Metabolism-associated Molecular Classification of Cervical Cancer

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Research

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Abstract

Objective Metabolic processes play an important role in cervical cancer. Here, we explored metabolic abnormalities in cervical cancer by analysing somatic mutations, copy number variations, and relationships with clinical parameters based on metabolism-related genes in cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC).

Methods Metabolism related genes were downloaded from Fluxer database. First, we analysed the impact of mutations and copy number changes of metabolism-related genes on CESC. Secondly, weighted gene co-expression network analysis (WGCNA) was used for analysing the potential functions of metabolism related genes.

Results We identified 3620 metabolism-related genes in CESC; 2493 genes contained related mutations. Among these genes, 15 were related to the prognosis of CESC. In addition, eight pairs of genes showed a co-occurrence relationship. Moreover, the inter-occurrence of three genes (*CUBN-KALRN-HERC1*) was related to the prognosis of CESC. Furthermore, we found that metabolism-related tumour mutation burden (TMB) and fraction genome altered (FGA) is closely related to CESC. In the expression analysis, 374 genes were related to the occurrence and prognosis of CESC. We then identified four metabolic pathway modules in WGCNA. Further analysis revealed that glycolysis/gluconeogenesis is related to endothelial cells and that the arachidonic acid metabolism module is related to cell proliferation. At the same time, these four modules were determined to be related to the prognosis of CESC. Finally, among CESC-related metabolic genes, we found that two might be regulated by miRNA and methylation. Two genes could thus be co-regulated by miRNA and mutation.

Conclusions: Among metabolism-related genes, 15 genes are related to the prognosis of CESC. The inter-occurrence of the three genes of *CUBN-KALRN-HERC1* is related to the prognosis of CESC. Glycolysis/gluconeogenesis is related to endothelial cells, and arachidonic acid metabolism module is related to cell proliferation.

Introduction

Cervical cancer is one of the most frequently encountered malignancies in women, and cervical squamous cell carcinoma and endocervical adenocarcinoma (CESC) are the most common histological types of cervical cancer. Because cervical cancer carries a high risk of morbidity and mortality in women in developing countries (Joshi et al., 2015; Lahue et al., 2015), it is necessary to elucidate the molecular mechanisms of CESC.

Metabolic activities have substantial effects on development, progression, and prognosis in various cancers (Baylin et al., 2011), including CESC (Zhang et al., 2019; Zhao et al., 2020). Specific metabolic activities can directly participate in the transformation process or support the biological process, which might cause tumour growth (Vander et al., 2017). Additionally, changes in cell metabolism can promote transformation and tumour progression (Vander et al., 2017). Therefore, elucidation of the mechanisms

mediating cancer metabolism might have implications in the exploration of basic cancer pathophysiology and clinical oncology.

Genetic and epigenetic abnormalities have central roles in cancer metabolism (Ojesina et al., 2014; Song et al., 2018; Zhou et al., 2014). Increased mutation rates are often observed in human cancers, leading to loss of function (LOF) caused by somatic mutations, copy number variations, and genomic rearrangements, among other mutations (Todd et al., 2019). In addition to genetic alterations, gene expression can be modulated by epigenetic mechanisms, such as DNA methylation, histone modification, and noncoding RNA regulation (Kwak et al., 2016). However, the associations between genetic and epigenetic alterations and metabolic processes in CESC are not fully understood.

Online public databases and high-throughput sequencing technology have provided us with platforms for comprehensive genome-wide gene expression analyses to improve our understanding of the impact of genetic compositions on various diseases (Frodsham et al., 2007). In this study, we aimed to identify metabolism-related genes in CESC and to explore the associations between genomic changes and clinical parameters, including overall survival, in patients. We also evaluated differentially expressed metabolism-related genes in CESC and used weighted gene co-expression network analysis (WGCNA) to construct a co-expression network of metabolism-related genes and screen for significant functional modules. Finally, we further explored the mechanisms mediating the effects of metabolism-related genes in cancer progression. Our results provide insights into metabolic functions in CESC and establish novel targets for clinical treatment.

Results

Genomic changes in metabolism-related genes in CESC

From the Fluxer database, we compiled a total of 3620 genes related to metabolism in CESC; these genes were regarded as metabolism-related genes for subsequent analyses. Additionally, 2493 of these genes harboured CESC-related mutations. As shown in Fig. 1A, the top three genes with the highest mutation frequency were *TTN* (31% mutation rate), *PIK3CA* (29% mutation rate), and *KMT2C* (19% mutation rate). Moreover, we found that nucleotide mutations of C > T were the most frequent (Fig. 1B) among all mutation types in CESC.

