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Down-regulation of NKD1 increase the ability of the invasion of non-small-cell lung cancer and correlates with the poor prognosis

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Abstract

Background: As a negative modulator of the canonical Wnt signaling pathway, Naked1 (NKD1) is widely expressed in many normal tissues. However, the expression pattern and clinicopathological significance of NKD1 in patients with non-small-cell lung cancer (NSCLC) is still unclear.

Methods: Immunohistochemical studies were performed on 35 cases of normal lung tissues and 100 cases of NSCLC, including 58 cases with complete follow-up records. The NKD1 protein and mRNA expressions were detected by western blot and RT-PCR, respectively. To examine the effect of NKD1 on the invasiveness of lung cancer cells, NKD1 was down-regulated by siRNA in lung cancer cell lines and the invasive ability was then evaluated by the Matrigel invasion assay. Besides, the expressions of Dishevelled-1 and β-catenin protein, as well as MMP mRNA were also examined in NKD1 knockdown cells.

Results: In 35 fresh lung cancer tissues examined, 27(79%) of them exhibited weaker levels of NKD1 protein in comparison with their corresponding normal tissue (P=0.009). However, the NKD1 mRNA level was significantly higher in cancerous lung tissues, compared to adjacent normal tissue (P=0.002). In 100 NSCLC tissues determined by immunohistochemical staining, NKD1 was significantly lower in 78 cases (78%), compared with the normal specimens. Besides, reduced NKD1 expression was correlated with the histological types (P=0.003), poor differentiation (P=0.004), lymph node metastasis (P = 0.013), TNM stage (P = 0.002) and poor survival (29.45 ± 3.24 vs 57.69 ± 7.14, P = 0.01). In addition, NKD1 knockdown could up-regulate Dishevelled-1 and β-catenin protein levels, as well as increased MMP-7 transcription and the invasive ability of lung cancer cells. Furthermore, when treated the NKD1-knockdown cells with Dishevelled-1 antibody, the invasive ability of the NKD1-knockdown cells reduced significantly.

Conclusion: NKD1 protein is reduced whereas NKD1 mRNA is enhanced in NSCLC. Reduced NKD1 protein is correlated with the poor prognosis of NSCLC. NKD1 might inhibit the activity of canonical Wnt pathway through Dishevelled-1.
Key Words: Naked; Dishevelled-1; β-Catenin; Invasion; Prognosis
**Background**

dNKD (Naked Cuticle Drosophila) was first found in Drosophila, and the mutation of Naked gene could induce the loss of segmentation of Drosophila [1]. Subsequently, NKD1 and NKD2, two homologues of drosophila naked cuticle, were also detected in mammalian [2]. These two genes are respectively located in chromosome 16q12.1 and 5p15.3 of human beings, and share 43.8% similarity amino-acid sequence with each other [2]. NKD1 was proposed to interact with Dishevelled (Dvl) through its conservative domain, which named EF-hand-like motif, and functioned as a negative regulator of the canonical Wnt/β-catenin signaling pathway [3-6]. Dishevelled (Dvl) is a positive regulatory factors located upstream of the Wnt pathway, which mediate at least two intracellular Wnt signal pathways, including the canonical Wnt/β-catenin pathway and JNK/PCP pathway [7].

NKD1 is an antagonist of the canonical Wnt/β-catenin pathway. When it directly interacts with PDZ domain of Dishevelled (Dvl) in the cytoplasm, the canonical Wnt/β-catenin signaling pathway is inhibited [5]. It is revealed that NKD1 could act as a switch that directs Dishevelled (Dvl) toward the JNK/PCP pathway and away from the canonical Wnt/β-catenin pathway [4], and thus inhibited the canonical Wnt pathway.

