

ORIGINAL ARTICLE

Molecular mechanism of aquaporin (AQP3) in regulating differentiation and apoptosis of lung cancer stem cells through Wnt/GSK-3 β / β -Catenin pathway

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Summary

Purpose: To explore the effect of aquaporin-3 (AQP3) on the functions of lung cancer stem cells (LCSCs), and its molecular mechanism in regulating the differentiation and apoptosis of LCSCs through the Wnt/glycogen synthase kinase-3 β (GSK-3 β)/ β -catenin pathway.

Methods: The stem cells were selected and the cell lines with low expression of AQP3 were constructed, followed by transcriptome sequencing. LCSCs were transfected with empty lentivirus in control group and transfected with AQP3 shRNA in interference group, and the low expression of AQP3 was inhibited using the Wnt pathway inhibitor XAV939 in interference + inhibitor group. The expressions of AQP3, Wnt/GSK-3 β / β -catenin pathway genes, stemness genes, differentiation-related markers and apoptosis proteins in LCSCs were detected.

Results: In interference group, the pathway genes were highly expressed. The genes in interference group were enriched in

the Wnt/GSK-3 β / β -catenin pathway. In interference group, the expressions of β -catenin, GSK-3 β and signal transducer and activator of transcription 3 (STAT3) were significantly higher, while the expression of adenomatous polyposis coli (APC) was significantly lower ($p < 0.05$). The expression of Wnt5a had no difference. In interference group, the expressions of stemness-related genes were obviously higher, while the expression of CDK2 had no difference ($p = 0.471$). Interference group had higher expressions of differentiation markers.

Conclusion: In conclusion, AQP3 can reduce the differentiation and inhibit the apoptosis of LCSCs through reducing the expressions of Wnt/GSK-3 β / β -catenin pathway-related genes such as β -catenin, GSK-3 β and STAT3, thereby affecting the tumor progression.

Key words: lung cancer stem cells, AQP3, apoptosis

Introduction

Lung cancer is a highly heterogeneous disease and prone to resistance to available drugs, with 5-year survival rate lower than 15% [1,2]. Despite a certain understanding of the genetic changes and aberrations in signaling pathways, it is still difficult to determine the causes of heterogeneity and drug resistance of lung cancer. To improve the therapeutic effect in lung cancer, it is necessary to better understand the cellular and molecular mechanisms of the occurrence and metastasis of

this disease, thereby designing new therapeutic strategies. In recent years, a new school of thought has emerged, which argues that tumors are derived from one part of cancer cells, known as cancer stem cells, that are in dormancy and able to evade drug therapy and metastasize [3,4]. Therefore, the function of LCSCs may have a great impact on the heterogeneity and drug resistance of lung cancer, thus affecting the progression and the therapeutic effect in lung cancer.

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The aquaporin (AQP) family can promote the rapid passive movement of water and regulate the osmotic balance [5]. Although AQP exists in a variety of tissues, it mostly has a unique tissue-specific expression pattern. AQP is extremely active in some cells mainly involved in liquid absorption and secretion [6]. In addition to the effects of transporting liquid and regulating osmotic balance, AQP also participates in regulating the cellular functions, including functional changes in cancer cells [7,8]. It can be seen that the AQP family may have an important regulatory effect on cancer cells.

AQP3 is a weakly active water-transporting protein, but it has a special effect on the transport of glycerol. AQP3 was originally cloned from the chief cells of the kidney, but it is also expressed in various epithelial cells. Recent studies have demonstrated

that there is a significant association between AQP3 expression and the occurrence, progression and prognosis of various malignant tumors, including breast cancer [9], colorectal cancer [10], prostate cancer [11], esophageal cancer [12] and lung cancer [13]. However, the specific role of AQP3 in LCSCs and its molecular mechanism affecting cellular functions have not been studied yet in China and other countries.

In the present study, therefore, the regulatory effect of AQP3 on the functions of LCSCs through the Wnt/glycogen synthase kinase-3 β (GSK-3 β)/ β -catenin pathway was studied, and its specific molecular mechanism in regulating the stemness, differentiation and apoptosis of LCSCs was explored.

Methods

Isolation and culture of LCSCs

The non-small cell lung cancer A549 cell line was purchased from the American Type Culture Collection (ATCC), and cultured in the RPMI 1640 medium containing 10% fetal bovine serum (FBS) + 1% penicillin/streptomycin. After amplification culture, one part of cells was cryopreserved in liquid nitrogen for later use, while the other part was passaged for further amplification. Then, LCSCs were selected from lung cancer A549 cells using the CD133 magnetic beads (BD, USA).

