Curcumin inhibits tumor epithelial-mesenchymal transition (EMT) via down-regulating wnt signaling pathway through up-regulating NKD2 expressing in colon cancer cell

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Abstract

Tumor invasion and metastasis is closely related to epithelial-mesenchymal transition (EMT). EMT refers to epithelial cells under physiological and pathological conditions specific to mesenchymal transition**.** Curcumin can inhibit EMT progress via Wnt signaling. Wnt signaling pathway is a conservative EMT-related signaling pathway and plays an important role in the development of a variety of tumors**.** In this study, MTS assays were employed to analyze the cell proliferation with Curcumin treated. NKD2, CXCR4 and some antibodies associated with EMT in the colorectal cancer cell lines by western blot and real-time qPCR. Finally, NKD2-small interfering RNA (siRNA) and CXCR4 expression plasmid was synthesized and transfected into colorectal cancer cell lines, then we detected NKD2 and CXCR4 expression levels. We also detected the methylated promoter of NKD2 by bisulfite sequencing(BSP).It was found that curcumin can significantly inhibit the proliferation of colorectal cancer cells and up-regulate the expression of NKD2 in the colorectal cancer cells SW620 and in the xeno graft which results in the downregulation of key markers in Wnt signaling crossing net. In addition we noticed the progress of epithelial-mesenchymal transition was inhibited due to the overexpression of E-cadherin as well as the downregulation of Viementin .Moreover we found curcumin can also inhibit tumor metastasis by down-regulate the expression of CXCR4 significantly. Meanwhile

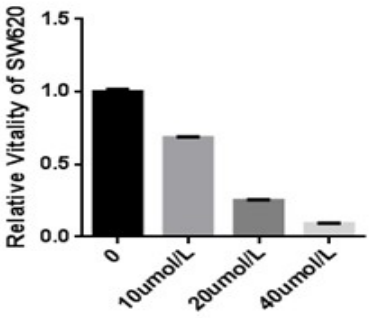
curcumin can inhibit the methylation of NKD2 in the CpG island of the promoter. Our research suggested a NKD2-Wnt-CXCR4 signaling pathway in colorectal cancer cells, moreover curcumin may upregulate this signaling via demethylation of the promoter of NKD2 so that inhibit the development of colorectal cancer.

Keywords: Curcumin; NKD-2; Wnt signaling; CXCR4; Epithelial-mesenchymal transition

Results

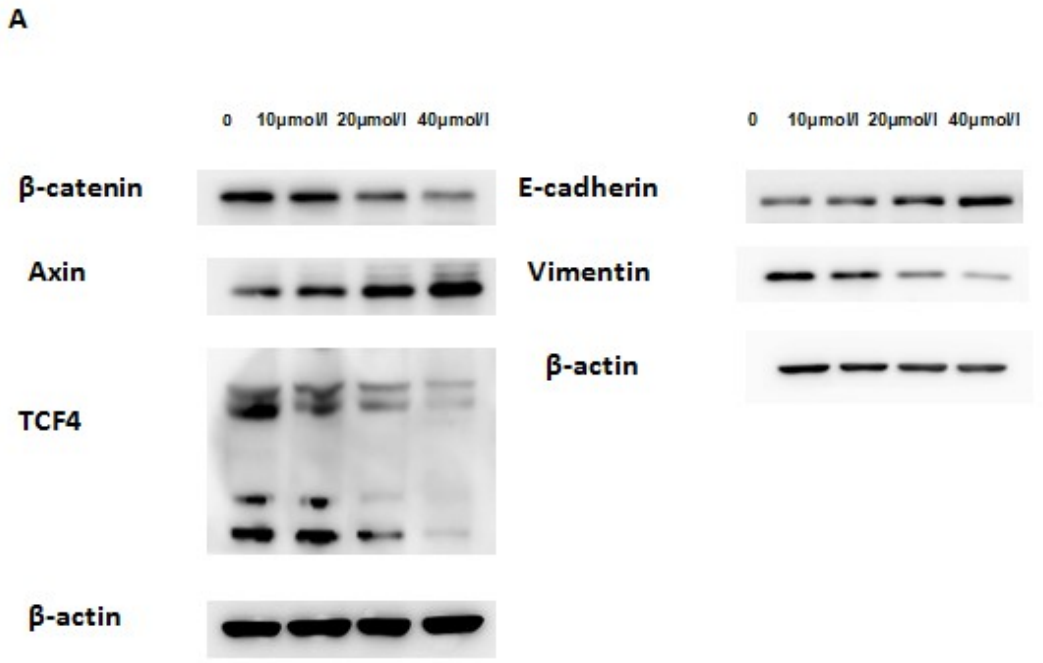
1.Effect of curcumin on the vitality of SW620 cell line