Although there are increasing reports of NKD1 today, the role of NKD1 in cancer progression still needs to be addressed. It has been reported that the NKD1 mRNA level was up-regulated in colorectal adenomas [8] and hepatoblastoma [9]. However, NKD1 protein expression was down-regulated in some gastric cancer tissues [2]. It raised the question that whether the expression level of NKD1 protein was consistent with the mRNA level in one tumor type. Besides, there is no report about the relationship between NKD1 expression and clinicopathological features in human tumor.

In current study, we examined the expression of NKD1 in 100 cases of non-small-cell lung cancer (NSCLC) and analyzed the correlation between the expression of NKD1 and clinicopathological factors. Meanwhile, the effect of NKD1 on prognosis of the
patients with NSCLC was also analyzed by inspecting follow-up data. In addition, we ablated NKD1 by siRNA technology in lung cancer cell lines to investigate alterations in Dishevelled-1 and β-catenin protein levels, MMP-7 transcription and the invasive ability of NKD1-knockdown lung cancer cells, to provide insight into the role of NDK1 in the progression of lung cancers.

**Methods**

**Patient and Specimens**

Paraffin specimens (n=100) were obtained from patients with lung cancer at the First Affiliated Hospital of China Medical University. Complete follow-up information of 58 patients was obtained from review of the patients’ medical records. None of these patients had received chemotherapy or radiotherapy before surgical resection. The mean age of these patients was 58 years. According to the WHO histological classification criteria [10], there were 33 cases of squamous cell carcinoma (SCC) and 67 cases of adenocarcinoma (ADC). The p-TNM staging system of the International Union Against Cancer in 2009[11] was used to classify specimens as stages I (n = 24), II (n = 20), III (n = 44), and IV (n = 12).

Besides, 35 cases of tumor and paired non-tumor portion (with >5 cm distance from the primary tumor's edge) of the same case were quickly frozen in liquid nitrogen and maintained at -70°C for mRNA and protein analysis.

**Immunohistochemistry**

As described previously [12-14], the tissues were fixed with 10% neutral formalin, embedded in paraffin, and 4-μm-thick sections were prepared. All the sections were stained by the streptavidin–peroxidase (S–P) method. The samples were incubated with NKD1 polyclonal antibody (1:100, Santa Cruz Biotechnology, Inc.) at 4°C overnight. Meanwhile the phosphate-buffered saline (PBS) was used as primary antibody for negative control. Biotinylated secondary antibody and diaminobenzidine (DAB) were purchased from Maixin Biotechnology (Fuzhou, China).

**Evaluation of Immunostaining**
Five views were examined per slide randomly, and 100 cells were observed per view. The expression of NKT1 was classified into five groups according to the percentage of positively staining cells: 0 = absent; 1 = 1-25%; 2 = 26-50%; 3 = 51-75%; 4 = ≥76%. The staining intensity was categorized as follows: 0 = negative; 1 = weak; 2 = moderate; and 3 = strong. The proportion and intensity scores were then multiplied to obtain a total score.

Since the scores in all 35 cases of normal lung tissues were all ≥3, we regarded the scores less than 3 as “reduced expression”, while scores of 3 or more were regarded as “normal expression”.

**Reverse Transcription (RT)-Polymerase Chain Reaction (PCR)**

Total RNA was extracted with Trizol regent (Invitrogen) according to the manufacturer’s instructions. And RT-PCR was performed with the AMV Ver3.0 kit (Takara, Shiga, Japan). PCR was carried out with the following primers (Table 1).

The PCR products were electrophoresed in a 2% agarose gel. And the results were analyzed using the BioImaging System (UVP, CA, USA). The relative mRNA levels were normalized to the relative amount of β-actin mRNA. Each experiment was performed three times independently.