To determine whether LOF mutations affect the prognosis of CESC, we analysed the number of mutations in more than five samples; this analysis yielded 616 samples with genetic mutations. From this prognostic analysis, LOF mutations in 15 genes including *KALRN*, *SLC18A2*, *ABCA8*, and *STK11*, were found to be closely related to prognosis in patients with CESC (Fig. 1C and Supplementary Table 1). Owing to the mutual influence between gene mutations, we further explored mutually exclusive/co-occurrence associations among these 15 prognosis-related genes. There was no mutual exclusion between prognostic-related gene mutations, but there were mutual occurrences between the eight pairs of genes (Fig. 1D). Therefore, we conducted a joint prognostic analysis of these eight gene pairs. Three gene

pairs, *KALRN-CUBN*, *HERC1-CUBN*, and *KALRN-HERC1*, were related to the prognosis of CESC (Supplementary Table 2). Since the three gene pairs represent the pairing between the three genes, we further conducted a prognostic analysis of the mutations in the three genes. Mutations in the three genes were related to prognosis (Fig. 1E).

To further assess the relationships between overall mutations in metabolism-related genes and CESC, we calculated the TMB score based on all mutations in metabolism-related genes and evaluated associations between TMB scores and clinical stage staging, histological typing, recurrence, and prognosis. The results showed that correlations between prognosis or clinical parameters of CESC and TMB were not meaningful (Supplementary Table 3). However, in an analysis based on histological grouping (except adenosquamous disease because of the small sample size), we found that clinical staging was associated with metabolism-related TMB in endocervical adenocarcinoma (Fig. 2A and Supplementary Table 4). In addition to genomic mutations, we also observed variations in copy number. Thus, we calculated the FGA, fraction genome gain (FGG), and fraction genome loss (FGL) based on changes in the copy numbers of metabolism-related genes. FGL exhibited differences among different histological types (Fig. 2B and Supplementary Table 5). In addition, all three factors were related to the prognosis of CESC (Fig. 2C).

Expression and functions of metabolism-related genes in CESC

In total, 234 up-regulated and 260 down-regulated genes in CESC were identified by differential expression analysis of metabolism-related genes (Fig. 3A and Supplementary Table 6). Additional analyses showed that 374 of these genes were significant for the prognosis of CESC (Supplementary Table 7). To elucidate the biological functions of these genes in CESC, we performed molecular clustering analysis using the WGCNA algorithm to construct a co-expression network. After evaluation of the preliminary data, we chose five as the candidate threshold for co-expression network construction (Fig. 3A). By fitting genes with similar expression patterns into a module, we obtained four modules, including 55–133 genes each (Fig. 3B). We performed functional annotations on the four modules through enrichment analysis and found that these four modules were related to arachidonic acid metabolism, carbon metabolism, glycolysis/gluconeogenesis, and purine metabolism. Therefore, the corresponding metabolic pathways were used to represent each module.

Next, we applied the MCP counter algorithm to evaluate the immune infiltration of each sample and to improve our understanding of the correlations between these modules and tumour-related immune cells. The results showed that the arachidonic acid metabolism module was related to most immune cells and that glycolysis/gluconeogenesis was related to endothelial cells (Fig. 3C). Additionally, using the GSVA algorithm, we evaluated the scores of tumour-related phenotypes (cell proliferation, apoptosis, and epithelial–mesenchymal transition) in patients with CESC. The results showed that the arachidonic acid metabolism module was related to cell proliferation (Fig. 3C).

The relationships between these modules and clinical parameters were then evaluated. The histological classification was mainly associated with carbon metabolism, purine metabolism, and arachidonic acid metabolism modules. Moreover, the correlation between tumour recurrence and the glycolysis/gluconeogenesis module was also significant (Fig. 3D). Finally, we conducted survival analysis for each metabolism module and showed that each metabolism-related module was related to the prognosis of CESC (Fig. 3E).

Possible regulatory mechanisms of metabolism-related genes

Through TCGA, we first analysed the negative regulatory effects of miRNAs on differentially expressed genes. The results showed that there were 11 genes negatively regulated by miRNAs. Furthermore, we evaluated the effects of mutations on gene expression and showed that the expression levels of six genes were affected by their mutations. Analysis of the effects of promoter methylation on gene expression revealed that 46 genes might be affected by methylation (Fig. 4). Thus, 58 genes were regulated by the aforementioned three mechanisms. Importantly, *NME5* and *SLC40A1* were influenced by methylation and miRNA regulation, whereas *HK3* and *JAK3* were regulated by both mutations and methylation.

