Original Research

Altered gene expression in glycolysis–cholesterol synthesis axis correlates with outcome of triple-negative breast cancer

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Impact statement

Although notable improvements have been made in diagnosis and treatment of triple-negative breast cancer (TNBC) in recent years, TNBC remains a refractory disease with high metastatic capability and mortality due to extreme heterogeneity and drug resistance. Therefore, new therapeutic strategies for TNBC treatment are urgently required. Deregulating cellular energetics is a hallmark of cancer. Different tumor subtype has its unique metabolic features and sensitivity to drugs. Classification of tumor subtypes based on metabolic characteristics may contribute to estimate prognosis, predict clinical efficacy, and design personalized treatment for patients with TNBC. We herein introduced a novel metabolic classification of TNBC into several subtypes according to the expression characteristics of glycolytic and cholesterogenic genes. Our results show that TNBC metabolic subgroup has a close relationship with survival, mutational, and expression features of prognostic genes, which may open a new window for improvement of individualized treatment based on TNBC metabolic profiles.

Abstract

Identification of molecular subtypes of clinically resectable triple-negative breast cancer (TNBC) is of great importance to achieve better clinical outcomes. Inter- and intratumor metabolic heterogeneity improves cancer survival, and the interaction of various metabolic pathways may affect treatment outcome of TNBC. We speculated that TNBC can be categorized into prognostic metabolic subtype according to the expression changes of glycolysis and cholesterol synthesis. The genome, transcriptome, and clinical data were downloaded from the Cancer Genome Atlas and Molecular Taxonomy of Breast Cancer International Consortium and subsequently analyzed by integrated bioinformatics methods. Four subtypes, namely, glycolytic, cholesterogenic, quiescent, and mixed, were classified according to the normalized median expressions of the genes involved in glycolysis and cholesterol synthesis. In the four subtypes, the cholesterogenic type was correlated with the shortest median survival (log rank $P = 0.044$), while patients with high-expressed glycolytic genes tended to have a longer survival. Tumors with PIK3CA amplification and dynein axonemal heavy chain 2 deletion exhibited higher expressions of cholesterogenic genes than other mutant oncogenes. The expressions of mitochondrial pyruvate carrier MPC1 and MPC2 were the lowest in quiescent tumor, and MPC2 expression was higher in cholesterogenic tumor compared with glycolytic or quiescent tumor (t-test $P < 0.001$). Glycolytic and cholesterogenic gene expressions were related to the expressions of prognostic genes in some other types of cancers. Classification of glycolytic and cholesterogenic pathways according to metabolic characteristics provides a new understanding to previously identified subtypes of TNBC and could improve personalized treatments based on tumor metabolic profiles.

Keywords: Glycolysis, cholesterol synthesis, bioinformatics, prognosis, triple-negative breast cancer, metabolic phenotype

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Introduction

Triple-negative breast cancer (TNBC), which is a highly aggressive and heterogeneous cancer,[1,2] accounts for approximately 10–20% of all breast cancer cases, with a low survival rate due to its high risk of recurrence and aggressiveness.[3,4] These features of TNBC make it the most challenging subtype of all breast cancers. With the rapid development of various "omic" techniques, systematic integrative identification of TNBC molecular subtypes shows great potential in the discovery of more effective strategies for treating TNBC,[5] and therefore attracts much research attention to determining the clinical-related molecular characteristics and actionable genomic alterations of the cancer.[6–12] However, a more comprehensive understanding on the specific carcinogenesis-related pathways involved in TNBC prognostic classification should be developed for designing personalized and effective therapy for TNBC patients.

Deregulating cellular energetics is a hallmark of cancer cells.[13] Driven by oncogenes or inactivated tumor suppressors, metabolic adaptation of cancer cells supports cancer progression in a complicated tumor microenvironment.[14]

The pan-cancer analysis on global metabolic pathway (iPath) demonstrated that cancer metabolic heterogeneity is related to survival rate, somatic driver gene mutations, and tumor subtypes.[15] However, whether the heterogeneity of different metabolic pathways can be applied to divide TNBC into clinical subgroups has not been fully determined.

MYC amplification and P53 loss-of-function mutation,[16] which are inducers of glycolytic pathway in cancer,[17–19] take place in most breast cancer cells, and glycolysis contributes to TNBC progression and chemotherapy resistance.[20–23] The effects of glycolysis on the occurrence and development of cancer can be reduced by metabolizing pyruvate from mitochondrial pyruvate carriers 1 and 2 (MPC1 and MPC2) to mitochondria and partially converting it to lactate.[24–26] In certain tumors, decreased mitochondrial pyruvate complex (MPC) activity is usually correlated with poor prognosis.[26] Pyruvate, which is an intermediate metabolic product of tricarboxylic acid cycle (TCA cycle), provides adipogenic precursor citrate for the biosynthesis of cholesterol and free fatty acids.[27]

The mevalonate–cholesterol biosynthesis pathway plays a critical role in the growth of cancer cells,[28] and accumulation of cholesterol in tumor is associated with tumor proliferation, metastasis, stemness, and drug resistance.[29,30] This suggests that pathway inhibitors could be explored as statins for cancer treatment. However, the relationship among statins, tumorigenesis, and treatment outcome still remained controversial,[31–34] and different therapeutic responses of statin are dependent on different molecular characteristics of a certain tumor.[32,35] The expressions of MPC1 and MPC2 influence tumor prognosis,[26] indicating that differences of pyruvate flow exist among different cancer types on one hand, and that variation between glycolysis and cholesterol synthesis could regulate cancer progression, on the other hand.

TNBC cell lines have unique glycolysis and adipogenic properties and have shown their responses to metabolic drugs. The combination of glycolysis inhibitor 2-deoxy-D-glucose (2-DG) or 3-bromo-pyruvate with the epidermal growth factor receptor (EGFR) inhibitor gefitinib significantly suppressed TNBC cell (MDA-MB-468 and BT549) proliferation. Co-administration of 2-DG and gefitinib caused a remarkably shrinkage of tumor size in an MDA-MB-468 xenograft tumor model in mice.[36] A fatty acid synthase (FASN) inhibitor G28 demonstrated a significant antiproliferative effect on TNBC cell line MDA-MB-231 (231) and its derivatives resistant to doxorubicin (231DXR) and paclitaxel (231PTR).[37] EGCG, another anti-FASN compound, plus cetuximab displayed strong antitumor activity against doxorubicin-resistant TNBC cell lines (231DXR and HCCDXR).[38] However, whether heterogeneity of gene profile in different metabolic pathways will affect clinical outcomes or regulate metabolic vulnerability in TNBC is unclear.

