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Research Article

Integrated in Silico and in Vitro Analysis of Peroxisome-Related Genes PEX16 and DHRS4 in Hepatocellular Carcinoma

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Abstract

Objective: Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related mortality worldwide, characterized by profound metabolic reprogramming and limited therapeutic options. Peroxisomes are essential metabolic organelles involved in lipid metabolism, redox homeostasis, and detoxification, and increasing evidence suggests that peroxisomal dysfunction contributes to tumor progression. However, the role of peroxisome biogenesis–related genes in HCC remains insufficiently characterized. This study aimed to investigate the potential involvement of the peroxisome-related genes PEX16 and DHRS4 in HCC by integrating bioinformatics analyses with experimental validation.

Methods: Differential gene expression analyses were performed using the GEO datasets GSE183349 and GSE14945 to identify peroxisome-associated genes altered in HCC. The expression levels of PEX16 and DHRS4 were subsequently validated in vitro using quantitative real-time PCR (qPCR) in the HepG2 hepatocellular carcinoma cell line and HUVEC cells as controls.

Results: Bioinformatics analyses revealed that PEX16 and DHRS4 were significantly upregulated in HCC datasets. Consistent with the in silico findings, qPCR analysis demonstrated significantly increased expression of both genes in HepG2 cells compared with HUVEC cells (PEX16: p=0.0021; DHRS4: p=0.038). The integrated in silico and in vitro analyses demonstrate that PEX16 and DHRS4 are consistently upregulated in hepatocellular carcinoma.

Conclusions: These findings suggest that both genes may serve as peroxisome-associated molecular biomarkers reflecting metabolic reprogramming in HCC. Further validation in patient-derived samples and functional studies are warranted to evaluate their diagnostic and prognostic potential.

Keywords: Hepatocellular carcinoma, Gene expression, Peroxisome, PEX16, DHRS4

1. INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common primary malignancy of the liver and represents a major global health burden due to its high incidence, poor prognosis, and limited treatment options.[1] Despite advances in surveillance strategies and systemic therapies, HCC-related mortality remains substantial, largely because the disease is often diagnosed at advanced stages and exhibits marked biological heterogeneity.[2] The development and progression of HCC are driven by complex molecular mechanisms involving chronic inflammation, oxidative stress, metabolic dysregulation, and genetic and epigenetic alterations.[3]

Metabolic reprogramming is a hallmark of cancer and plays a particularly critical role in hepatocarcinogenesis, given the central metabolic functions of the liver.[4] In addition to mitochondria, other metabolic organelles have emerged as important regulators of tumor biology. Among these, peroxisomes are single-membrane–bound organelles that contribute to essential cellular processes, including very-long-chain fatty acid β-oxidation, ether lipid biosynthesis, reactive oxygen species (ROS) metabolism, and detoxification of harmful metabolites.[5] Dysregulation of peroxisomal function has been implicated in oxidative imbalance, chronic inflammation, and altered lipid metabolism, all of which are key features of liver disease progression and HCC development.[6]

Peroxisome biogenesis and maintenance are tightly regulated by a group of peroxisome biogenesis factors encoded by PEX genes.[7] Alterations in the expression or function of these genes can disrupt peroxisomal homeostasis and lead to profound metabolic consequences. *PEX16* is a critical regulator of early peroxisomal

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membrane assembly and de novo peroxisome formation.[8] Emerging evidence suggests that aberrant *PEX16* expression may contribute to cancer-associated metabolic reprogramming by modulating peroxisome abundance and inter-organelle communication, particularly between peroxisomes and mitochondria.[9] However, the relevance of *PEX16* dysregulation in HCC has not yet been clearly elucidated.

In parallel, the dehydrogenase/reductase family member *DHRS4* has been implicated in retinoid and lipid metabolism, oxidative stress regulation, and cellular differentiation.[10] Altered expression of *DHRS4* has been reported in several solid tumors, including prostate and breast cancers, suggesting a potential role in tumor biology.[11] Nevertheless, data regarding *DHRS4* expression and function in hepatocellular carcinoma are scarce, and its possible association with peroxisome-related metabolic pathways in HCC remains unexplored.

In this context, the present study aimed to investigate the involvement of peroxisome-related genes in hepatocellular carcinoma by focusing on *PEX16* and *DHRS4*. By integrating bioinformatics analyses of publicly available gene expression datasets with experimental validation in HCC cell lines, we sought to clarify whether these genes are differentially expressed in HCC and to provide preliminary insights into their potential contribution to hepatocarcinogenesis. This integrated approach may help to identify novel molecular targets linked to peroxisomal dysfunction and metabolic alterations in HCC.