Construction of AQP3 shRNA lentiviral expression vectors and cell lines with low expression of AQP3

The AQP3 shRNA lentiviral expression vectors were constructed: LCSCs in logarithmic growth phase were digested with trypsin and inoculated into 6-well plates overnight, so that the cell density could reach 2×10^5 /well before lentiviral transfection. On the next day, the original medium was replaced with fresh medium containing polybrene (6 μ g/mL), and the lentivirus suspension was added, followed by incubation for 24 h.

The original medium was replaced again. At 72 h after transfection, the expression of green fluorescent protein in cells, namely the transfection efficiency, was observed under a fluorescence microscope.

LCSCs transfected with empty lentivirus were used as control group, and those with the AQP3 shRNA transfection efficiency above 80% were used as interference group. Some LCSCs stably transfected in interference group were treated with the Wnt pathway inhibitor XAV939 (1 μ mol/mL) for 24 h, as interference+inhibitor group.

Table 1. qPCR primer sequences

Gene	Primer sequence 5'→3'	Tm
AQP3		
Forward	GGGGAGATGCTCCACATCC	61.5
Reverse	AAAGGCCAGGTTGATGGTGAG	62.3
β -catenin		
Forward	AAAGCGGCTGTTAGTCACTGG	62.6
Reverse	CGAGTCATTGCATACTGTCCAT	60.2
GSK-3 β		
Forward	GGCAGCATGAAAGTTAGCAGA	60.6
Reverse	GGCGACCAGTTCCTCTGAATC	62.5
Wnt5a		
Forward	ATTCTTGGTGGTTCGCTAGGTA	60.3
Reverse	CGCCTTCTCCGATGTACTGC	62.7
STAT3		
Forward	CAGCAGCTTGACACACGGTA	62.4
Reverse	AAACACCAAAGTGGCATGTGA	60.6
APC		
Forward	AAAATGTCCCTCCGTTCTTATGG	60.3
Reverse	CTGAAGTTGAGCGTAATACCAGT	60.0
CyclinD1		
Forward	GCTGCGAAGTGGAAACCATC	61.6
Reverse	CCTCCTTCTGCACACATTTGAA	60.8
Axin2		
Forward	CAACACCAGGCGGAACGAA	62.8
Reverse	GCCCAATAAGGAGTGTAAGGACT	61.4
Bcl-2		
Forward	GGTGGGGTCATGTGTGTGG	62.6
Reverse	CGGTTCCAGGTACTCAGTCATCC	61.8
LRP6		
Forward	TTTATGCAAACAGACGGGACTT	60.2
Reverse	GCTCCAACACTACAATCGTAGC	60.5
CDK2		
Forward	CCAGGAGTTACTTCTATGCCTGA	60.9
Reverse	TTCATCCAGGGGAGGTACAAC	60.9
GAPDH		
Forward	GGAGCGAGATCCCTCCAAAAT	61.6
Reverse	GGCTGTTGTCATACTTCTCATGG	60.9

Transcriptome sequencing (RNA-seq) and gene set enrichment analysis (GSEA) in the interference group and control group

The total RNA was extracted using TRIzol in the interference group and control group and sent for transcriptome sequencing, followed by bioinformatics analysis and GSEA of the sequencing results.

Detection of mRNA level

The mRNA levels of AQP3, β -catenin, GSK-3 β , Wnt5a, signal transducer and activator of transcription 3 (STAT3), adenomatous polyposis coli (APC), CyclinD1, Axin2, B-cell lymphoma-2 (Bcl-2), low-density lipoprotein receptor-related protein 6 (LRP6) and cyclin-dependent kinase 2 (CDK2) were detected via real-time fluorescence quantitative polymerase chain reaction (qRT-PCR) with GAPDH as an internal reference. The total RNA [absorbance (A)₂₆₀/A₂₈₀=1.8-2.0] was extracted using TRIzol from the three groups, reversely transcribed into cDNA and stored at -20°C. The gene primers were designed using Primer Premier 5.0 and synthesized by Sangon Biotech (Shanghai). The primer sequences and T_m values are shown in Table 1.

