width of 30 ppm and 98304 data points. The number of scans was set to 16, relaxation delays to 10s and temperatures to 310K for serum samples. All obtained spectra of serum samples are Fourier-transformed with TopSpin software version 4.0 (Bruker Biospin, Billerica, USA) [6].

3.3 Data Processing and Statistical Analysis

All spectrum data pre-processing analysis was done using NMRProcFlow software. It involved calibration, normalization, baselining, scaling and bucketing process before subjected to statistical analysis using MetaboAnalyst 5.0 software. This was followed next step by performing multivariate statistical analysis, which involved principal component analysis (PCA) and partial squares-discriminant analysis (PLS-DA) to identify important feature that discriminate the treated and un-treated groups. Statistical analysis was critical in understanding the NMR metabolomic profiling of sh-TINCR in MDA-MB-231 and HS578T.

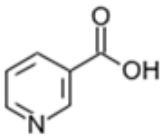
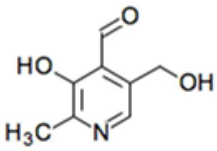
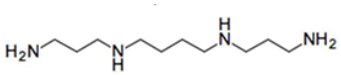
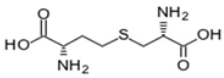
3.4 Network Analysis

The list of metabolites that was identified using Biological Magnetic Resonance Data Bank (BMRB) then underwent metabolite-protein interaction analysis using Human Metabolome Database (HMDB). All listed protein/gene was then further analyzed to identify the hub gene using GeneMANIA software which freely available online.

4.0 Findings

Metabolic pathway perturbation plays a critical role in the progression and treatment of Triple Negative Breast Cancer (TNBC) cell lines. Several metabolites are known to be involved in these metabolic pathways, including spermine, cystathionine, nicotinic acid (also known as niacin), and pyridoxal.

Table 1. Metabolite that most affected during sh-TINCR process.

Analysis	MDA-MB-231	HS578T
	Nicotinic acid	Pyridoxal
Metabolite Fingerprinting	 C00253	 C00250
	Spermine	Cystathionine
Metabolite Footprinting	 C00750	 C02291

Spermine, a polyamine essential for cellular growth, redox regulation and DNA synthesis, can be disrupted in TNBC, leading to altered cell growth [7]. In the meantime, cystathionine metabolism can affect the balance of sulfur-containing amino acids, which can lead to oxidative stress that resulted to TNBC progression. Both Nicotinic acid and Pyridoxal play a critical role in metabolic pathways, including the synthesis of NAD^+. Modulating nicotinic acid metabolism can affect the redox balance within TNBC cells and potentially be used for therapeutic purposes. While targeting pyridoxal-related pathways may offer strategies to disrupt TNBC progression. Interestingly, metabolite that involve in fingerprinting analysis insinuate the energy production is insulted by the sh-TINCR process and for the footprinting portray that DNA methylation process was inflicted and cellular ROS homeostasis fluctuation occur.

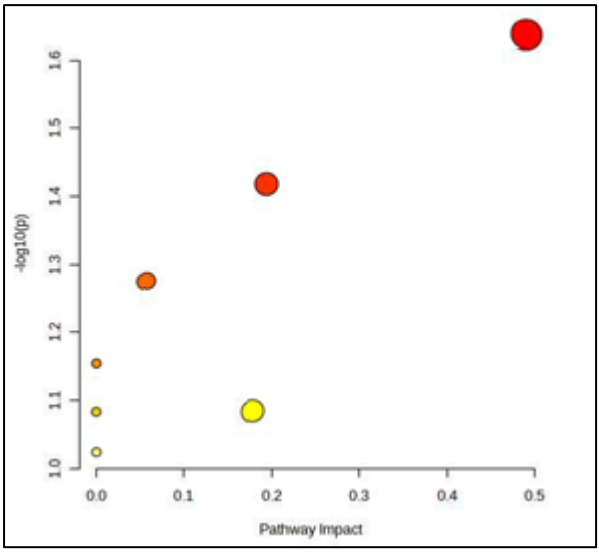


Fig. 1. Metabolic pathway perturbation sn_TINCR TNBC cell lines. Red – high impact, White – Less impact.