The current study classified TNBC into several subtypes according to the expression characteristics of glycolytic and cholesterogenic genes and examined their relationships with survival, mutational, and expression features of prognostic genes.

Materials and methods

Data acquisition and processing

The data in the present study were obtained from Molecular Taxonomy of Breast Cancer International Consortium (METABRIC)[39] and the Cancer Genome Atlas (TCGA) database (https://www.cancer.gov/tcga). TCGA (BRCA-US) data (sequence-based gene expression, GRCH37) for all available BRCA-US samples ($n = 1100$) were downloaded from the ICGC Portal (http://dcc.icgc.org/releases/PCAWG/) on 2 January 2020 (ICGC data release 28). BRCA_METABRIC data (Illumina Human v3 microarrays with expression log-intensity levels) were collected from cBioPortal (http://www.cbioportal.org) ($n = 2506$). Subsequently, any samples labeled as metastatic, xenografts, cell lines, normal, or non-laser microdissected enriched were removed.

Somatic mutation data of all the screened samples, including CRCh37 and those both with copy number variation (CNV) or single-nucleotide variants/indels (SNV/Indels), were downloaded from the ICGC Portal on 2 January 2020. TNBC samples were filtered out from breast cancer samples by using characteristic receptors of ER, PR, and HER2 as the screening criteria. The final sample sizes are listed in Table 1. The present study does not require ethical approval or informed consent because all the data were downloaded from the internet databases.

For RNA-seq data, quantitative data were downloaded and the counts were standardized using the TMM.[40] Standardized gene expression values of TNBC-US were original count values, and METABRIC standardized gene expression values of TNBC were log-transformed ($\log 2$ [normalized_count + 1]). We utilized ESTIMATE R package (DOI: 10.1038/ncomms3612) to screen all the samples, and those with cancer content less than 30% were excluded. Finally, the batch effects of the TNBC-US and TNBC METABRIC data sets were removed using Training Distribution Matching method.[41]

Identification of metabolomic subgrouping

To identify molecular subgroups related to breast cancer metabolism, we first retrieved the gene sets "REACTOME_GLYCOLYSIS" ($n = 29$) and "REACTOME_CHOLESTEROL_BIOSYNTHESIS" ($n = 72$) pathways from the genes pertaining to molecular features database (mSigDB[42]), and the genes included in the two pathways were genes involved in glycolysis and cholesterol production. The two type of genes were subjected to consensus clustering using ConsensusClusterPlus[43] v1.38 (parameters: reps = 100, pItem = 0.8, and pFeature = 1). Euclidean

Table 1. The number of TNBC samples in the TCGA and METABRIC data sets.

Sample	Sample size (TCGA)	Sample size (METABRIC)
TNBC	115	299
Non-TNBC	985	2207

TNBC, triple-negative breast cancer; TCGA, the Cancer Genome Atlas; METABRIC, Molecular Taxonomy of Breast Cancer International Consortium.

distance and hierarchical clustering with $K = 5$ were considered as distance metric and clustering algorithm, respectively. Each sample was allocated cholesterogenic (glycolysis ≤ 0, cholesterol > 0), glycolytic (glycolysis > 0, cholesterol ≤ 0), quiescent (glycolysis ≤ 0, cholesterol ≤ 0), or mixed (glycolysis > 0, cholesterol > 0) metabolic subgroup according to the median level of co-expressed glycolytic and cholesterogenic genes.

Comparison with the existing molecular subgroups

Previous research showed that characteristics of TNBC gene expressions were correlated with patients' survival. Subtypes associated with poor prognosis included the classification were discovered by Liu *et al.*, Jang *et al.*, and Pinto *et al.* Liu subtyping was based on one-gene feature of the original version;[44] Jang subtyping was based on one-gene feature of the original version;[45] and Pinto subtyping was based on three-gene features of the original version.[46] The overlapping relationship between metabolic subgroups proposed in the current study and previous molecular subtypes was systematically compared in order to better understand the relationship of the two. Specifically, for each processing of the subtype, the samples were clustered based on each classifier genes, followed by semi-automatic subtype distribution according to gene expression patterns. Finally, the sample intersection of different subtypes was counted.

Association between MPC-related genes MPC1/2 and metabolic subgroups

In cancer cells, MPC regulates mitochondrial pyruvate flow, inhibits the expressions of MPC1 and MPC2, and promotes glycolytic activity and lactic acid generation.[26] To investigate the relationship between MPC1 and MPC2 and glycolysis and cholesterol-generating phenotypes, we first compared the mutation frequencies and expressions of the two genes in the metabolic subsets. Next, Spearman rank correlation coefficients of all genes associated with MPC1 or MPC2 were calculated, so as to find cell pathway related to MPC1/2 expression, and the gene set significantly positively or negatively associated with MPC1/2 was screened with false discovery rate (FDR) < 0.01 as the threshold. Finally, these genes were used for GO functional enrichment analysis for identifying the biological pathway significantly associated with MPC1/2.

Association analysis of genomic variation and molecular subtypes

All the data were obtained from hg19. SNV/Indels and CNV in TNBC-TCGA, and TNBC METABRIC samples were identified as previously described. As to TNBC METABRIC tumor ploidy, DNA fragments with CNV ≥ 1 or ≤ -1 were considered as having amplifications or deletions, respectively. TNBC-TCGA CNV data were downloaded from GDC Data Portal (https://portal.gdc.cancer.gov/) on 2 January 2020. Following a previous study.[47] TNBC-US copy number events were screened for those with at least 10 probes and a mean value of fragment > 0.2 (enlargement) or < -0.2 (deletion). Bedtools v2.26 was used to map the coordinates of copy number events into gene coding region. The SNV and CNV of each gene were examined before contingency analysis. In every subgroup, each of the 12 genes was tested and calculated by Fisher's exact test to determine whether there was a loss-of-function mutation or copy number amplification/deletion. The resulting P values were corrected using Benjamini–Hochberg method.

Pan-cancer analysis

Driven by mutant environment and expression of organ-specific enzyme, different cancers exhibit unique metabolic features that may affect clinical treatment outcomes.[15,48] To clarify the relationship among glycolytic, cholesterogenic expression subtypes, and other organ sites, we performed consensus clustering analysis of glycolytic and cholesterogenic expressions in 25 cancer types. RNA-seq for all non-BRCA TCGA samples was downloaded from GDC Data Portal, and the data type was set as TPM. For non-BRCA TCGA cancer, 26 cancer types incorporating at least 100 samples were identified. The gene expressions were log-transformed ($\log 10[\mathrm{TPM} + 1]$), and gene orientation scale was used for batch correction in every type of cancer. In addition, gene expression value belonging to "REACTOME_GLYCOLYSIS" ($n = 29$) or "REACTOME_CHOLESTEROL_BIOSYNTHESIS" ($n = 72$) was subjected to repeated consensus clustering (consensus clustering parameter: reps $= 100$, pItem $= 0.8$, pFeature $= 1$; Ward.D2 and Euclidean distance, $k = 5$). The proportion of glycolytic and cholesterogenic genes was calculated for each gene cluster, and a core gene cluster was defined when a gene cluster contained more than 90% CHOLESTEROL gene or more than 30% GLYCOLYSIS gene. For these cancer types with multiple core clusters in a same gene set, the most homogeneous clusters were defined as core. Metabolic subtype of each cancer type was distributed based on the median value of each core glycolytic and cholesterogenic gene.