**Western Blot**

As described previously [14], total protein was extracted with RIPA lysis buffer and quantified using the Bradford method. Protein lysates (100 µg) were separated by 10% SDS–PAGE and transferred to the polyvinylidene fluoride (PVDF) membranes. After blocking, the blots were incubated with primary antibodies directly against NKT1 (1:200) and β-catenin (1:800) at 4°C overnight. After that, the blots was incubated with the secondary antibody labeled with HRP at room temperature for 2 h, protein bands were visualized using enhanced chemiluminescence (ECL) and detected using the BioImaging System. The relative protein levels were calculated by comparison to the amount of β-actin protein. The experiments were repeated three times independently.

**Cell Culture and Transfection**

HBE (the normal human bronchial epithelial cell lines) was obtained from the Cell
Bank of Chinese Academy of Science (Shanghai, China). And the lung carcinoma cell lines, BE1 and LH7 were kindly provided by Professor Jie Zheng (College of Medicine, Peking University, China). The cells were cultured in RPMI-1640 (GIBCO, Inc, Grand Island, NY) containing 10% fetal calf serum (Invitrogen), 100 IU/mL penicillin (Sigma, St. Louis, MO, USA), and 100 µg/mL streptomycin (Sigma) in a humidified atmosphere with 5% CO₂.

NKD1 siRNA (SC-93414) and negative control siRNA (Cat. SC-36869) were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For transient transfection, cells were cultured in a 24-well plate 24 h before the experiment. Then cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Following the transfection, the mRNA and protein levels were collected after 48 h.

Matrigel Invasion Assay

The cells' invasive abilities were examined using a 24-well Transwell with 8-µm pore polycarbonate membrane inserts (Corning Inc., Corning, NY, USA) according to the manufacturer’s protocol. Cells that appeared on the lower surface of the filter were counted in five random ×200 fields using an inverted microscope (Olympus IX51; Olympus America Inc., Melville, NY, USA). The experiments were performed in triplicate and got means.

Statistical analysis

SPSS version 13.0 for Windows was used for all analyses. The chi-square test was used to examine possible correlations between NKD1 expression and clinicopathological factors. The Student’s t-test was used to analyze the results of RT-PCR and western blotting. The Kaplan–Meier method was used to estimate the probability of patient survival. The Cox’s proportional hazard regression model was used to estimate the possible prognostic significance of clinicopathological variables. P < 0.05 was considered to indicate statistical significance.

Results
The protein expression of NKD1 in NSCLC is lower, while mRNA expression is higher than that in normal lung tissue

Total protein of 35 pairs NSCLC tissues and adjacent noncancerous lung tissues were extracted and the protein expression of NKD1 was detected by Western blot. The results showed that the expression of NKD1 protein in NSCLC is lower than that in normal lung tissue (P=0.009, Figures 1A).

To further examine the expression of NKD1 in cell lines, two lung cancer cell lines (named BE1 and LH7, respectively) were chosen in our study. BE1 cells have a high metastatic potential, whereas LH7 cells have a low metastatic potential. Besides, HBE, a normal human bronchial epithelial cell line, was used as a control. The results showed that the NKD1 protein expression of HBE was higher than LH7 and BE1. Moreover, NKD1 expression in BE1 cells (high metastatic potential) was lower than that in LH7 cells (low metastatic potential) (Figures 1B).

Above results implied that NKD1 expression might associated with invasiveness of lung cancer cells. However, RT-PCR results showed that NKD1 mRNA levels were obviously higher in lung cancer in comparison with corresponding normal lung tissue (P=0.002, Figures 2A). And same results also can be found in cell lines (Figures 2B).

Reduced NKD1 expression was associated with clinicopathological factor and poor prognosis of NSCLC

In 35 cases of normal lung tissues which were examined by immunohistochemistry, NKD1 was mainly expressed in the cytoplasm of bronchial epithelial cells (≥3 score, According to our evaluation criteria, they were judged as normal expression) (Figure 3A). However, in the 100 NSCLC specimens, NKD1 showed reduced expression (<3 score) in 78 samples (78%) and normal expression (≥3 score) in 22 cases (Figure 3B and C) (Table 2).