One of the most affected metabolic pathways for the sh-TINCR TNBC cell lines identified is Vitamin B6 metabolism, shown in Figure 1. This metabolic pathway is essential for amino acid metabolism and neurotransmitter synthesis also this finding supports the study done by Xiao et al. [8] who found out that metabolism of cofactors and vitamins is altered in TNBC patients. Dysregulation of pyridoxal level can disrupt the availability of active vitamin B6, impairing enzymes that rely on it [9]. This can lead to imbalances in essential amino acids and neurotransmitter synthesis. In addition, the dysregulation of nicotinic acid and nicotinamide can disrupt NAD^+ synthesis, potentially promoting oxidative stress and DNA damage [10]. Another interesting find is the, beta-alanine metabolism that can hinder carnosine synthesis, impacting muscle function and endurance. Another finding that can be of interest based on comparing the silenced cell lines and the parental types are on cysteine and methionine metabolism which act as essential amino acids for protein synthesis, antioxidant defense, and one-carbon metabolism. These imbalances further lead to DNA methylation and cell proliferation.

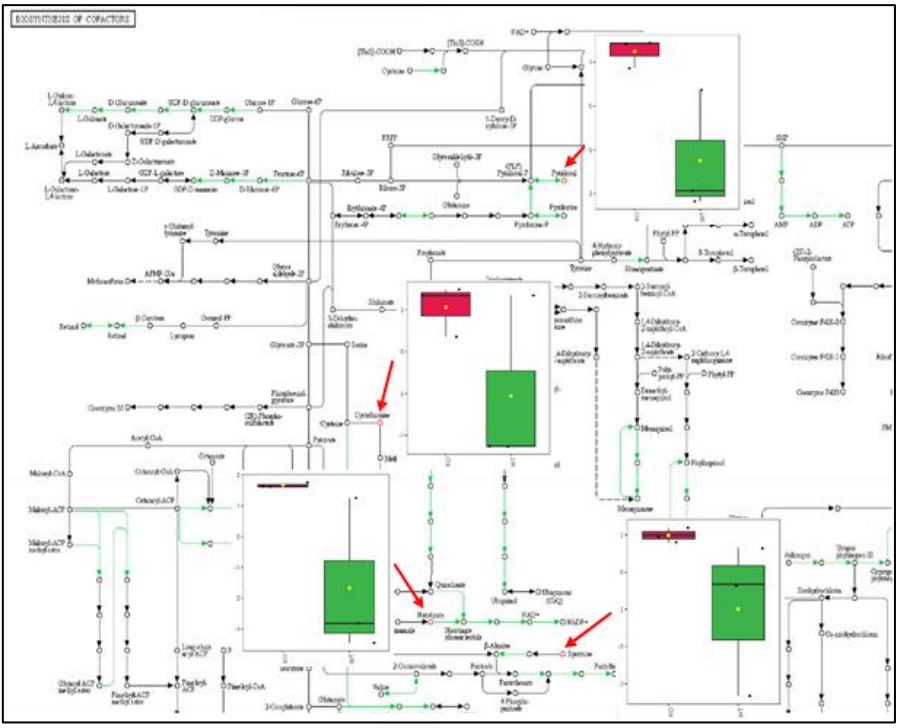


Fig. 2. Biosynthesis of cofactors metabolic pathway that involved in synthesize many vitamins in humans. (a) Pyridoxal (b) Cystathionine (c) Nicotinic acid (d) Spermine. Red – Knock-out cell, Green – Wild-type cell.