Statistical and survival analysis

Kaplan–Meier survival curve was plotted using the R package "survival" v.2.4.2 (https://CRAN.R-project.org/package=survival) and "survminer" v.0.4.2 (https://cran.r-project.org/web/packages/survminer/index.html). Samples with overall survival period shorter than one month were excluded from the survival analysis.

Results

Analysis of glycolytic and cholesterogenic gene expression identified four distinct subgroups of TNBC

To stratify TNBC subgroups, after removing samples with low tumor content ($< 30\%$ per sample), a total of 414 samples (TCGA $n = 115$, METABRIC $n = 229$) were obtained based on the relative expressions of cholesterogenic and glycolytic genes, and of these genes those belonged to responsive genome "cholesterol biosynthesis" ($n = 72$)

and "glycolysis" ($n = 29$) were selected for further analysis. To detect co-regulated genes of each pathway and were related to TNBC biology, consensus clustering was performed to identify a robust co-expression metabolic subtype gene in both glycolysis ($n = 13$) and cholesterol biosynthesis pathways ($n = 8$) (Figure 1(a)). The median expressions of co-expression cholesterogenic and glycolytic genes in each sample were calculated and applied to one of the following four curves particularly relevant to these two pathways: quiescent, glycolytic, cholesterogenic, and mixed (Figure 1(b)). Figure 1(c) demonstrates the expression levels of glycolytic and cholesterogenic genes in metabolic subgroups. Quiescent phenotype defined the largest cluster(138/414; 33.3%), mixed type (105/414; 25.36%), cholesterogenic type (85/414; 20.5%), and glycolytic type (86/414; 20.7%). Genetic characteristics of metastatic and non-metastatic TNBC cases were subjected to perform cluster analysis in order to determine whether matrix type affected

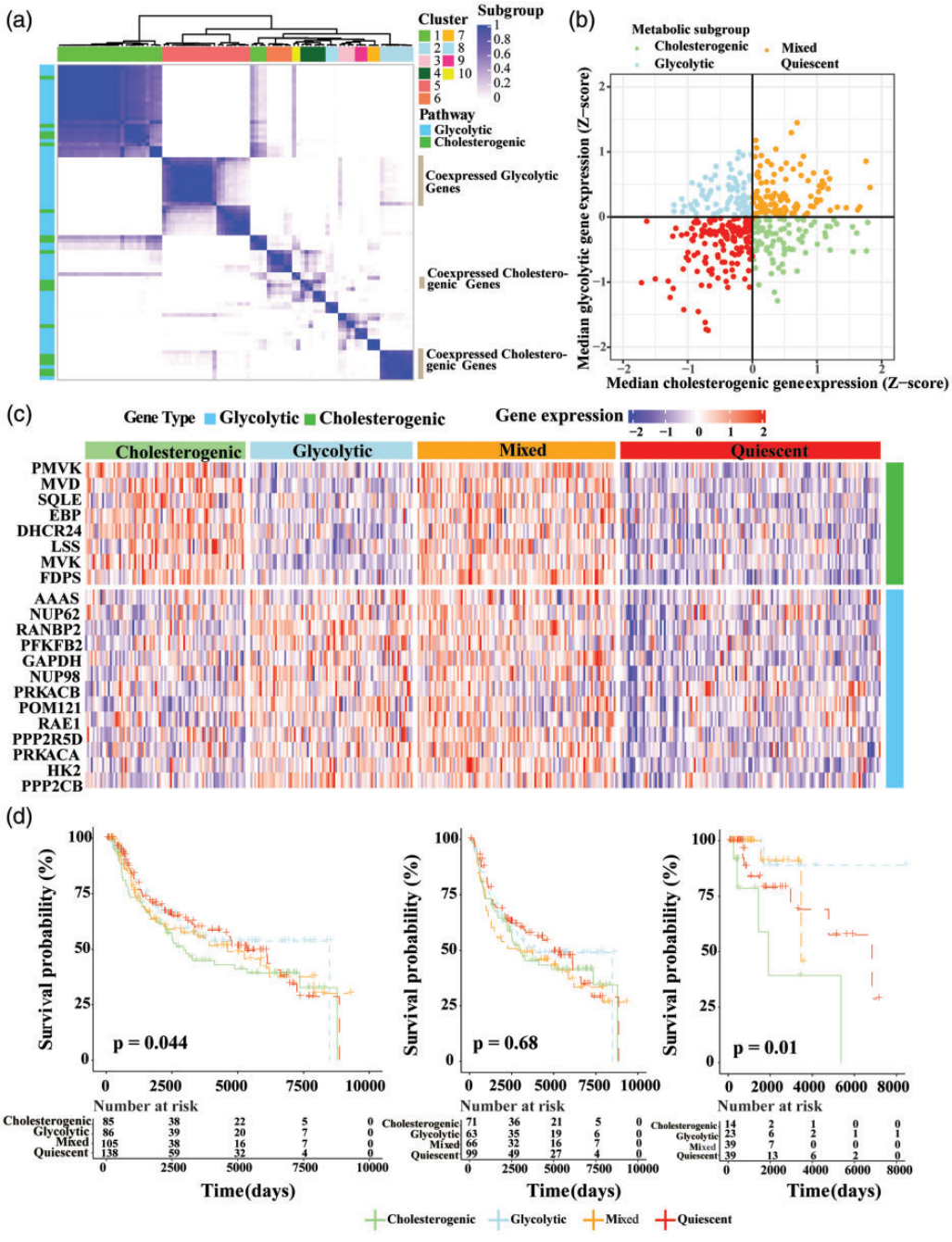

Figure 1. Stratification of TNBC tumors based on the expressions of glycolytic and cholesterogenic genes. (a) Heatmap showing consensus clustering solution ($k = 5$) for glycolytic and cholesterogenic genes in resected and metastatic TNBC samples ($n = 414$). (b) Scatter plot showing median expression levels of co-expressed glycolytic (*x*-axis) and cholesterogenic (*y*-axis) genes in each TNBC sample. Metabolic subgroups were assigned on the basis of the relative expression levels of glycolytic and cholesterogenic genes. (c) Heatmap showing the expression levels of co-expressed glycolytic and cholesterogenic genes across each subgroup. (d) Kaplan–Meier survival analysis of patients with all (left), metastatic (middle), and non-metastasic (right) TNBC stratified by metabolic subgroup. Log-rank test *P* values are shown. (A color version of this figure is available in the online journal.)