The PCR system was 25 μ L in total, including 1 μ L of primers, 0.5 μ L of template cDNA, 12.5 μ L of SYBR premix Ex Taq and 10 μ L of dH₂O. The PCR conditions were as follows: 95°C for 3 min, and 95°C for 30 s, 58°C for 40 s, and 72°C for 35 s \times 40 cycles.

Western blotting

The protein expressions of AQP3, Oct4, Sox2, Caspase3 and Bcl-2 were detected using Western blotting. The RIPA lysis solution was added with 1% PMSF protease inhibitor and prepared into protein lysis working solution, by which the total protein was lysed on ice for 15 min in the interference group and interference+inhibitor group, followed by centrifugation at 14000 rpm for 15 min. Then, the protein was added with loading buffer, denatured via boiling water bath and stored at -20°C. After SDS-PAGE for 1 h, the protein was transferred onto a PVDF membrane, sealed with 5% skim milk for 2 h, and incubated with primary antibodies of AQP3 and Oct4 (Abcam, USA),

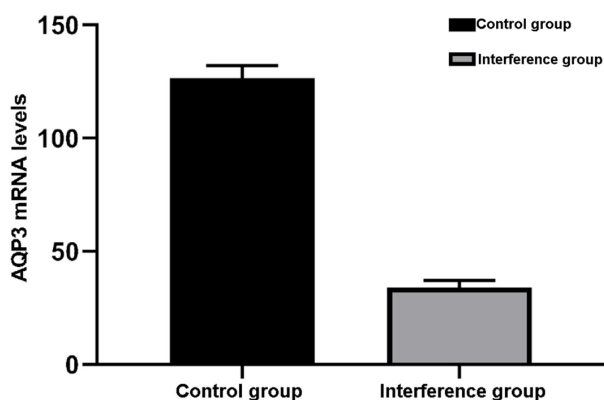


Figure 1. AQP3 mRNA levels in the control and interference group. There is a significant difference in the AQP3 mRNA expression between the two groups ($p < 0.05$).

Sox2, Caspase3 and Bcl-2 (R&D, USA) at 4°C overnight. On the next day, the protein was incubated again with HRP-labeled secondary antibodies for 1 h and washed, followed by exposure using Bio-Rad gel doc XR+imager.

Statistics

SPSS 20.0 software was used for statistical analysis. T-test and one way analysis of variance (ANOVA) were performed to evaluate the differences among groups. $P < 0.05$ suggested statistically significant difference.

Results

Expression of AQP3 in control group and interference group

There were differences in the mRNA and protein expressions of AQP3 between control group and interference group ($p < 0.05$) (Figures 1 and 2). The expression of AQP3 in the interference group was significantly lower than that in the control group, indicating that the cell line stably transfected with AQP3 shRNA lentivirus was successfully constructed.

Transcriptome sequencing in the control group and interference group

According to the mRNA sequencing results in the control and interference group, some genes

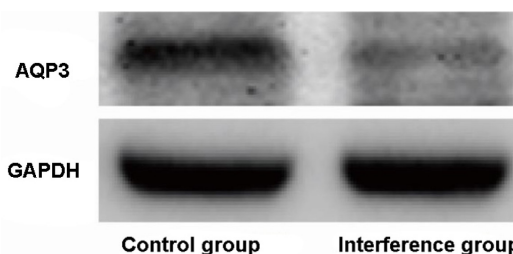


Figure 2. AQP3 protein levels in the control and interference group. There is a significant difference in the AQP3 protein expression between the two groups ($p < 0.05$).

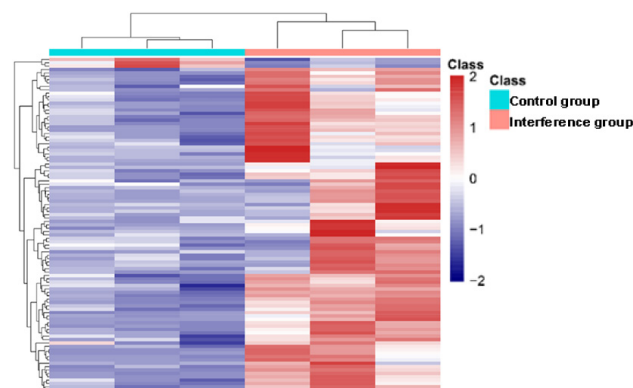


Figure 3. Transcriptome sequencing heat map in the control and interference group.