Next, we analyzed the relationship between NKD1 expression and clinicopathological factors in 100 NSCLC samples. The normal expression rate of NKD1 in squamous carcinoma (13/33, 39.4%) was higher than that in adenocarcinoma (9/67, 13.4%) (P=0.003); the expression rate of NKD1 in stages I–II (16/44, 36.3%) was also higher than in stages III–IV (6/56, 10.7%) (P=0.002); and it was higher in cases without
lymphatic metastasis (15/45, 33.3%) than in cases with lymphatic metastasis (7/55, 12.7%) (P=0.013). Besides, the expression rate of NKD1 in well-moderate differentiation (17/50, 34%) was higher than poor differentiation (5/50, 10%) (P=0.004). There was no significant correlation between NKD1 expression and gender or age (P>0.05, Table 2).

As shown in Figure 4, Kaplan–Meier survival curves showed that the average survival time of patients with NKD1 normal expression was longer than that of patients with NKD1 lower expression (57.69 ± 7.14 versus 29.45 ± 3.24 months)( P = 0.01).

In order to clarify whether the reduced NKD1 expression was associated with patients’ prognosis, we employed the Cox’s proportional hazard regression model. The result showed that reduced expression of NKD1 was a hazard factors for the prognosis of patients with lung cancer (P=0.017, Table 3).

**NKD1 depletion could up-regulate Dishevelled-1, β-Catenin protein expression and enhance the invasive ability of lung cancer cells**

siRNA technology was used to knock-down NKD1 expression in LH7 cell lines, which expressing relative high levels of NKD1. After transfection of siRNA-NKD1, the NKD1 protein expression was down-regulated approximately 60% in comparison with control cells (P<0.05). Meanwhile, the protein expressions of Dishevelled-1 and β-catenin were obviously up-regulated (Figure 5).

In order to examine the effect of NKD1 on the invasiveness of lung cancer cells, we down-regulated NKD1 expression in the LH7 cell lines and evaluated the invasive ability correspondingly by the Matrigel invasion assay. The results showed that the invasive ability of the LH7 cells was significantly enhanced when NKD1 was down-regulated by siRNA. However, we found that treated the NKD1-depletion LH7 cells with Dvl-1 specific antibody(200 ng/ml), which was used to block Dvl-1 function, reduced the invasive ability of the NKD1-depletion LH7 cells significantly (P<0.05, Figure 6).

These results showed that NKD1 down-regulation could up-regulate Dvl-1, β-catenin protein expression and enhance the invasive ability of lung cancer cells. Since Dvl-1
and β-catenin are functioned as positive regulators of the canonical Wnt signaling pathway, it is plausible that NKD1 increased the invasive ability of lung cancer cells through activating the canonical Wnt pathway. To examine this supposition, we examined the mRNA levels of MMP-7 (a target gene of canonical Wnt pathway) in NKD1-depletion LH7 cells and found that MMP-7 mRNA levels was significantly enhanced accordingly (P < 0.05, Figure 7).

**Discussion**

NKD1, a negative regulator of the canonical Wnt signaling pathway [3, 5], is widely expressed in many normal tissues. It was shown that NKD1 protein was down-regulated in gastric cancer [2], whereas its mRNA expression was up-regulated in colorectal adenomas and hepatoblastoma. However, it is still unclear whether NKD1 protein expression is consistent with the mRNA expression in one tumor type. Besides, the correlation between NKD1 and clinicopathological factors also need to clarify. In order to ascertain this question, we used NSCLC tissues and cell lines to clarify this issue.