We initially investigated the effect of curcumin on the proliferation of SW620 cells.SW620 cell lines was treated for 24h with graded concentrations of curcumin (0-40 μmol/l) and cell vitality was measured by MTS assay. As shown in Fig. 1, the resulting demonstrated that the vitality of cells was inhibited by curcumin. Upon increasing the concentration of curcumin, the rate of inhibition changed more significantly. The results suggest that curcumin can effectively inhibit SW620 cell's vitality.



2.Curcumin inhibits Wnt signaling and expression of markers of EMT in SW620 cells

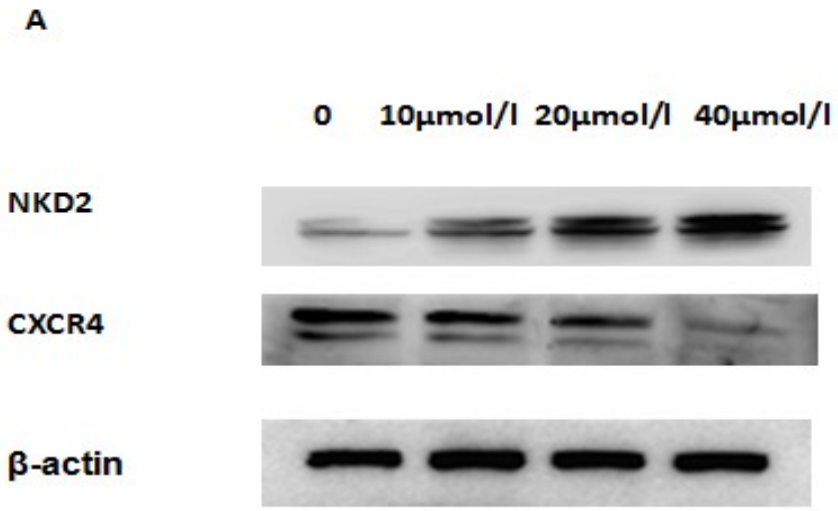
The phenotypic alterations occurring suggested that SW620 cells had undergone EMT, thus there are two expression of specific EMT markers, including the epithelial marker E-cadherin and the mesenchymal marker vimentin. Meanwhile Wnt signaling pathway as one of the conservative EMT-related signaling pathway, we chose the three genes (β-catenin, Axin, TCF4) which related the Wnt signaling pathway. The β-catenin is the hub of the molecule in Wnt signaling pathway and Axin is a negative regulator of the Wnt signaling pathway . TCF4 is one of the Wnt pathway transcription factor, it is highly expressed in colorectal cacner. In order to demonstrate the effect of curcumin on the Wnt signaling pathway and EMT, we used different concentrations of curcumin to treat SW620 cells for 24h, and measured the five genes protein expression by western blot analysis. We found that β-catenin, TCF4 and vimentin protein expression were significantly reduced, while the Axin and E-cadherin protein expression were increased in the curcumin group (Fig. 2 A). By increasing the concentration of curcumin, its protein expression changes more significantly. Thus, our results demonstrated that curcumin can inhibit the Wnt signaling pathway and EMT in SW620 cells.