2. MATERIALS AND METHODS

2.1. Bioinformatics identification of candidate genes

Publicly available gene expression datasets related to hepatocellular carcinoma were retrieved from the NCBI Gene Expression Omnibus (GEO) database.[12] Based on the availability of appropriate control groups, comparable sample sizes, and experimental design quality, the datasets GSE183349 and GSE14945 were selected for analysis. Raw expression data were imported into the R statistical software environment[13] and processed using standard Bioconductor[14] differential expression analysis workflows. Genes exhibiting statistically significant differential expression between tumor and control samples ($p<0.05$) were identified. Among the significantly altered genes, *PEX16* and *DHRS4* were selected as candidate peroxisome-related genes for further experimental validation.

2.2. Cell culture

Human Hepatocellular Carcinoma cell line (HepG2, ATCC HB-8065) and Human Umbilical Vein Endothelial cell line (HUVEC, ATCC CRL-1730) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and used for all in vitro experiments. HepG2 cells were used as the cancer group whereas HUVEC cells were used as the control group. Cells were cultured in high-glucose Dulbecco's Modified Eagle Medium (DMEM; Gibco, Thermo Fisher Scientific, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-glutamine, and 1% penicillin–streptomycin (100 U/mL penicillin and 100 µg/mL streptomycin). Cells cultered in sterile culture flasks at 37°C in a incubator containing 5% CO_2 and 95% air atmosphere. Culture medium was replaced every 2 days to ensure optimal nutrient supply and removal of metabolic waste products for cell growth. Cell morphology, adherence and proliferation were monitored using an inverted microscope to confirm healthy growth and absence of contamination. We used early-passage cells for experiments to minimize phenotypic and genomic unstability associated with prolonged in vitro culture. Cells were subcultured at approximately 80% confluence using 0.25% trypsin–EDTA. Trypsin activity was neutralized by adding complete culture medium and cells were collected by centrifugation for downstream applications. Cells were cultured across five consecutive passages, and total RNA was extracted from three independent replicates at each passage (15 sample for each group) for gene expression analysis.

2.3. RNA isolation and cDNA synthesis

Total RNA was isolated from cultured cells using the All-In-One DNA/RNA Mini-Preps Kit (Cat. No: BS88203, BioBasic, Canada) according to the manufacturer's protocol. The purity and concentration of RNA samples were measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA)

Complementary DNA (cDNA) synthesis was performed using the OneScript® Plus cDNA Synthesis Kit (Cat. No: G236, ABM, Canada) following the supplier's instructions. Reverse transcription reactions were carried out under recommended thermal conditions. The resulting cDNA was diluted appropriately and stored at −20°C for quantitative real-time PCR analyses.

2.4. Primer design and Quantitative Real-Time PCR (QRT-PCR)

Gene-specific primers for *PEX16* and *DHRS4* with the reference gene *ACTB,* were designed using the Primer3Plus online tool.[15] Primer sequences and amplicon characteristics are provided in Table 1. Primer specificity was verified in silico prior to experimental use.

Tablo 1.

Primer pairs for PEX16 and DHRS4

Gene	Primer type	Sequence (5′–3′)
PEX16	Forward	ACCATCCTGCTGCTCTACTA
PEX16	Reverse	ATGGATTACTTGCCCACCTG
DHRS4	Forward	TCTCCAATGCTGCTGTCAAC
DHRS4	Reverse	AACTGCCTAGCACCTGGACT
ACTB	Forward	TGGTGATGGAGGAGGTTTAGTAAGT
ACTB	Reverse	AACCAATAAAACCTACTCCTCCCTTAA

For QRT-PCR, a suitable reaction mixture was prepared and dispensed into 96-well plates at a volume of 20 µL per well using a multichannel pipette. The QRT-PCR reaction mix included 10 µL of 10X Sensifast SYBR No-ROX Kit Mix, 0.2 µL of forward primer (100 µM), 0.2 µL of reverse primer (100 µM), 6.6 µL of dH2O, and 3.0 µL of the sample (cDNA) for each well.