metabolic classification, and we observed that the distribution of metastatic subsets was not significantly different between these two subtypes. Survival curve of cholesterogenic and glycolysis group showed a significant survival difference (log rank $P = 0.044$) in all the TNBC samples. The survival prognosis was remarkably worse in cholesterogenic group than that in glycolytic group (Figure 1(d), left). In the comparison with non-metastatic groups, significant difference in prognosis was observed between cholesterogenic and glycolytic group (log rank $P = 0.01$). In metastatic groups, no significant difference has been observed between glycolytic and cholesterogenic groups (log rank $P = 0.68$) (Figure 1(d), middle and right). Noticeably, a more favorable survival was found in cases with increased glycolytic gene expression. Apart from these, multiple metabolic phenotypes related to glycolysis–cholesterol synthesis axis in TNBC were identified, among them, tumor with higher glycolytic rate and lower cholesterol synthesis was less aggressive and more chemotherapy-sensitive than tumor with more cholesterogenic phenotypes.

Relation between tumor genome of metabolic subtypes and known TNBC subtypes

To determine oncogenic events in different subtypes, we examined SNV, Indels, and CNV affecting mutation frequency of frequently mutated genes in TNBC[6,49] in the metabolic subgroups (Figure 2(a)). The data revealed that although there was no significant difference about mutation frequency of each gene among the subtypes (Fisher's exact test and BH correction, adjusted $P > 0.05$), we noted that the median expression of cholesterogenic genes increased significantly in samples with PIK3CA amplification and dynein axonemal heavy chain 2 (DNAH2) deletion (Figure 2(b)). The expressions of cholesterogenic genes were negatively correlated with DNAH2 and positively correlated with PIK3CA (Figure 2(c)). These findings were consistent with the fact that PIK3CA promotes glycolysis metabolism of TNBC and suggested that tumors with enhanced replication of DNAH2 and PIK3CA may be more dependent on the use of cholesterol and are more susceptible to cholesterol inhibition.

To examine whether the expression model of glycolysis–cholesterol synthesis axis could screen the difference among previously classified subtypes, we identified various TNBC subtypes in each sample ($n = 7$) and studied their overlap degree with metabolic phenotype (Figure 3 (a)). Quiescent group mainly included good-prognosis cases (51.1%) (Liu *et al.*), which were significantly different from the good-prognosis cases (Fisher's exact test and BH correction) in mixed group (0.2%, adjusted P value = 0.003)

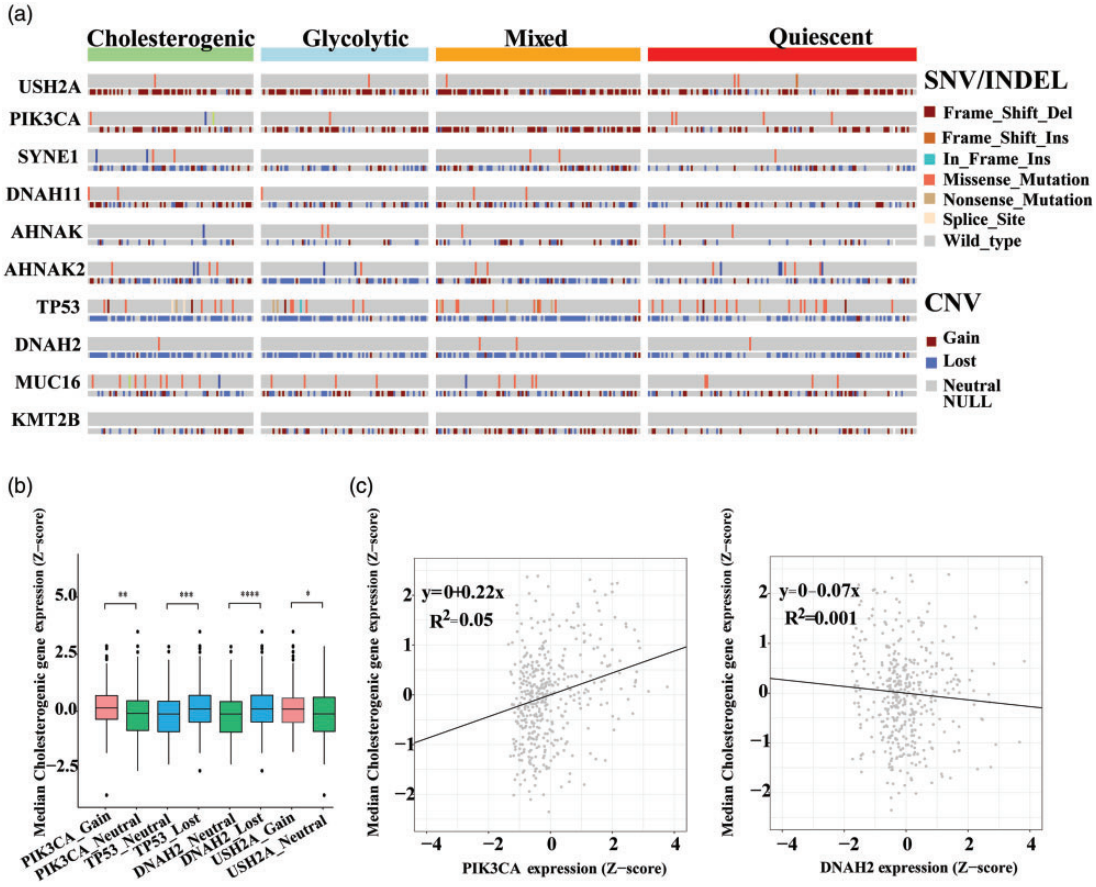

Figure 2. Mutational landscape across metabolic subgroups of TNBC. (a) Oncoprint showing the distribution of somatic mutation (SNV/Indel) and CNV events affecting frequently mutated genes in TNBC across the metabolic subtypes. (b) Box plot showing median expression of glycolytic genes in samples with DNAH2 and/or PIK3CA copy number. (c) Scatter plot showing the correlation between median cholesterogenic gene expression and PIK3CA (left) and DNAH2 (right) expression. (A color version of this figure is available in the online journal.)

and cholesterogenic group (18.57%, adjusted *P* value = 4.4e-3) (Figure 3(b)). Using Pinto classification, the sample number of quiescent group was significantly different from that of mixed and cholesterogenic group (*P* value = 7.8e-6 and *P* value = 4.0e-4,respectively).