Discussion

Cancer metabolism is essential for tumour growth, and altered metabolic activities are a hallmark of cancer (Hoang et al., 2019), leading to the modulation of tumour progression, metastasis, and prognosis (Danhier et al., 2017; DeBerardinis et al., 2016). In the current study, we focused on analysing the characteristics of metabolism-related genes in cervical cancer. We also assessed the roles of these genes in carcinogenesis and further explored their genetic and epigenetic alterations. Our results provide potential directions for studying the overall metabolism of cervical cancer.

Gene function is affected by genomic variation. Therefore, we analysed the changes in metabolism-related genes in the genome. Genomic analysis identified 2493 genes with mutations that might be involved in modulating metabolic activities. Among these genes, *TTN*, *PIK3CA*, and *KMT2C* showed the highest mutation frequency, and C > T was the most common nucleotide mutation in CESC. The *PIK3CA* gene has been reported to be frequently mutated in patients with cervical cancer in the United States of America (Femi et al., 2018). Mutations in *PIK3CA* affect glucose metabolism and proliferation in cervical cancer by activating the AKT/glycogen synthase kinase 3 β / β -catenin pathway signalling pathway (Jiang et al., 2018), thereby reversing radio-resistance (Jiang et al., 2020). Notably, Vasilios et al. showed that patients with a mutated *PIK3CA* genotype tend to have poor survival outcomes compared with patients harbouring wild-type *PIK3CA*, particularly in patients with CESC (Pergialiotis et al., 2020). Similarly, *KMT2C* mutations have been shown to be dominant suppressor gene alterations in cervical cancer (Scholl et al., 2020). In the current study, we identified mutations in *TTN* in CESC for the first time and showed that these mutations were relevant for metabolism in this disease.

To determine whether LOF mutations affect prognosis in patients with CESC, we analysed 616 genes mutated in more than five samples and identified 15 significant genes. In the subsequent gene mutual exclusion/co-occurrence analysis, we found that *CUBN-KALRN-HERC1* has a co-occurrence relationship and affects the prognosis of CESC. Although there is no research on these three genes and CESC, in Gronhøj's research, it was found that *HERC1* can be mutated with human papilloma virus infection (Gronhøj et al., 2018). Therefore, it is possible that HPV infection related to cervical cancer might affect the prognosis of CESC by causing mutations in these three genes.

In this study, we further evaluated the effects of metabolism-related gene mutations on clinical parameters using all available gene mutation information to calculate the TMB for each sample. TMB is the total number of mutations present in a tumour specimen and is an emerging biomarker for the response to immunotherapy (Chalmers et al., 2017). We found no significant correlations between TMB and clinical parameters (including overall survival), which is consistent with Huang's research (Huang et al., 2021). However, we found that clinical staging could be associated with metabolism-related TMB in CESC. Except in stage IV disease, metabolism-related TMB was increased with the progression of endocervical adenocarcinoma. Cervical squamous cell carcinoma and endocervical adenocarcinoma belong to two different histological subtypes in cervical cancer. However, the pathogenesis of the two is not the same. Due to different tissue types, TMB is different in different clinical stages. Therefore, for the treatment of two different tissue types, personalised therapeutics can be provided based on different TMB and clinical types.

We also observed changes in copy numbers (Cejas et al., 2020). Importantly, an evaluation of changes in copy numbers of metabolism-related genes showed that FGA, FGG, and FGL were related to recurrence and overall survival in patients with CESC. Similarly, Peng et al. found that *EBP50* plays important roles in cell proliferation and that copy number variations in *EBP50* could affect the prognosis of patients with cervical cancer (Peng et al., 2016). In addition to genomic changes in metabolism-related genes, we also assessed the expression of the aforementioned genes in CESC samples based on TCGA database. In total, 494 metabolism-related genes were aberrantly expressed in CESC compared with levels in normal tissues, 374 of which were related to prognosis.