The QRT-PCR reaction conditions consisted of an initial denaturation step at 95°C for 30 seconds, followed by 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, and a melting curve analysis with 95°C for 10 seconds, 60°C for 20 seconds, and a final cooling step at 40°C for 30 seconds. The results were analyzed using the Basic Relative Quantification program within the LightCycler 480 software. Additionally, the amplification of the target product was validated through melting curve analysis.

All qPCR reactions were conducted in technical triplicate and mean Ct values were used for further calculations. Relative gene expression levels were determined using the comparative Ct ($2^{-\Delta\Delta Ct}$) method after normalization to ACTB expression.

2.5. Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.0 (Dotmatics, USA). Data are presented as mean ± standard deviation (SD) derived from five independent biological replicates. For each replicate, technical triplicates were averaged prior to statistical evaluation. Differences in relative gene expression between HepG2 and HUVEC cells were assessed using an unpaired two-tailed Student's t-test, as comparisons were made between two independent cell populations. A p value ≤ 0.05 was considered statistically significant.

3. RESULTS

3.1. Differential expression of *PEX16* and *DHRS4* identified by bioinformatics analysis

Bioinformatic analysis of the GEO datasets GSE183349 and GSE14945 demonstrated a distinct dysregulation of peroxisome-associated genes in hepatocellular carcinoma compared with non-tumor controls. Among the identified candidates *PEX16* and *DHRS4* were consistently upregulated across datasets, exhibiting log fold change values of 0.40 (p=0.026) and 0.51 (p=0.026) respectively. These findings provided a rationale for selecting *PEX16* and *DHRS4* for subsequent experimental validation.

3.2. Relative expression of *DHRS4* and *PEX16* in HepG2 cells

Validation of *PEX16* and *DHRS4* expressions was assessed in HepG2 and HUVEC cell lines using quantitative real-time PCR (qPCR) (n=15 per group) (Figure 1, Table 2). qPCR analysis demonstrated that both DHRS4 and PEX16 were significantly upregulated in HepG2 cells compared with HUVEC controls. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with HUVEC cells used as the control group and ACTB as the endogenous reference gene for normalization. *DHRS4* expression was significantly increased in HepG2 cells (p=0.038), while *PEX16* showed a more pronounced upregulation (p=0.0021).

Figure 1.

Relative expression levels of PEX16 and DHRS4 in HepG2 and HUVEC cells

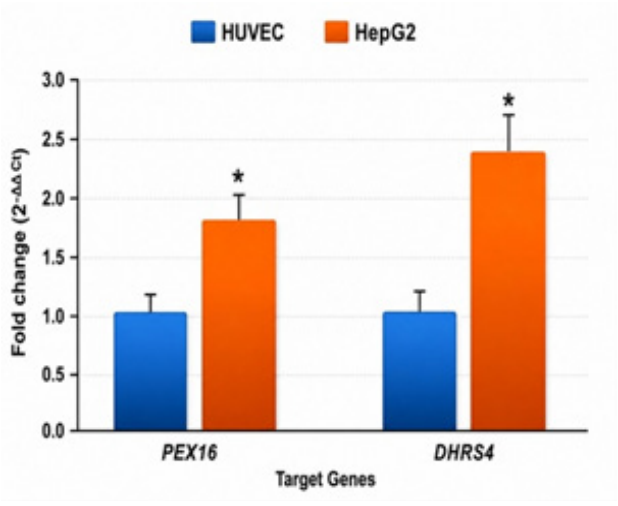


Table 2.

Relative expression levels of DHRS4 and PEX16 in HepG2 cells compared to HUVEC controls

Gene	HUVEC	HepG2	p value
PEX16	1.00 ± 0.23	3.27 ± 0.74	0.0021
DHRS4	1.00 ± 0.45	5.40 ± 1.62	0.038

As summarized in Table 2, *DHRS4* expression increased in HepG2 cells (mean fold change: 5.40 ± 1.62) compared to HUVEC cells (1.00 ± 0.45). Statistical analysis using an unpaired t-test confirmed that this increase was statistically significant (p=0.038). Similarly, *PEX16* expression was significantly elevated in HepG2 cells, with a mean fold change of 3.27 ± 0.74 relative to HUVEC controls (1.00 ± 0.23). This difference was also statistically significant (p=0.0021; Table 2). Overall, these results indicate that both *DHRS4* and *PEX16* are consistently and significantly upregulated in hepatocellular carcinoma–derived HepG2 cells, supporting their potential association with peroxisome-related metabolic alterations in HCC.