Significant differences were found in the number of poor-prognosis samples between quiescent group and cholesterogenic group (*P* value = 3.3e-3). Furthermore, a correlation analysis was conducted between the gene expression of cholesterol biosynthesis/glycolysis pathway and gene-dependent expression based on Liu and Jang's classifications. We found that based on Jang classification, in poor-prognosis samples, the expression of genes by characterized grouping was positively correlated with the expression of genes in the cholesterol biosynthesis pathway. However, in good-prognosis samples, according to Liu classification, the expression of genes by characterized grouping was negatively correlated with those in the cholesterol biosynthesis pathway. There was a high consistent correlation of the genes in the cholesterol biosynthesis pathway and glycolysis pathway in the above two clinical types (Figure 3(c)). The above data indicated that different tumor metabolic pathways play a role in the prognostic effects of the TNBC subtypes and determined that glycolysis and cholesterol biosynthesis can serve as potential metabolic-targeted active sites for different TNBC subtypes.

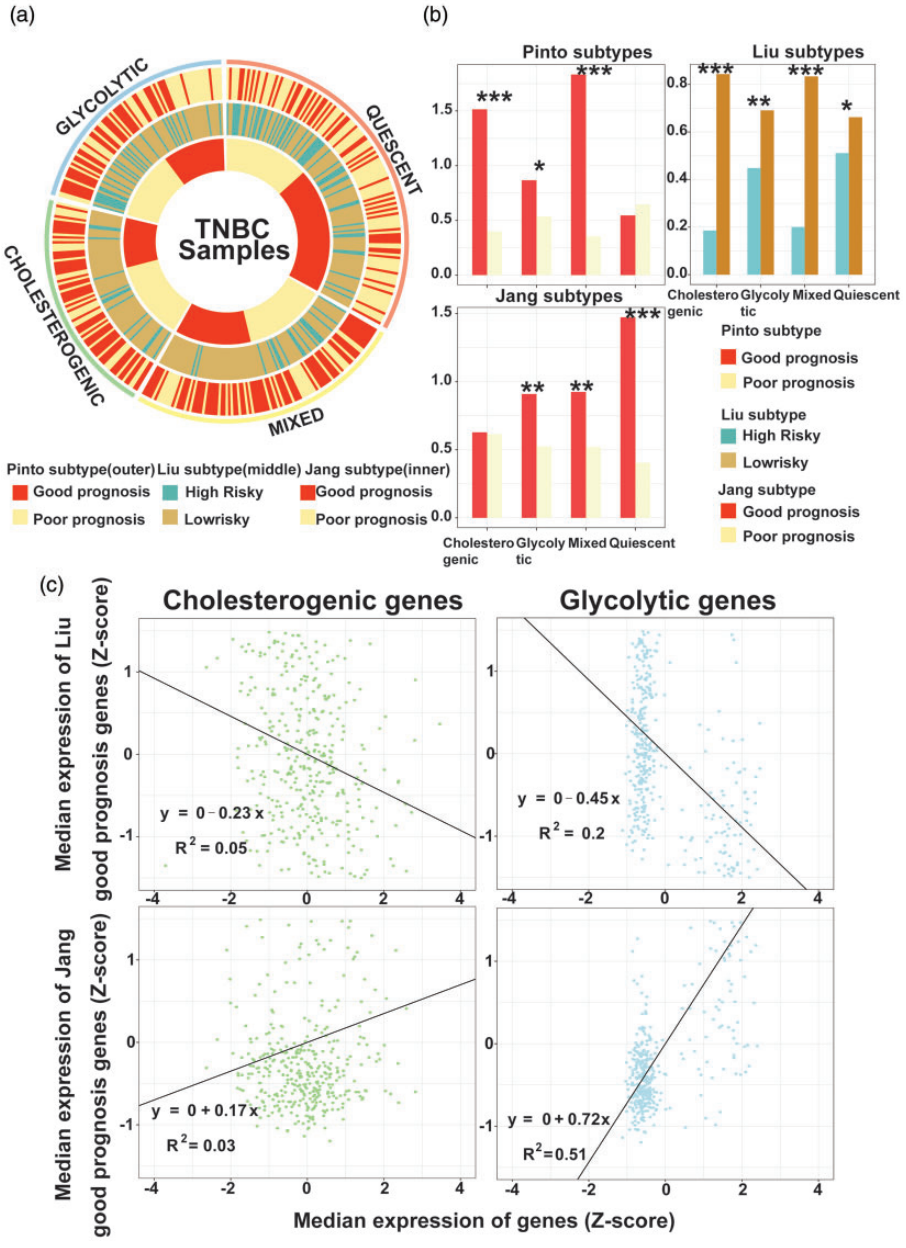

Figure 3. Alignment of TNBC metabolic subgroups with known gene expression subtypes. (a) Overlap of metabolic profiles with TNBC expression subtypes based on the Liu, Jang, and Pinto classifications. (b) Bar plots showing the proportion of TNBC expression subtypes across each metabolic subgroup. (c) Scatter plot showing reciprocal correlations between expression of glycolytic and cholesterogenic genes and genes associated with the Liu and Jang classifications. (A color version of this figure is available in the online journal.)

MPC as a potential regulator of glycolysis–cholesterol synthesis axis in TNBC

To study the relationship between MPC1/2 and phenotype of glycolysis–cholesterol synthesis, we compared the mutation frequency and the expression of the two genes in metabolic subgroups of TNBC. A specific relationship of CNVs in each gene was found, among the CNVs those affects MPC1 showed specific deletions, whereas the CNVs affects MPC2 were mostly amplified (Figure 4(a)). Significant differences existed between the expressions of MPC1 and MPC2 in metabolic subgroups. Compared with cholesterol group, the expression of MPC2 gene was significantly reduced in glycolytic samples, while the expression of MPC1 was significantly higher in mixed and cholesterogenic group than that in quiescent group, and MPC2 expression was significantly increased in cholesterogenic group compared with that in quiescent group (Figure 4(b)). These results indicated that the dysfunction of mitochondrial pyruvate transport at mRNA level might be correlated with metabolic tumor subgroups.