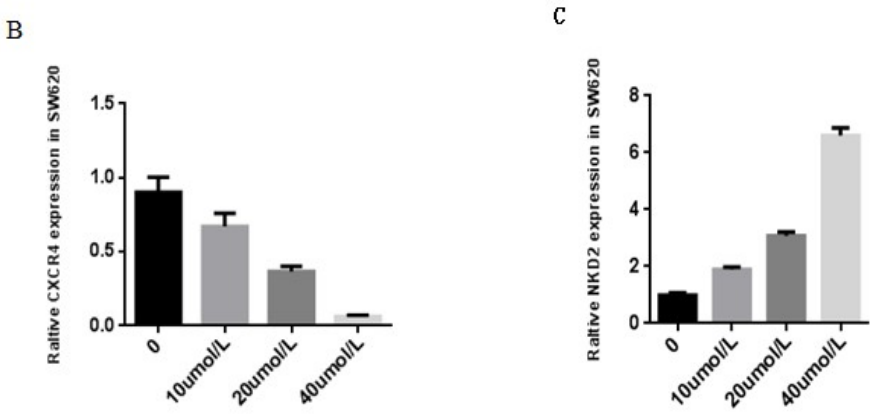


3. Curcumin can increase expression of NKD2 and inhibit expression of CXCR4 in SW620 cells

NKD2 acts as inhibitor of Wnt signaling pathway, plays an important role in tumor of EMT, chemokine receptor CXCR4 acts as an alpha-chemokine receptor specific for stromal-derived-factor-1 is highly expressed in a variety of tumors and it can promote tumors growth and metastasis [35]. Therefore, in order to clarify whether curcumin significantly affect the genes, we carried out a biological analysis. We used different concentrations of curcumin to treat SW620 cells for 24h and measured the genes protein expression by western blot analysis and mRNA expression by Real-time qPCR analysis. We found that NKD2 protein expression were significantly increased, while the CXCR4 protein expression were reduced in the curcumin group (Fig. 3A). The CXCR4 mRNA expression were reduced in the curcumin group (Fig. 3B) and the

NKD2 mRNA expression were significantly increased in the curcumin group (Fig. 3C).Thus, our results demonstrate that curcumin can increase the expression of NKD2 in WNT signaling pathway and inhibit the expression of CXCR4 in the SW620 cells.



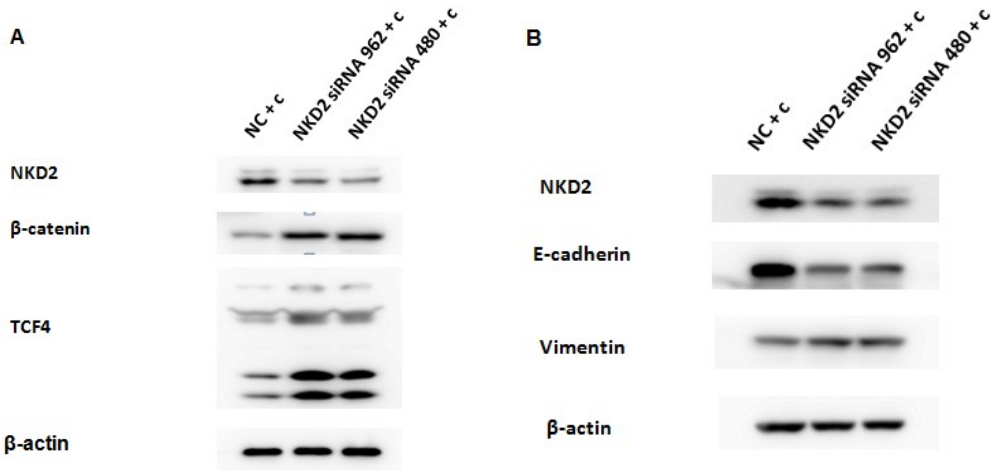


4. NKD2 siRNA transfection and Wnt pathway activator can promote the Wnt signaling pathway

To demonstrate NKD2 siRNA and Wnt pathway activator can affect the Wnt signaling pathway. Firstly, we treated the SW620 cells with NKD2 siRNA transfection for 48h and Wnt pathway activator for 24h respectively, then detect the Wnt signaling pathway genes protein expression levels. Western blotting (Fig. 4A-B) showed that the expression of β-catenin and TCF4 protein were significantly upregulated in the NKD2 siRNA and Wnt pathway activator group compared to the control group. These results further suggest that NKD2 siRNA can promote the Wnt signaling pathway by silencing NKD2 gene.