4. DISCUSSION

Hepatocellular carcinoma is characterized by profound metabolic alterations that support tumor growth, survival, and progression.[4,16] In recent years, increasing attention has been directed toward non-mitochondrial metabolic organelles as contributors to cancer biology. Among these, peroxisomes have emerged as key regulators of lipid metabolism, redox balance, and cellular stress responses.[9,17] The present study provides integrated *in silico* and *in vitro* evidence demonstrating that the peroxisome-related genes *PEX16* and *DHRS4* are significantly upregulated in hepatocellular carcinoma, suggesting a potential association between peroxisomal dysregulation and hepatocarcinogenesis. Bioinformatics analysis of independent GEO datasets revealed consistent upregulation of *PEX16* and *DHRS4* in HCC samples compared with non-tumoral controls. The concordance between these *in silico* findings and the experimental validation in HepG2 cells strengthens the robustness of the results and reduces the likelihood that the observed expression changes are dataset-specific artifacts. This integrated approach supports the relevance of both genes in the molecular landscape of HCC.

PEX16 plays a central role in peroxisome biogenesis by regulating peroxisomal membrane assembly and de novo organelle formation.[8,18] Altered expression of *PEX16* has been reported in several malignancies and has been linked to metabolic reprogramming through modulation of peroxisome abundance and peroxisome–mitochondria crosstalk.[9] Similarly, peroxisome-associated gene signatures in breast cancer (e.g., those containing *PEX16*) show promise in predicting immunotherapy response,[19] supporting the potential prognostic value of the upregulation we observed in HCC. Given the high metabolic demands of hepatocellular carcinoma cells, increased *PEX16* expression may reflect an adaptive response that enhances peroxisomal capacity to support lipid metabolism and redox homeostasis. In this context, the marked upregulation of *PEX16* observed in HepG2 cells suggests that peroxisome biogenesis may be selectively enhanced in HCC, potentially contributing to tumor-associated metabolic flexibility.

Similarly, *DHRS4*, a member of the short-chain dehydrogenase/reductase family, is involved in retinoid and lipid metabolism and has been implicated in oxidative stress regulation.[10] Previous studies have reported dysregulated *DHRS4* expression in several solid tumors, including prostate and breast cancers, supporting a potential role in tumor biology.[11,20,21] However, its involvement in hepatocellular carcinoma has remained largely unexplored. On the other hand, the antisense transcript of *DHRS4*, *DHRS4*-AS1, is downregulated in HCC, suppressing proliferation and promoting apoptosis, suggesting that the upregulation of *DHRS4* mRNA may represent an epigenetic escape mechanism.[22,23] The significant upregulation of *DHRS4* observed in both bioinformatics analyses and *in vitro* experiments in the present study suggests that this gene may also contribute to metabolic adaptations in HCC cells, possibly through altered lipid handling or redox-related pathways.

The concurrent upregulation of *PEX16* and *DHRS4* points toward a coordinated alteration of peroxisome-associated metabolic pathways in hepatocellular carcinoma.[24,25] Peroxisomes are known to generate and detoxify reactive oxygen species, and enhanced peroxisomal activity may provide tumor cells with a selective advantage by maintaining redox balance under conditions of increased metabolic stress.[9,26-28] Furthermore, peroxisome dysregulation may facilitate immune escape by fueling the immunosuppressive tumor microenvironment.[29] In this regard, elevated expression of peroxisome-related genes may reflect a compensatory mechanism that supports tumor cell survival and proliferation.

In hepatocellular carcinoma, dysregulation extends beyond peroxisomes to other metabolic organelles, further underscoring the multifaceted nature of tumor-associated metabolic reprogramming. Mitochondria, for instance, exhibit profound alterations in electron transport chain activity and oxidative phosphorylation,

facilitating a shift toward glycolysis (Warburg effect) that supports rapid proliferation under hypoxic conditions.[4,30] Similarly, endoplasmic reticulum (ER) stress responses are upregulated in HCC, promoting unfolded protein response (UPR) pathways that enhance protein folding capacity and mitigate proteotoxic stress, thereby conferring survival advantages.[31] Lysosomal dysfunction has also been implicated, with impaired autophagic flux contributing to the accumulation of damaged organelles and aggregated proteins, which in turn fuels inflammatory signaling and tumor progression.[32,33] These organelle-specific changes often intersect with peroxisomal functions; for example, peroxisome-mitochondria tethering influences fatty acid flux and ROS signaling, suggesting that *PEX16*-mediated peroxisomal biogenesis may amplify mitochondrial metabolic overload in HCC.[34,35] Collectively, these observations highlight the need for multi-organelle targeted therapies to disrupt the interconnected metabolic network in HCC.