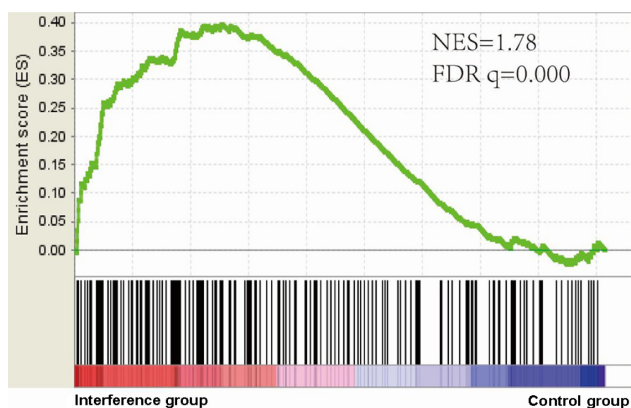


Figure 4. GSEA of Wnt/GSK-3β/β-catenin pathway in the control and interference group showing that the genes in the interference group were enriched in the WNT/GSK-3β/β-catenin pathway.

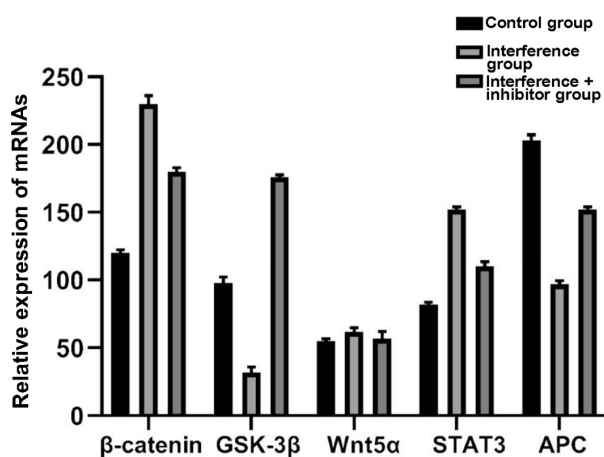


Figure 5. Analysis of Wnt/GSK-3β/β-catenin pathway-related genes. In the interference group the expressions of β-catenin, GSK-3β and STAT3 are significantly higher than those in the other two groups ($p < 0.05$), while the expression of APC is significantly lower than that in the other two groups ($p < 0.05$).

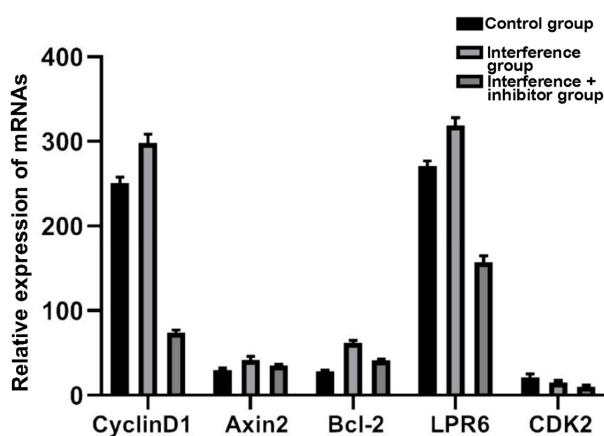


Figure 6. Expressions of stemness genes. In the interference group the expressions of stemness-related genes CyclinD1, Axin2, Bcl-2 and LRP6 are evidently higher than those in the other two groups ($p < 0.05$), while the expression of CDK2 has no difference among the three groups ($p = 0.471$).

and pathways, including TMEM106A, ALG10, TTC39C, NEXN, RAX2, MFAP3, EYA3, GIMAP1, KLK8, Wnt/GSK-3β/β-catenin pathway and Hippo pathway, were enriched in the interference group (Figure 3).

GSEA of sequencing results in the control and interference group

According to the GSEA of sequencing results in the control and interference group using GSEA 3.0 software, the genes in the interference group were enriched in the Wnt/GSK-3β/β-catenin pathway (NES=1.78, FDR $q = 0.000$) (Figure 4).

Analysis of Wnt/GSK-3β/β-catenin pathway-related genes in the three groups

In the interference group, the expressions of β-catenin, GSK-3β and STAT3 were significantly higher than those in the other two groups ($p < 0.05$), while the expression of APC was significantly lower than in the other two groups ($p < 0.05$). The expression of Wnt5α had no difference among the three groups ($p = 0.231$) (Figure 5). The results above demonstrate that the decline in AQP3 will obviously affect the Wnt/GSK-3β/β-catenin pathway in LCSCs, and obviously up-regulate the expressions of most genes, which can be restored by the Wnt pathway inhibitor XAV939.