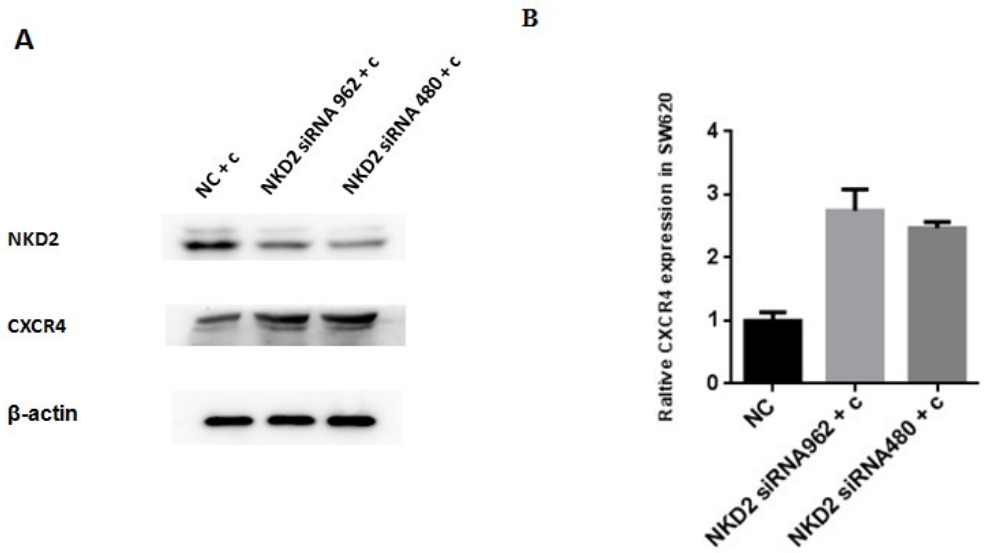
5.NKD2 siRNA transfection can reverse curcumin inhibition of Wnt signaling pathway and EMT in the SW620 cells

Previous results showed that curcumin can inhibit Wnt signaling pathways and EMT in the SW620 cells. Therefore, we explore whether curcumin inhibit this pathway by effecting the expression of NKD2. Firstly we treated the SW620 cells for 48h with NKD2 siRNA transfection and then treated with curcumin (10μmol/l) for 24h.. We found that β-catenin and TCF4 protein expression were significantly increased (Fig. 5A). The E-cadherin protein expression was reduced while the vimentin protein expression was increased in the NKD2 siRNA transfection group(Fig. 5B).Thus, according to the previous results and coupling with the present results, it demonstrate that curcumin can inhibit Wnt signaling pathway and EMT in SW620 cells by increasing the expression of NKD2.



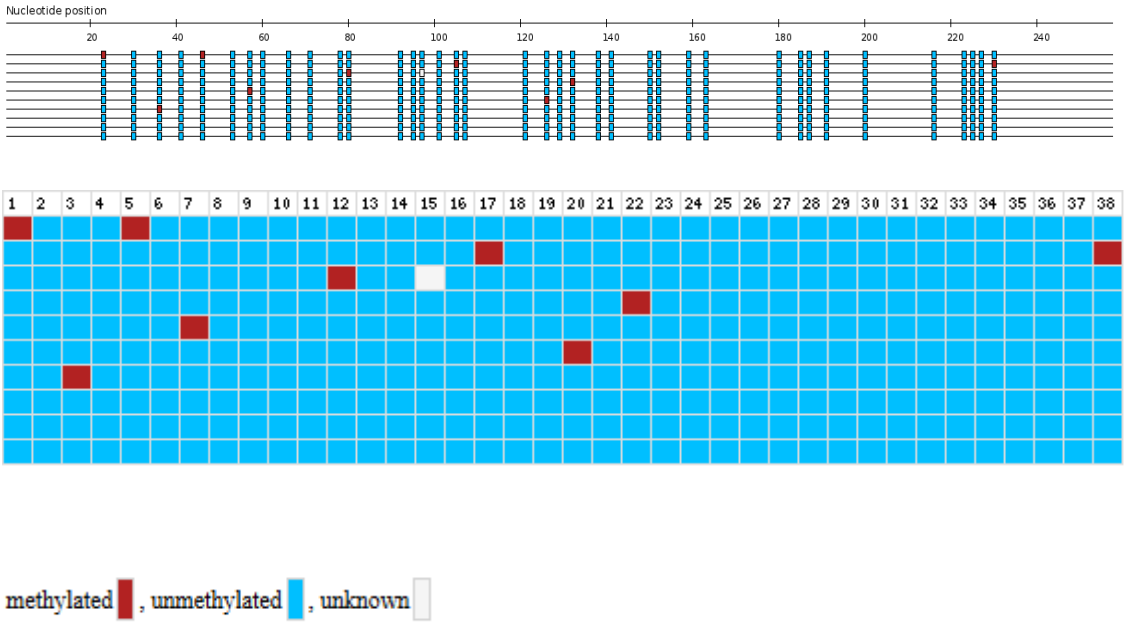
6.NKD2 siRNA transfection can increase the expression of CXCR4 in the SW620 cells which has treated with curcumin.