Figure 2 shows the 4 metabolites that were identified to be involved in biosynthesis of cofactors which flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD^+) synthesis were affected by the spermine concentration level. Inept of spermine levels can disrupt the synthesis of these cofactors, impacting various metabolic processes. Pyridoxal, the active form of vitamin B6, is also involved in cofactor biosynthesis, converting tryptophan to niacin (NAD^+), a precursor of NAD^+, deficiency can lead to disruptions in amino acid levels and impair the production of essential cofactors [11]. Cystathionine, an intermediate in the transsulfuration pathway, is essential for maintaining cellular redox balance and protecting against oxidative stress. Its dysregulation can disrupt cysteine production and affect glutathione availability, impacting cellular defense mechanisms against oxidative damage [12]. Nevertheless, nicotinic acid metabolite is essential for cellular metabolism and maintaining sufficient NAD^+ and $NADP^+$ pools for processes like glycolysis, the citric acid cycle, and DNA repair [10]. In a nutshell, putatively, all 4 upregulated metabolites that were identified can be used as a potential metabolic key to reduce or stop the progression of TNBC or its chemoresistance attribute.

Furthermore, metabolite-protein interactions are crucial in regulating metabolic pathways, including those involved in cofactor biosynthesis based on Table 2. The dysregulation of these interactions can lead to alterations in enzyme activity, potentially reducing the production of essential cofactors and impacting cellular metabolism. Many protein/gene were listed that interact with those 4 metabolites abovementioned. About 44 genes were identified to be involved and Spermine was the major contributor to the metabolic pathway perturbation accounting for 26 protein/gene that interacted with it. Meanwhile, Cystathionine only affects the metabolic pathway perturbation with 2 protein/gene that can be determined. Overall, metabolite-protein interactions play a pivotal role in extrapolating the potential metabolic pathway perturbations in sh-TINCR TNBC cell lines.

Table 2. Metabolite-Protein interaction (inhibitors/inducer/substrate) for sh-TINCR TNBC cell lines.

Metabolite	Gene Name	Protein Name	Locus
Pyridoxal	AOX1	Aldehyde oxidase	2q33
	PNPO	Pyridoxine-5'-phosphate oxidase	17q21.32
	GLDC	Glycine dehydrogenase [decarboxylating], mitochondrial	9p22
	PDXK	Pyridoxal kinase	21q22.3
	PDXP	Pyridoxal phosphate phosphatase	22q12.3
	PHOSPHO2	Pyridoxal phosphate phosphatase PHOSPHO2	2q31.1
Spermine	SAT2	Diamine acetyltransferase 2	17p13.1
	SAT1	Diamine acetyltransferase 1	Xp22.1
	XDH	Xanthine dehydrogenase/oxidase	2p23.1
	CHDH	Choline dehydrogenase, mitochondrial	3p21.1
	DMGDH	Dimethylglycine dehydrogenase, mitochondrial	5q14.1
	SMS	Spermine synthase	Xp22.1
	SRM	Spermidine synthase	1p36-p22
	ALD9A1	trimethylaminobutyraldehyde dehydrogenase	1q23.1
	BBOX1	Gamma-butyrobetaine dioxygenase	11p14.2
	ABP1	Amiloride-sensitive amine oxidase [copper-containing]	7q36.1
	MTAP	S-methyl-5'-thioadenosine phosphorylase	9p21
	AMD1	S-adenosylmethionine decarboxylase proenzyme	6q21
	GATM	Glycine Amidinotransferase, mitochondrial	15q21.1
	BHMT	Betaine—homocysteine S-methyltransferase 1	5q14.1
	SARDH	Sarcosine dehydrogenase, mitochondrial	9q33-q34
	HDC	Histidine carboxylase	15q21-q22
	ODC1	Ornithine decarboxylase	2p25
	SLC22A4	Solute carrier family 22 member 4	5q31.1
	SLC22A1	Solute carrier family 22 member 1	6q26
	SMOX	Spermine oxidase	20p13