The NKD1 protein expression in NSCLC tissues was lower than that in normal lung tissue in our study. Reciprocally, the NKD1 mRNA level was up-regulated in NSCLC tissues in comparison with adjacent normal lung tissue. These data were consistent with previous reports [8, 9], and demonstrated that NKD1 protein expression was not consistent with its mRNA expression at least in NSCLC. It is easy to understand that down-regulated NKD1 protein in cancer would result in NKD1 lost its inhibition function on the canonical Wnt pathway. Besides, our immunohistochemical results demonstrated that reduced NKD1 expression was associated with poor differentiation, high pTNM stage, lymph node metastasis and poor prognosis of NSCLC. This interpretation is supported by results obtained from our experiments on BE1 and LH7 cells, which were derived from one lung cancer cell line. The BE1 cells that have high metastatic potential show much lower levels of NKD1 than the LH7 cells that have low metastasis. Furthermore, our NKD1 knockdown results using the siRNA
technology on LH7 cells resulted in enhanced invasive ability further suggested that loss of NKD1 might critically contribute to the formation of metastatic potential in lung cancer cells.

In order to explain why NKD1 knockdown could increase the invasive ability of lung cancer cells, we detected the protein expressions of Dvl-1, Dvl-3 and β-catenin, mRNA expression of MMP-7 in NKD1 down-regulated cells. The results showed that NKD1 knockdown caused dramatically up-regulated protein expressions of two positive modulators of the canonical Wnt pathway, Dvl-1 and β-catenin protein [15-18]. Thus, it is plausible that NKD1 knockdown might trigger the activity of Wnt signal pathway. Indeed, we observed that the mRNA level of one Wnt/β-catenin responsive gene, MMP-7, was significantly increased in NKD1 knockdown cells. Recently, Yan D et al found that NKD1 could up-regulate β-catenin protein expression in human colon tumors[6], and Hu T et al reported NKD2, one homologue of NKD1, could accelerate the degradation of Dishevelled via increasing its ubiquitinoylation [19]. Although our results are not contradictory to their reports, whether same mechanism is involved in degradation of Dishevelled accelerating by NKD1 and NKD2 remains a curious question to be addressed.

Using a loss-of-function approach with a Dishevelled-1 specific antibody, the transcription of MMP-7 and the invasive ability were effectively down-regulated in NKD1 knockdown LH7 cells. The result further confirmed that NKD1 was a negative regulator of the canonical Wnt signaling pathway. Moreover, we found that NKD1 knockdown only markedly up-regulated Dvl-1 protein expression but not changed Dvl-3 protein expression. Because we has demonstrated that Dvl-1 mainly involved in the canonical Wnt pathway, while Dvl-3 mainly regulated the activity of non-canonical Wnt pathway in lung cancer cell[20], that is why MMP-7 was chosen as a marker to present the activation of classical Wnt signal pathway in our study.

It is very curious that NKD1 protein expression in NSCLC is lower than that in normal lung tissue, whereas the mRNA expression in NSCLC is higher than that in normal lung tissue. We speculated the enhanced NKD1 mRNA level might due to the elevated activity of Wnt pathway in NSCLC. It has been reported that one of the
target genes of the Wnt signaling cascade is NKD1, whose activity is required to restrict Wnt signaling, thus generating a negative-feedback loop [21]. It is plausible that enhanced activity of Wnt/β-catennin pathway caused elevated transcription of a series of Wnt-target genes, including NKD1. How NKD1 protein expression was reduced in NSCLC with up-regulated NKD1 mRNA remains a central question to be clarified. The reasons may involve in the post-translation modification of NKD1, and/or the procedure of protein degradation. Alternatively, as a negative-feedback loop exists to control the level of NKD1 protein [4], the high expression of NKD1 protein might inhibit the classical Wnt signal pathway and thereby inhibiting the transcription of NKD1. Reciprocally, reduced NKD1 protein might enhance the activity of classical Wnt pathway, and the transcription and translation of NKD1, thus elevate the NKD1 protein expression. However, this balance might be destroyed in cancer. The NKD1 protein degradation speed might be accelerated or easier to degradation due to the changes existing in post-translation process. Thus, the NKD1 protein is always in low level and the classical Wnt pathway is continuously activated. Certainly, more experiment may need to carry out to confirm our supposition.