Some studies found that CXCR4 is closely related to the EMT and they can induce and promote each other. So in order to confirm whether curcumin inhibit the expression of CXCR4 by effecting the expression of NDK2. We transfected NKD2 siRNA for 48h and then treated with curcumin (10μmol/l) for 24h in SW620 cells. We measured the gene protein expression by western blot analysis and the gene mRNA expression by Real-time qPCR analysis. We found that the CXCR4 protein expression was significantly increased in the NKD2 siRNA transfection group (Fig. 6A). The CXCR4 mRNA expression was increased in the NKD2 siRNA transfection group (Fig. 6B). Thus, our results demonstrate that curcumin can inhibit the expression of CXCR4 by increasing the expression of NDK2 in the SW620 cells.

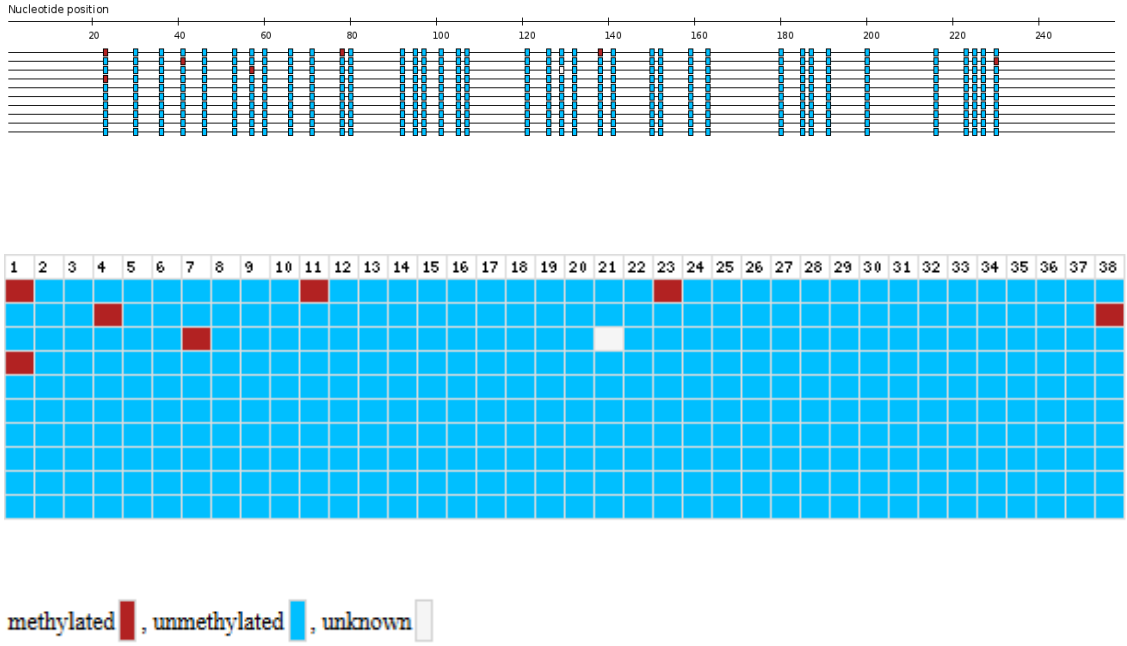


7.Curcumin can demethylate the promoter of NKD2.