WGCNA corresponds to an unsupervised classification method, establishing connections among co-expressed genes and grouping them into modules (Langfelder et al., 2008; Niemira et al., 2019). For example, Tian et al. identified two co-expressed functional modules involved in the process of metastasis of lung cancer through WGCNA (Tian et al., 2017). Using WGCNA, eight candidate biomarkers were proposed for the progression and metastasis of breast cancer (Wang et al., 2019). In this study, we identified four metabolism-related modules for CESC according to WGCNA and pathway enrichment analysis, including arachidonic acid metabolism, carbon metabolism, glycolysis/gluconeogenesis, and purine metabolism modules, and each of these modules was verified to be significantly related to prognosis. The arachidonic acid pathway is a metabolic process involved in cervical carcinogenesis that functions by targeting multiple pathways (Yarla et al., 2016), including the cyclooxygenase (COX)-2 pathway; frequent COX-2 expression is closely related to poor prognosis in patients with cervical cancer

(Mandic et al., 2014). Moreover, the metabolic process of glycolysis was reported to be correlated with worse prognosis in patients with cervical cancer (Cai et al., 2020; Li et al., 2019). WGCNA is also known to be particularly suitable for identifying co-expressed modules significant to clinical variables and biological tumour behaviour (Choi et al., 2018; Ohue et al., 2016). Hence, we evaluated the relationship between immune cells and metabolism-related modules. Previous studies have shown that fibre cells are related to glycolysis/gluconeogenesis (Funes et al., 2007). In our research, we also found a positive correlation between the two ($R^2 = 0.4$). In addition, as a metabolic pathway closely related to the occurrence of tumours, arachidonic acid metabolism is positively related to most tumour immune cells. Among them, it has the strongest correlation with T cells. This article by Kato also confirmed the relationship between arachidonic acid and T cells (Kato et al., 1983). Moreover, after further exploring the correlations between these metabolism-related modules and clinical parameters, we speculated that the modules of carbon metabolism, purine metabolism, and arachidonic acid metabolism might be related to different histological classifications and could affect the overall survival patients with CESC. Glycolysis/gluconeogenesis can influence tumour recurrence and has been proven in previous studies to be an independent prognostic factor for tumour recurrence in advanced CESC patients treated with definitive chemoradiotherapy (Hong et al., 2016). Furthermore, we also discovered that the arachidonic acid metabolism module was related to cell proliferation using the GSVA algorithm and that this might attenuate cell proliferation via a mechanism independent of calcium entry, thereby contributing to poor prognosis (Cantonero et al., 2020).

Genetic and epigenetic anomalies, responsible for genetic dysregulation, are the most common aberrations that determine the underlying heterogeneity of tumour cells. Therefore, we also explored potential epigenetic regulatory factors affecting the identified differentially expressed genes. We found that 11 genes were negatively regulated by miRNAs and that the expression levels of six genes were affected by their mutations. Additionally, the expression of 46 genes might be affected by promoter methylation. Interestingly, we identified 58 genes that were regulated by the aforementioned three mechanisms. *NME5* and *SLC40A1* were affected by methylation and miRNA regulation, whereas *HK3* and *JAK3* were regulated by both mutation and methylation. Promoter methylation in the *SLC40A1* gene has been reported to affect differential expression (Ju et al., 2020), and the expression of *HK3* has been shown to be upregulated in CpG island methylator phenotype-high tumours (Fedorova et al., 2019), supporting our findings. Our findings thus provide evidence to further study the association between genetic variation and the expression of differentially expressed genes in CESC.

Conclusion

In summary, in this study, we identified genomic alterations in metabolism-related genes in CESC and evaluated their expression levels and biological functional modules. Furthermore, we explored the potential mechanisms regulating metabolism-related genes. Our findings provide insights into the metabolic activities of CESC and establish potential biomarkers for the carcinogenesis and prognosis of cervical cancer.

Materials And Methods

Data collection

Metabolism-related genes were obtained from the Fluxer database (<https://fluxer.umbc.edu/>). RNA sequencing, copy-number variants, mutation variants, methylation, microRNA (miRNA) expression data, and clinical data for patients with CESC in The Cancer Genome Atlas (TCGA) datasets were downloaded from UCSC XENA (<https://xena.ucsc.edu/>). The level of gene expression was measured as transcripts per million reads. Clinical data included histological type, cancer stage, recurrence, and survival.

Somatic mutation and copy number analysis

Since gene mutations do not necessarily affect gene function, we selected loss of function (LOF) mutations for the following analysis in order to illustrate the importance of mutations. Among the mutation types, we defined FrameShiftIns, FrameShiftDel, NonsenseMutation, NonstopMutation, SpliceSite, and TranslationStartSite to describe mutations that are LOF. Among the mutation types, we evaluated frame shift insertions and deletions, nonsense mutations, nonstop mutations, splice site mutations, and translation start site mutations as those related to LOF. First, we selected mutated genes identified in at least five cases for prognostic analysis. The effects of these genes on prognosis in patients with CESC were observed using Kaplan–Meier log-rank tests. In addition, we performed comet Exact Tests to identify prognosis-related mutated genes with mutual exclusivity, and mutually exclusive mutations were defined as groups for prognostic analysis to assess the effects of different mutation types on prognosis. To further elucidate the effects of metabolism-related gene mutations on patients with CESC, we used the aforementioned gene mutation information to calculate the tumour mutation burden (TMB) and fraction genome altered (FGA) for each sample. We then evaluated the associations between these factors and clinical parameters. Finally, in the analysis of the mechanism of the effect of mutation on its own gene expression, we grouped the samples according to whether they are mutations or not. Use non-parametric tests to analyse whether gene mutations affect the expression of their genes.