A comprehensive correlation analysis was performed on MPC1/2 and all the other tested genes ($n = 25{,}483$) for the detection of cellular pathways correlated with MPC1/2 expression. MPC1/2 (Spearman correlation BH correction $P < 0.01$) (Figure 4(c)) was found to be positively correlated with a total of 1147 genes and negatively correlated with 95 genes. The molecular functions of positively correlated gene also showed a positive correlation with cellular

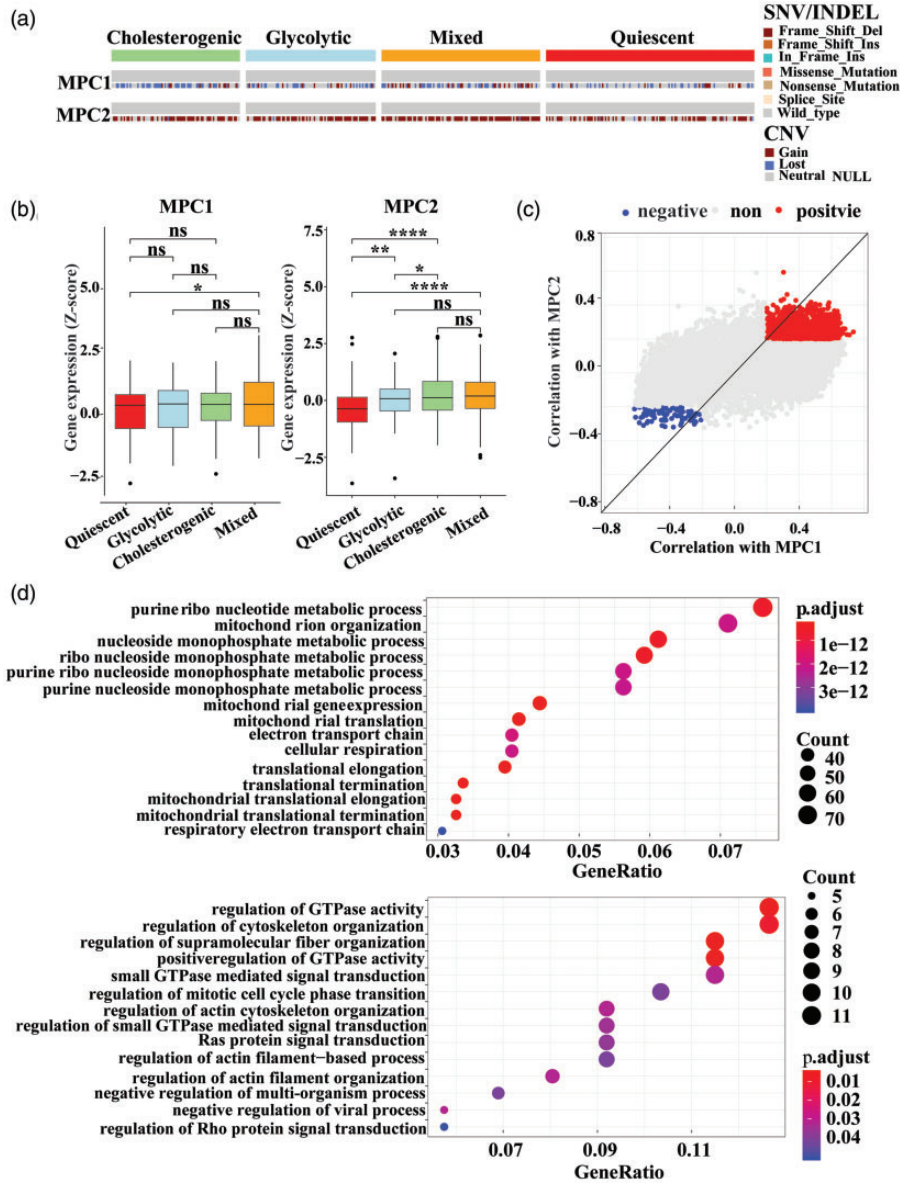

Figure 4. Association of MPC1 and MPC2 expressions with TNBC metabolic subgroups and cell signaling pathways. (a) Oncoprint showing the distribution of MPC1 and MPC2 SNVs and CNVs across the metabolic groups. Only one case was found with an SNV in MPC2. (b) Box plots showing significant (*t*-test $P < 0.001$) differences in expression levels of MPC1 and MPC2 across TNBC metabolic subgroups. (c) Scatter plot showing the correlations between MPC1 (*x*-axis) and MPC2 (*y*-axis) and each of 25,483 genes. A total of 1147 and 95 genes were found to be positively (Spearman correlated BH-adjusted $P < 0.01$; $\rho > 0$) and negatively (adjusted $P < 0.01$; $\rho < 0$) correlated with MPC1 and MPC2 expressions, respectively. (d) The most significantly enriched (hypergeometric test BH-adjusted $P < 0.05$) gene sets among genes positively (up) and negatively (bottom) associated with MPC1/2 expression. (A color version of this figure is available in the online journal.)

energy metabolism (hypergeometric test, BH correction $P < 0.05$) (Figure 4(c)). The pathways enriched in negatively correlated genes were cell cycle transition and phosphorylation (Figure 4(d)). These data suggested that MPC activity is involved in cellular network related to tumor progression of TNBC, at least partially, through affecting the balance between glycolysis and cholesterol synthesis of TNBC.

Relationship between glycolytic/cholesterogenic gene cluster and other cancer types

The relationship among glycolytic, cholesterogenic expression subtypes and other organ sites was determined by repeating consensus clustering analysis of glycolytic and cholesterogenic expressions in 25 cancer types obtained from TCGA (tumor content $\geq 30\%$). We discovered that specific gene clusters of co-expressed pathways were in discrete state. Many genes have consistent expression pattern in a majority of cancer types, but it may also vary as certain genes are unique to a few cancers in co-expressed glycolytic and cholesterogenic pathways, suggesting that some genes have cell-type-specific function in metabolic process of each cancer (Figure 5(a)). Based on TNBC classification system constructed by Pinto *et al.*, the expressions of cholesterogenic genes were significantly positively correlated with the expression of poor-prognosis genes (Spearman correlation BH correction $P < 0.05$), suggesting that gene signatures indicative of a poor outcome were related to increased cholesterol synthesis activity, with a wider coverage of tumor types (Figure 5(b)). In some cancer types, we found median expression of glycolytic genes was positively correlated with expression of KRAS (COAD,ESCA,STAD,LGG,OV,THYM,READ,LUAD, KIRC,LUSC,UCEC,PCPG,LAML,HNSC,GBM,PRAD,KIRP, THCA) and MYC (STAD,THCA,LUSC,LUAD,UCEC, KIRP, COAD,KIRC,HNSC,PCPG,SKCM) (BH correction $P < 0.05$). Similar to TNBC, the expression of MPC1 (LUAD,COAD,PRAD,READ,TGCT,THCA,CESC,LAML,