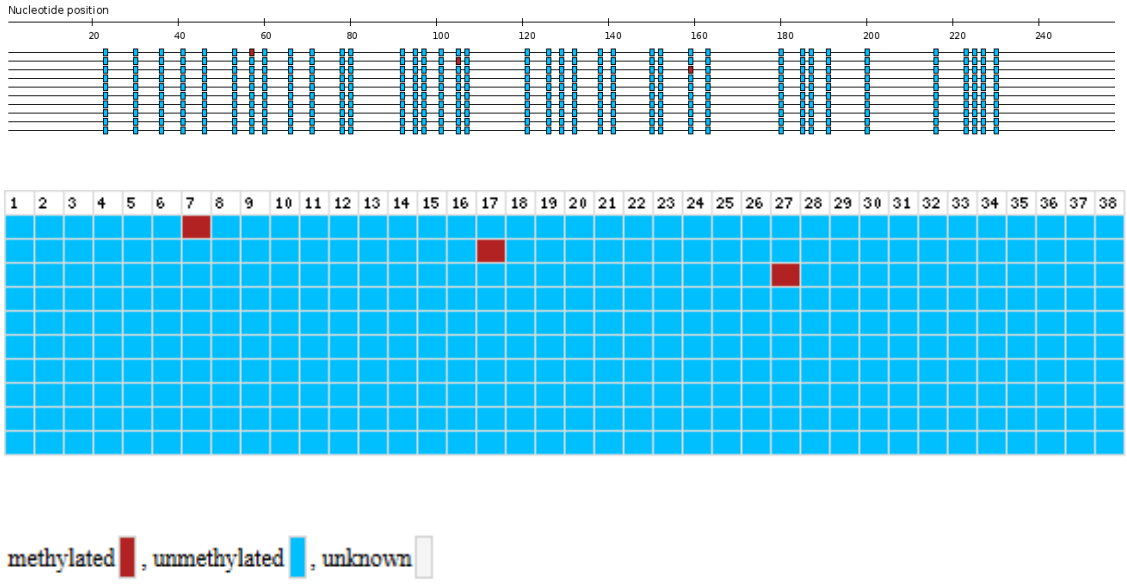
We detected the methylation of NKD2 in SW620 cells after curcumin treated by BSP. We found NKD2 maintain the hypermethylation of CpG island in SW620 cells.After different concentration of curcumin treated(0μmol/l ,10μmol/l and 20μmol/l), the CpG island showed the demethylation variously.



Methylation status of CpG-sites cytosine in the sequence context(after treated by 0μmol/l)



Methylation status of CpG-sites cytosine in the sequence context(after treated by 10μmol/l)

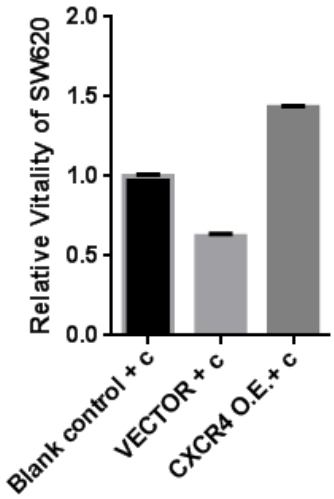


Methylation status of CpG-sites cytosine in the sequence context (after treated by 20μmol/l)

7.CXCR4 expression plasmid transfection can increase the vitality of SW620 cells which has treated with curcumin.

CXCR4 as chemokine receptor, it can reflect the invasion and metastasis of tumor

cells directly. Therefore, we investigate whether CXCR4 expression plasmid can affect the vitality of SW620 cells which has treated by curcumin in vitro. We transfected CXCR4 expression plasmid for 72h and then treated the SW620 cells for 24h with curcumin (10μmol/l). Cell vitality was measured by MTS assay. As shown in Fig. 7, the resulting demonstrated that the vitality of cells was increased by CXCR4 expression plasmid. The results suggested that CXCR4 expression plasmid can effectively increase cell viability, it means curcumin inhibits the vitality of SW620 in vitro by reducing CXCR4 gene expression.



8.CXCR4 expression plasmid transfection can reverse curcumin inhibition of EMT and promote the expression of CXCR4 in the SW620 cells

Previously we have mentioned that in the process of tumor invasion and metastasis, CXCR4 is highly expressed in metastatic tissues, and the tumor invasion and metastasis are closely related with the EMT. Our previous experiments have confirmed that curcumin can inhibit EMT in SW620 cells. So we transfected CXCR4

expression plasmid to observe whether it can reverse curcumin inhibition of EMT. We transfected CXCR4 expression plasmid for 72h in SW620 cells and then treated with curcumin (10μmol/l) for 24h. We measured the gene protein expression which related EMT and CXCR4 expression by western blot analysis and Real-time qPCR analysis. We found that The E-cadherin protein expression was reduced while the vimentin and CXCR4 protein expression was increased in the CXCR4 expression plasmid transfection group (Fig. 8A). The CXCR4 mRNA expression was significantly increased in the CXCR4 expression plasmid transfection group (Fig. 8B). It explains that CXCR4 expression plasmid can induce EMT and promote the expression of CXCR4 in SW620 cells.