	AGMAT	Agmatinase, mitochondrial	1p36.21
	TMLHE	Trimethyllysine dioxygenase, mitochondrial	Xq28
	BHMT2	S-methylmethionine—homocysteine S-methyltransferase BHMT2	5q13
	PDPR	Pyruvate dehydrogenase phosphatase regulatory subunit, mitochondrial	16q22.1
	OAZ1	Ornithine decarboxylase antizyme 1	19p13.3
	PMF1	Polyamine-modulated factor 1	1q12
Nicotinic Acid	QPRT	Nicotinate-nucleotide pyrophosphorylase [carboxylating]	16p11.2
	NNMT	Nicotinamide N-methyltransferase	11q23.1
	SLC16A1	Monocarboxylate transporter 1	1p12
	CYP2D6	Cytochrome P450 2D6	22q13.1
	SLC22A5	Solute carrier family 22 member 5	5q31
	SLCO2B1	Solute carrier organic anion transporter family member 2B1	11q13
	GPR109B	G-protein coupled receptor 109B	12q24.31
	GPR109A	G-protein coupled receptor 109A	12q24.31
	PNP	Purine nucleoside phosphorylase	14q13.1
	NAPRT1	Nicotinate phosphoribosyltransferase	8q24.3
Cystathionine	CTH	Cystathionine gamma-lyase	1p31.1
	CBS	Cystathionine beta-synthase	21q22.3

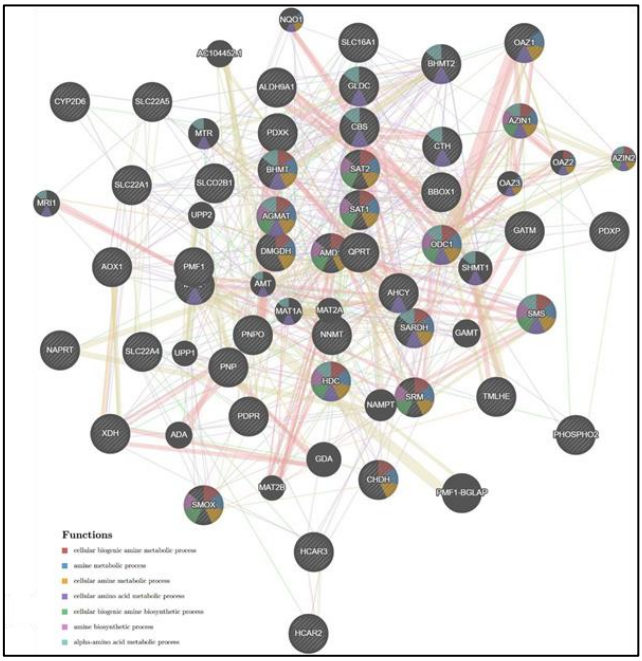


Fig. 3. Gene-gene interactions show BHMT gene as a hub gene and play crucial role in the sh-TINCR TNBC cell lines.

In Figure 3, BHMT gene was selected to become hub genes due to (i) no. of nodes connected = 19 and (ii) no. of functions involve = 5, which encodes the enzyme betaine-homocysteine methyltransferase, plays a vital role in one-carbon metabolism and indirectly in cofactor biosynthesis plus has significant implications for the metabolism of triple-negative breast cancer (TNBC) patients [13]. Nevertheless, BHMT gene encodes the enzyme betaine-homocysteine methyltransferase, which is primarily involved in the methionine cycle. Methionine is a precursor for S-adenosylmethionine (SAM), a critical cofactor involved in numerous methylation reactions in the cell, including DNA methylation [14].

DNA methylation plays a pivotal role in gene regulation and epigenetic modifications, influencing the expression of genes involved in various cellular processes [15]. Dysregulation or mutations in the BHMT gene can impact the methionine cycle, affecting the availability of methionine and SAM [16]. Hence, alteration of methionine and SAM levels could lead to changes in DNA methylation patterns in TNBC cells, potentially influencing the development, progression, and treatment response of TNBC.

Understanding the role of the BHMT gene in cofactor biosynthesis and epigenetic regulation may provide insights into potential therapeutic strategies for TNBC. All in all, by targeting BHMT gene it is the same as targeting the methionine cycle or epigenetic modifications, such as DNA methylation, could be explored as therapeutic approaches to modulate TNBC cell behavior and improve treatment outcomes.

5.0 Discussion

The results of the NMR metabolomic analysis on sh-TINCR of MDA-MB-231 and HS578T cell samples have identified BHMT as a potential biomarker in triple-negative breast cancer (TNBC). The study found that the knockdown of TINCR in two TNBC cell lines resulted in identification of BHMT. The association between TINCR and BHMT could be important in the approach to halt the progression of growth for TNBC. These provide valuable insights into the role of TINCR in TNBC and shed light on the potential use of BHMT as a therapeutic value for TNBC patients.