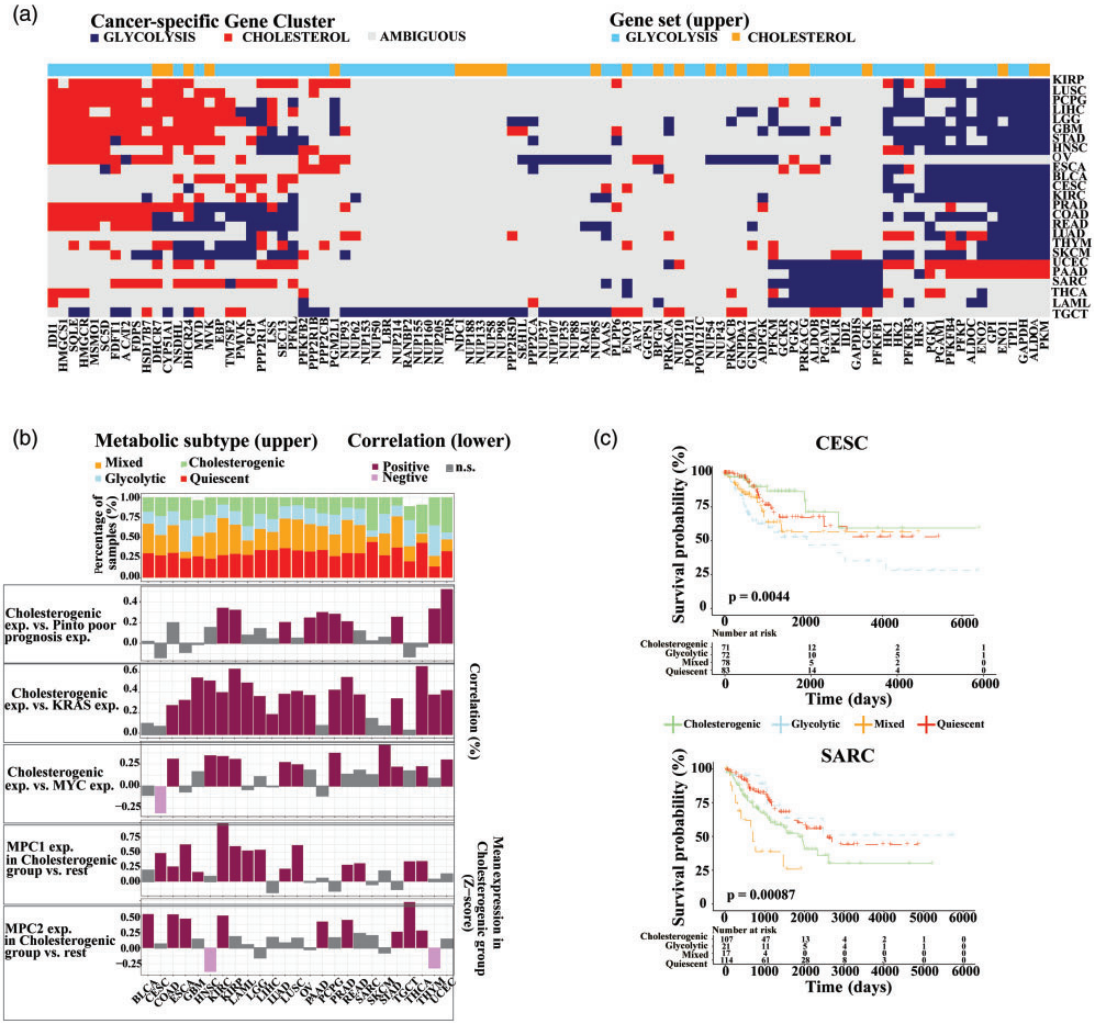

Figure 5. Glycolytic and cholesterogenic gene profiling of other cancer types. (a) Heatmap showed that glycolytic and cholesterogenic genes were robustly co-expressed when consensus clustering was applied to each individual cancer type. (b) Bar plots showing the proportions of metabolic subgroups across the various cancer types (top) and correlation between CHOL subgroups and expression of Hoshida poor subtype, KRAS, MYC, and MPC1/2 in each cancer type (bottom). Median glycolytic gene expression was positively (Spearman $\rho > 0$, BH-adjusted $P < 0.05$) correlated with basal-like gene expression in all cancer types. The correlation between MPC1/2 expression and the glycolytic subgroup was measured using Wilcoxon rank sum tests followed by BH correction. (c) Kaplan–Meier survival analysis curves showing differences in median overall survival across metabolic subgroups in CESC and SARC.

LGG,KIRP,LUSC,ESCA,KIRC), MPC (READ,STAD,THCA, PAAD,PRAD,ESCA,KIRC,COAD, BLCA,TGCT), and both (ESCA,KIRC,COAD,PRAD,READ,TGCT,THCA) were increased significantly in cholesterogenic group (Figure 5 (b)). These results further supported the finding that that changes in mRNA expressions of these genes contribute to cholesterol synthesis of tumor. Survival rates of the four metabolic subtypes showed significant differences in CESE (log rank $P = 0.0044$) and SARC ($P = 0.00087$, Figure 5(c)). In CESE, significant survival differences were compared between mixed and cholesterogenic group, and mixed group showed a poor prognosis. In SARC, however, the prognosis of quiescent and glycolytic groups was worse than those of cholesterogenic and mixed groups. Taken together, these results indicated that tumor metabolism dependency varies according to different genomic features and cancer-type-specific tumor microenvironment factors.

Discussion

A comprehensive understanding of clinically relevant tumor subtype develops personalized therapies of TNBC. In the current study, TNBC showed distinctive metabolic signature according to the expressions of genes related to glycolysis and cholesterol synthesis, which were two biological processes affecting TNBC prognosis. Due to structural variations, chromosomal rearrangement events, epigenetic modification, and gene expression signatures, high degree of molecular heterogeneity in TNBC results in tumor subtypes of the cancer with distinctive differences.[2,6,9,50,51] According to unique molecular characteristics of each tumor subtype, such a phenomenon also aroused great interest in applying related knowledge to clinical practice to estimate prognosis, predict clinical efficacy, and design personalized treatment of patients. TNBC transcriptomic subclasses are currently used to predict survival,[3,4,52] but their prediction accuracy in actual clinical management and development of new therapies is relatively low. Previous studies demonstrated that heterogeneity of metabolic gene expression, including isoenzyme in specific pathways, varies according to specific cancer types,[15,48] and metabolic gene expression reprograming is correlated with the changes in metabolites.[15] In the present study, we found that the expressions of glycolytic and cholesterogenic genes and TNBC-specific oncogenes were closely related to each other, and such a finding provides a deeper understanding of applying TNBC subtypes to targeting metabolic vulnerabilities of aggressive tumors.