RNA-seq analysis

For comparisons of tumour samples with normal tissues, we performed differential expression analysis using the Deseq2 package. Differentially expressed genes were defined as having a false discovery rate less than 0.05 and a $|\log_2(\text{fold-change})|$ greater than 1.

WGCNA

To elucidate the roles of metabolism-related genes in CESC, we used metabolically-related differentially expressed genes for WGCNA analysis. A gene co-expression network was constructed using the WGCNA package of R. Power values were screened using the WGCNA algorithm in the construction of co-expression modules. Scale independence and average connectivity analyses of modules with different power values were performed using gradient tests (power values ranging from 1 to 20). The appropriate power value was determined when the scale independence value was equal to 0.9. The WGCNA algorithm

was then used to construct a co-expression network and extract gene information from the most relevant module. After clustering gene expression, we determined the specific metabolic pathways of each module through pathway enrichment analysis. Further, the correlation between each metabolic module and immune cells and clinical phenotype, evaluated by the MCP counter and GSVA algorithms, was determined using Pearson correlation. Finally, we determined whether the metabolic module affects the prognosis of CESC.

miRNA expression analysis

We obtained normalised miRNA expression data from Genomic Data Commons. To study the mechanisms underlying dysregulated metabolism-related genes in cancer, we identified master miRNA regulators for metabolism-related genes based on two criteria. First, an miRNA had at least one seed region (2–8-mer) matched to the 3' untranslated region of a metabolism-related gene. Second, the Pearson correlation of the miRNA with the expression of the target gene was statistically significant ($P < 0.05$ and $R^2 < -0.5$). Cytoscape was used to visualise the network of miRNAs and metabolism-related genes.

DNA methylation analysis

For the methylation probe, a sequence in the promoter region was selected. For each gene, one DNA methylation probe was selected based on the correlation with its mRNA expression level. If multiple probes for a gene were available, the one with the largest negative correlation value was selected. We identified master methylation regulators for metabolism-related genes according to the Pearson correlation between methylation and the expression of the target gene ($P < 0.05$ and $R^2 < -0.3$).

Declarations

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Ethical approval and consent to participate

Not applicable.

Conflict of interest

All authors have no conflicts of interest to declare.

Consent for publication

All the authors in this paper consent to publication of the work.

Availability of data and material

The datasets used and/or analyzed during the current study are available from the first author on reasonable request.

Author contributions

All authors have seen and approved the final version of this article. DM: designed and executed the study, gathered, analysed and interpreted the data, drafted the manuscript, and contributed to the critical discussion. LH: gathered, analysed and interpreted the data, drafted the manuscript, and contributed to the critical discussion. ZX: contributed to the critical discussion. TJC: acted as the supervisor, contributed to the study design, study execution, critical discussion, revised the manuscript, and approved of the final submitted version.

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Figures

Figure 1

Mutation status of metabolism-related genes in cervical cancer. A. Mutation types of the top 10 most frequently mutated genes in cervical cancer. B. Nucleotide changes in metabolism-related genes. C. Top four mutation-related genes that affect prognosis. D. Co-occurrence analysis of gene mutations related to the prognosis of cervical cancer. E. Prognostic analysis of combined mutations in CUBN-KALRN-HERC1 genes.

Figure 2

Genomic characteristics of metabolism-related genes in cervical cancer. A. Relationships between tumour staging and tumour mutation burden (TMB). B. Association between metabolism-related fragment

changes and histological type. C. Association between metabolism-related fragment changes and tumour prognosis.

Figure 3

Metabolism-related gene functional analysis. A. Differential expression of metabolism-related genes in cervical cancer. B. Module grouping of metabolism-related genes. C. Relationship between metabolism-related modules and tumour immunity and related phenotypes. D. Relationship between metabolism-

related modules and clinical features. E. Association between metabolism-related modules and prognosis in patients with cervical cancer.

Figure 4
Potential regulatory mechanism of metabolism-related genes in cervical cancer.

Supplementary Files

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- [SupplementaryTable1.xlsx](#)
- [SupplementaryTable2.xlsx](#)
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