5.1 Differential Metabolites Identified by 1H-NMR analysis in sh-TINCR Knockdown Cells

Differential metabolites were identified through 1HMR analysis in sh-TINCR knockdown cells. NMR analysis revealed significant in the levels of several metabolites in sh-TINCR knockdown cells compared to control cells. The identified differential metabolites were primarily involved in amino acid metabolism, energy metabolism, and nucleotide metabolism. Notably, the levels of betaine homocysteine S-methyltransferase (BHMT), key enzyme involved in homocysteine metabolism [17], were found to be significantly altered in sh-TINCR knockdown cells. This finding

suggests that BHMT could be a potential biomarker for triple- negative breast cancer (TNBC) patients since TNBC is known to be associated with aberrant of methionine and homocysteine metabolism. The results of this study provide valuable insights into the metabolic alterations associated with TINCR down in breast cancer cells.

5.2 Central Dogma Network Interaction Analysis

The use of network analysis is a powerful tool in understanding biological systems, capturing complex interactions and identifying key players [18]. Even though only top 4 metabolites were identified and carried out to identify the metabolic pathway perturbation on TNBC cell lines, the core of the mechanism can be putatively drawn and subjected to further analysis using inhibitor and inducer to validate any initial inference. Hub gene was determined not merely based on the connected nodes, but the degree of functionality of the nodes also must be identified hence we get BHMT as the most connected node and most functional based on the interaction list.

5.3 BHMT as A Potential Therapeutic Biomarker for TNBC

Betaine-homocysteine methyltransferase (BHMT) has been identified as a potential biomarker for triple-negative breast cancer (TNBC) NMR metabolomic analysis on sh-TINCR of MDA-MB-231 and HS578T cells. BHMT is an enzyme involved in the regulation of homocysteine, which has been linked to various diseases, including cancer and cardiovascular disease [19].

6.0 Conclusion & Recommendations

The clinical application of BHMT as a potential therapeutic biomarker in TNBC patients presents both opportunities and challenges. On the one hand the development of a reliable biomarker for TNBC patients could greatly improve diagnosis and treatment options for this aggressive and difficult-to-treat cancer. BHMT has shown promise as a potential biomarker, as expression has been found to be significantly different in TNBC cells compared to non TNBC cells. However, on the other hand, there are challenges in clinical implementation of BHMT as a biomarker, such as reliable and accurate tests for its detection, ensuring appropriate patient selection and overcoming potential variability in expression among different TNBC patients Despite these challenges, the potential impact of BHMT as a therapeutic biomarker in TNBC is significant and warrants further.

NMR metabolomic analysis provides a powerful tool for the investigation of metabolic pathways in cancer cells. As the application of NMR metabolomics becomes increasingly widespread, the resolution and sensitivity of NMR techniques continue to improve, enabling deeper insights into networks in cancer cells. In recent years, increased efforts towards understanding metabolic alterations in TNBC have led to the identification of several biomarkers and therapeutic targets. The future perspectives of

NMR metabolomic analysis in TNBC research remain bright, with continued exploration metabolic pathways and identification of potential biomarkers for diagnostic and therapeutic purposes. The identification of BHMT as a potential biomarker for TNBC patients highlights the importance of NMR metabolomic analysis in the field of TNBC research and its potential impact on patient outcomes.

In conclusion, the NMR metabolomic analysis conducted on sh-TINCR of MDA-MB-231 and HS578T cells has shed light on the potential role of BHMT on TNBC cell lines. The findings of this study highlight the importance of understanding the metabolic pathways associated with sh-TINCR and the potential implications for the management and treatment of TNBC. Further investigations and validation studies are needed to confirm the potential of BH as a biomarker and to explore its clinical utility in TNBC patients. With increasing interest in personalized medicine and targeted therapies, the identification of reliable biomarkers like BHMT could pave the way for more effective and personalized treatment strategies for TNBC patients.

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