Glycolysis facilitates tumor growth, immune escape, and drug resistance.[14] Previous study indicated that cancer patients with increased expressions of glycolytic genes tend to have a shorter overall survival, pointing to the role of glycolysis in promoting the progression of TNBC.[53,54] Similarly, cholesterol metabolism also facilitates tumor cell growth, and the function of tumor-suppressor AMPK is partially mediated by the inhibition of cholesterol synthesis.[55–57] Interestingly, the current study found that in TNBC cases high-expressed glycolytic genes benefit patient survival, suggesting although both glycolysis and cholesterol synthesis stimulates tumor cell growth, they do not necessarily exert the same synergistic effect on the progression of certain cases of TNBC. The relationship between molecular heterogeneity of glycolysis-cholesterogene axis and prognostic performance of different TNBC subtypes suggests that different treatment methods should be specifically designed to target its corresponding metabolic vulnerabilities. Therefore, blocking dependency of tumor on glycolysis and cholesterol synthesis according to the subtype of TNBC patient can translate into clinical benefit.

The potential mechanisms leading to the poor outcome of cholesterogenic tumor may be explained by the tumor-promoting effect of cholesterol metabolites, as a majority of cholesterol metabolites and derivatives can promote tumor growth.[58] Cholesterol ester (CE), a cholesterol metabolites, is found up-regulated in glioma and prostate cancer,[59] and the abnormal accumulation of CE is related to a poor prognosis in pancreatic cancer.[60] Caveolin-1 is a regulator of intracellular cholesterol homeostasis, and Caveolin-1-mediated pathway may play an important role in tumor metastasis promoted by CE.[61] Acyl-coenzyme A: cholesterol acyltransferase-1 can activate sterol regulatory element-binding protein-1 (SREBP1) through excessive CE synthesis, thereby promoting caveolin-1-mediated MAPK signaling pathway and ultimately leading to metastasis.[60] 27-Hydroxycholesterol (27-HC) is a hydroxylated metabolite of cholesterol and can be used as selective estrogen receptor modulator[62] and LXR agonist in the body. A study showed that compared with normal breast tissue, 27-HC concentration is increased significantly in patients with breast cancer, and that cholesterol 27-hydroxylase is positively correlated with higher grade of breast cancer.[63] In addition, 27-HC can elevate the expression of mouse double minute 2 protein (MDM2) and enhance MDM2-mediated P53 ubiquitination and degradation, resulting in accelerated proliferation of estrogen receptor-positive breast cancer cells.[64] Moreover, it has been reported that the proportion of metastatic tumor nodules is increased significantly in 27-HC-pretreated breast cancer mouse model compared with that in the placebo group.[65]

Molecular events are able to drive metabolism reprograming of cancers, including in TNBC.[17,66] Hyperactivation of PI3K/AKT/mTOR signaling pathway is one of the most widely studied mechanisms in cholesterol metabolic reprograming. In an mTOR complex1-dependent manner, PI3K/AKT signaling pathway activates SREBP pathway-mediated cholesterol endogenous synthesis and LDLR-mediated exogenous input, and simultaneously inhibits ABCA1-mediated reverse cholesterol transport, resulting in increased intracellular cholesterol.[67,68] DNAH2 is microtubule-associated motor protein complex with ATPase activity.[69] DNAH2 is mainly expresses in bronchus and testis, and it is reported that DNAH2 mutation is involved in bronchial diseases, sperm flagella defects, Fanconi anemia, and chronic myelomonocytic leukemia.[70–74] In this study, we found that TNBC tumor with PIK3CA amplification and DNAH2 deletion had higher cholesterogenic gene expression, suggesting that this TNBC subtype may have a cholesterogenic dependency and susceptibility to cholesterogenic inhibition. Thus, a potential therapeutic strategy targeting

cholesterogenic-dependent tumor with PIK3CA amplification and DNAH2 deletion could be to transfer the metabolic phenotype to activation of the glucose metabolism pathway.

MPC is composed of pyruvate carriers 1 and 2 (MPC1 and MPC2), and loss of activity in any single subunit of the complex can result in dysregulated MPC, which subsequently leads to decreased mitochondrial pyruvate translocation and utilization.[24,25] It has been previously found that suppression of pyruvate transport greatly inhibits both glucose and pyruvate oxidation, surprisingly, oxygen consumption, TCA metabolism, and cell growth are well maintained. MPC knockdown significantly increases lipogenic AcCoA pool, thus inducing substrate to convert to De Novo Lipogenesis.[75] In this study, we found that CNVs affect MPC1 through specific deletions, and for MPC2, through amplification, which will directly induce the dysfunction of MPC. Cholesterogenic subgroup showed a poor prognosis, possibly because MPC mutation caused expansion of lipogenic AcCoA pool, consequently promoting more cholesterol synthesis and thus accelerating the occurrence and development of cancer. In the same context, survival benefit in glycolytic subgroup may be due to the relatively low glucose and pyruvate oxidation and low-expressed intrinsic cholesterogenic genes. From the above analysis, it can be observed that MPC2 expression is negatively correlated with cell genetic programing, leading to aggressive tumor subtype. These findings define the MPC as a latent target for changing cancer metabolic profiles. Based on this, in cholesterogenic TNBC cases, down-regulation of MPC2 expression may be able to attenuate the effects of tumor cholesterol synthesis through converting the tumor into a glycolytic subtype. Moreover, MPC2 can be a potential target for reducing the degree of malignancy of TNBC with or without MPC1 deletion. Correlation analysis found that the genes negatively correlated with MPC2 expression were mainly enriched in cell cycle transition and phosphorylation process, which points out an important direction for further study on the action mechanism of MPC2 in tumor metabolic reprograming.

Taken together, the findings presented in this study not only provide a new perspective for prognostic prediction of TNBC, but also suggest new treatment strategies for TNBC metabolic subtypes. In TNBC cases, increased glycolytic gene expression is statistically correlated with more favorable prognosis, by contrast, high-expressed cholesterogenic gene has the opposite effect. As such, this finding offers an alternative and complementary approach for improving outcome predictions in TNBC. Except for poor prognosis, TNBC subtype with higher cholesterogenic gene expression is associated with PIK3CA amplification and DNAH2 deletion. Thus, cholesterogenic inhibition treatments may offer a novel therapeutic approach for this tumor type.

AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data, and review of the manuscript. Y-JH and P-CZ designed the study. RS and H-WW carried out data collection. P-CZ and Z-WL performed data analysis and interpretation. X-LS contributed to supervising the study and was involved in making figures and tables. P-CZ wrote the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

ETHICAL APPROVAL

The present study was granted an exemption from requiring ethics approval by the Human Research Ethics Committee of Guangzhou University of Chinese Medicine (Guangzhou, China).

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