Peroxisomal alterations are not unique to HCC but represent a conserved feature across various solid tumors, where they similarly drive oncogenic metabolic adaptations. In prostate cancer, peroxisomal β-oxidation is upregulated, leading to increased ether lipid synthesis that enhances membrane fluidity and signaling for androgen-independent growth.[36] Breast cancer cells exhibit peroxisome proliferation driven by PPAR signaling, which correlates with stemness and therapeutic resistance, as evidenced by reduced mammosphere formation upon peroxisomal inhibition.[37] Specifically, PPARγ inhibitors that modulate peroxisome biogenesis may provide therapeutic benefit by inhibiting adhesion and anoikis in HCC cells,[38] opening a new window for strategies targeting *PEX16* upregulation. In colorectal cancer, peroxisomal dysfunction contributes to bile acid metabolism dysregulation, promoting inflammation and epithelial-mesenchymal transition (EMT) that facilitates metastasis.[39] These parallels suggest that the *PEX16* and *DHRS4* upregulation observed in our HCC study may reflect a broader peroxisomal "signature" in epithelial-derived malignancies, potentially amenable to pan-cancer therapeutic strategies targeting peroxisome biogenesis.[17] Such cross-tumor insights could inform the development of peroxisome-modulating agents with enhanced specificity for HCC.

From a translational perspective, the consistent upregulation of *PEX16* and *DHRS4* in *in silico* datasets and *in vitro* models highlights their potential utility as molecular biomarkers for peroxisome-related genes in hepatocellular carcinoma, as reported in the literature.[40] Although the present study does not address diagnostic or prognostic performance directly, the observed expression patterns suggest that these genes may serve as indicators of peroxisome-related metabolic reprogramming in HCC. To more comprehensively evaluate biomarker potential, future studies should test peroxisome-mitochondrial interactions in vivo HCC models (e.g., xenografts) using CRISPR-based knockdown, validate changes at the protein level, and also include patient-derived tissues and clinical outcome data.

To understand if these genes can be used as biomarkers or therapeutic targets in HCC, the limitations of this study must be acknowledged. First, the experimental validation was performed using a single hepatocellular carcinoma cell line and an endothelial control cell line, which may not fully capture the heterogeneity of HCC. Second, gene expression was assessed at the mRNA level, and corresponding changes at the protein level were not evaluated. Third, functional assays were not performed to directly elucidate the mechanistic roles of *PEX16* and *DHRS4* in HCC biology. Finally, it is necessary to conduct validation studies with larger patient samples to ensure the accuracy and validity of the results. Despite these limitations, the consistency between bioinformatics and experimental findings supports the validity of the results and provides a foundation for further investigation.

5. Conclusion

In conclusion, this study demonstrates for the first time that *PEX16* and *DHRS4* are significantly upregulated in hepatocellular carcinoma through an integrated *in silico* and *in vitro* approach. These findings support a potential link between peroxisomal dysregulation and HCC pathogenesis and suggest that *PEX16* and *DHRS4* may represent promising candidate biomarkers or molecular targets related to peroxisome-associated metabolic pathways in the diagnosis or treatment of hepatocellular carcinoma.

Article Information Form

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Conceptualization: SŞ, FKT, MK, MT; Methodology: SŞ, FKT, MK, TS, MT; Software: SŞ, FKT, HED, MEG, TS; Validation: SŞ, FKT, HED, MEG, TS; Formal

analysis: SŞ, FKT, MK; Investigation: SŞ, FKT, MK, MT; Resources: SŞ, FKT, MK, HED, MEG, TS; Data curation: SŞ, FKT, HED, TS, MT; Writing – original draft: SŞ, FKT, MK, MT; Writing – review & editing: SŞ, FKT, MK, HED, TS, MT; Visualization: SŞ, FKT, MK, MT; Supervision: FKT, MT; Project administration: MT; Funding acquisition: MT.

The Declaration of Conflict of Interest/ Common Interest

No conflict of interest or common interest has been declared by authors.

Data Availability Statement

The data supporting the findings of this study are available from the corresponding author upon reasonable request.

Artificial Intelligence Statement

No artificial intelligence tools were used while writing this article.

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