Expressions of stemness genes in the three groups

In the interference group, the expressions of stemness-related genes CyclinD1, Axin2, Bcl-2 and LRP6 were evidently higher than those in the other two groups ($p < 0.05$), while the expression of CDK2 had no difference among the three groups ($p = 0.471$) (Figure 6), indicating that the decline in AQP3 will evidently affect the stemness of LCSCs.

Expressions of differentiation-related molecules Sox2 and Oct4 in LCSCs

The interference group had higher mRNA and Sox2 and Oct4 protein expressions than the other two groups ($p < 0.05$) (Figures 7 and 8). The increase in Sox2 and Oct4 in the interference group indicated that AQP3 affected the differentiation of LCSCs, which could be restored by the Wnt pathway inhibitor XAV939. It can be seen that AQP3 affects the differentiation of LCSCs through the Wnt/GSK-3β/β-catenin pathway.

Apoptosis-related molecules in LCSCs

The interference group had higher Caspase3 and Bcl-2 protein expressions than the other two groups ($p < 0.05$) (Figure 9). The increase in Caspase3 and Bcl-2 in the interference group suggests

that AQP3 affected the apoptosis of LCSCs, which could be restored by the Wnt pathway inhibitor XAV939. It can be seen that AQP3 affects the apoptosis of LCSCs through the Wnt/GSK-3 β / β -catenin pathway.

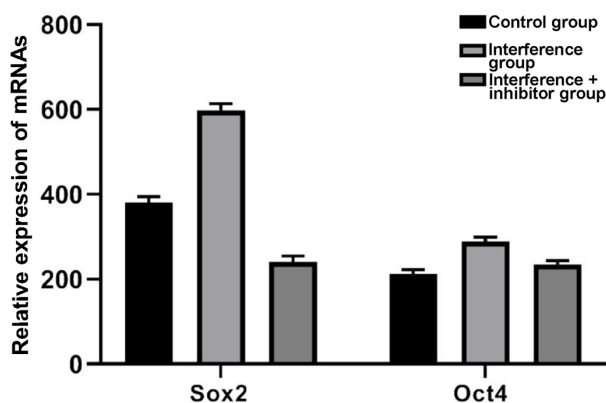


Figure 7. Sox2 and Oct4 mRNA levels. The interference group has remarkably higher mRNA expressions of Sox2 and Oct4 than the other two groups ($p < 0.05$).

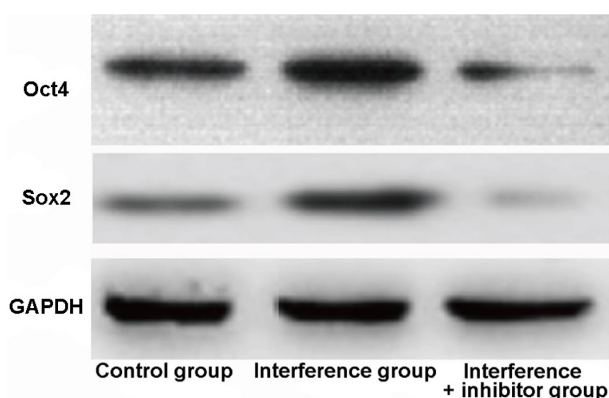


Figure 8. Sox2 and Oct4 protein levels detected via Western blotting. The interference group has remarkably higher protein expressions of Sox2 and Oct4 than the other two groups ($p < 0.05$).

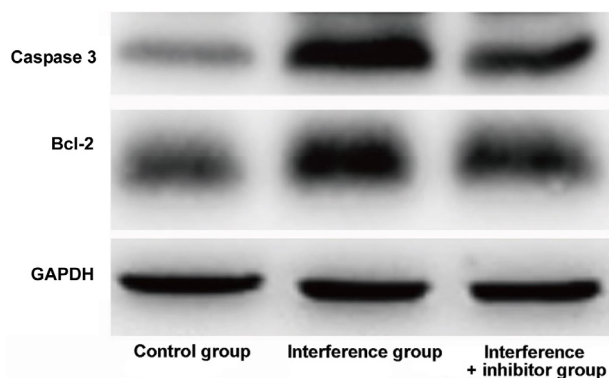


Figure 9. Apoptosis proteins detected using Western blotting. The interference group has remarkably higher protein expressions of Caspase3 and Bcl-2 than the other two groups ($p < 0.05$).

Discussion

Recently, related studies on LCSCs has attracted considerable attention in the context of lung cancer, and it has been found that LCSCs are associated with the tumor invasion, metastasis, progression, resistance to treatment and recurrence. The vague nature of specific markers for LCSCs may be due to the intercellular and intracellular heterogeneity in stem cell-like cancer cells, which depends on the pathological type of lung cancer. The lack of specific identification markers for a variety of stem cell-like cancer cells may be a major obstacle to the transition of the concept of cancer stem cell into the therapeutic strategy for lung cancer. In this paper, the mRNA and protein expressions of AQP3 were determined in LCSCs, and it was found that AQP3 was highly expressed, so it may be a new specific marker for LCSCs, which extends the treatment means of lung cancer to stem cells.

The roles of several AQPs in cancer have been reported in recent studies [14,15]. According to current research, AQP3 affects the material transport and metabolic pathways in tumor cells, including intracellular and extracellular transport of glycerol [16,17]. The results of studies on AQP3-knockout mice showed that AQP3 is essential for the development of skin tumor, because skin tumor will not occur in AQP3-knockout mice after receiving tumor cells [18]. In addition, overexpression and ectopic expression of AQP3 are observed in several cancers, which contribute to the epithelial-mesenchymal transition (EMT) in cancer cells, promote the differentiation of tumor cells into cells with stronger proliferation ability, and inhibit the apoptosis of cancer cells [19,20].

It was found in this study that after interference in the AQP3 gene, the expressions of TMEM106A, ALG10, TTC39C, NEXN, RAX2, MFAP3, EYA3, GIMAP1, KLK8, Wnt/GSK-3 β / β -catenin pathway and Hippo pathway in LCSCs were up-regulated (Figure 3), suggesting that AQP3 affects the expressions of multiple genes and pathways, thereby influencing the functions of LCSCs. At the same time, AQP3 may also influence the expressions of downstream signaling molecules through these genes and pathways, and make them secrete cytokines, thereby affecting the proliferation and metastasis of lung cancer cells.

According to the GSEA results, AQP3 obviously affected the Wnt/GSK-3 β / β -catenin pathway (NES=1.78, FDR $q = 0.000$) (Figure 4), and the same results were obtained in the verification of the pathway-related genes β -catenin, GSK-3 β and STAT3 (Figure 5), demonstrating that AQP3 may affect the cell functions of LCSCs through the Wnt/

GSK-3 β / β -catenin pathway. GSK-3 β modifies some loci of β -catenin, leading to inactivation via ubiquitination. In this study, the expression levels of β -catenin and GSK-3 β were increased after interference in AQP3 expression, which may be related to the changes in the phosphorylated GSK-3 β level, inactivating and reducing GSK-3 β . The above data clearly indicate that AQP3 affects GSK-3 β through increasing the β -catenin protein level. The specific Wnt pathway inhibitor XAV939 can reduce the expression of β -catenin in LCSCs with low expression of AQP3. To sum up, AQP3 regulates the stemness of human LCSCs through the Wnt/GSK-3 β / β -catenin pathway, thereby enhancing the renewal capacity of cancer tissues and making it more difficult to radically treat the tumor.

Finally, the results of differentiation markers and apoptosis-related markers for LCSCs in the interference group and interference+inhibitor group revealed that AQP3 affected the differentiation and regulated the apoptosis of LCSCs through the Wnt/GSK-3 β / β -catenin pathway. In the interference group, the decline in Sox2 and Oct4 suggested that AQP3 affects the differentiation of LCSCs, while the decline in Caspase3 and Bcl-2 suggested that AQP3 affects the apoptosis of LCSCs. The above two cell

phenotypes caused by the low expression of AQP3 could be restored by the Wnt pathway inhibitor XAV939, demonstrating that AQP3 influences the differentiation and apoptosis of LCSCs through the Wnt/GSK-3 β / β -catenin pathway. It can be inferred that the differentiation of LCSCs can be inhibited by AQP3, and the AQP3 activator or an appropriate amount of AQP3 recombinant protein can be injected into the mouse model of lung cancer, so as to inhibit the differentiation of LCSCs. In addition, the combined application of Wnt/GSK-3 β / β -catenin pathway activator may greatly facilitate the apoptosis of LCSCs, reduce the risk of lung cancer recurrence, and improve the therapeutic effect.

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Conflict of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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