Summary, our data showed that NKD1 protein in NSCLC was lower than that in normal lung tissue, whereas the mRNA expression in NSCLC is higher than that in normal lung tissue. Besides, reduced NKD1 expression is associated with poor differentiation, high pTNM stage, lymph node metastasis and poor prognosis of NSCLC. Down-regulation of NKD1 protein could up-regulate Dvl-1, β-catennin protein expression, MMP7 mRNA expression, and increase the invasive ability of lung cancer cells.

**Conclusion**

Above all, we found that NKD1 is mainly expressed in the cytoplasm of lung cancer tissues, which is associated with poor differentiation, high pTNM stage, lymph node metastasis and poor prognosis of NSCLC. Interestingly, NKD1 protein in NSCLC was lower than that in normal lung tissue, whereas the mRNA expression in NSCLC is higher than that in normal lung tissue. Besides, we found that NKD1 knockdown
by siRNA technology could obviously up-regulate Dishevelled-1 and β-catenin protein levels. Meanwhile, the transcriptional activity of MMP-7 and the invasiveness of lung cancer cells were also enhanced in NKD1-knockdown cells. Furthermore, when treated the NKD1-knockdown cells with Dishevelled-1 antibody, the invasive ability of the NKD1-knockdown cells reduced significantly.
Abbreviations

NKD1: Naked1; Dvl: Dishevelled; NSCLC: non-small cell lung cancer; MMP-7: matrix metallopeptidase 7;

Competing interests

None of the authors of this manuscript have a financial interest related to this work.

Authors' contributions

WEH designed research, evaluated immunohistochemical results. DSD performed the statistical analysis of all data and wrote the paper. ZS carried out prepared pathology samples, follow-up and immunohistochemical study cell culture, and invasive assay. The manuscript has been read and approved by all the authors.

Acknowledgements

All lung tissue samples were obtained from the first affiliated hospital of China Medical University. This study was supported by grants from the National Natural Science Foundation of China (No. 81071905 to En-Hua Wang and No. 81071717 to Shun-Dong Dai).
References


Dishevelled-1 and dishevelled-3 affect cell invasion mainly through canonical and noncanonical Wnt pathway, respectively, and associate with poor prognosis in nonsmall cell lung cancer. *Mol Carcinog* 2010, 49(8):760-70.

Figure legends

Figure 1. Expression of NKD1 in lung cancer tissues and cell lines.

(A). Compared with normal lung tissue (N1-N3), NKD1 protein expression was significantly increased in lung cancer tissues (T1-T3) (P<0.05).

(B). NKD1 protein expression in two lung cancer cells, BE1 and LH7 cells, were lower than that in HBE cells, a normal human bronchial epithelial cell line (P<0.05). Besides, NKD1 protein expression in BE1 cells (high metastatic potential) was lower than that in LH7 cells (low metastatic potential) (P<0.05)
Figure 2. Expression of NKD1 mRNA in lung cancer tissues and cell lines.

(A). The NKD1 mRNA expression in the lung cancer tissues (T1–T3) were significantly higher than corresponding nontumorous tissues (N1–N3) (P<0.05).

(B). NKD1 mRNA expression in both BE1 and LH7 cells were higher than that in HBE cells (P<0.05). NKD1 mRNA expression in BE1 cells (high metastatic potential) were higher than LH7 cells (low metastatic potential) (P<0.05)
Figure 3. Immunohistochemical staining of NKD1 in NSCLC.

NKD1 expression was expressed in the cytoplasm of normal bronchial epithelial cells (Normal expression) (A). NKD1 expression was significantly decreased in lung squamous cell carcinoma (B) and adenocarcinoma (C) (Reduced expression).
Figure 4. Reduced NKD1 expression was correlated with poor prognosis in patients with NSCLC (P=0.01).
Figure 5. Knockdown of NKD1 could up-regulate Dishevelled-1 and β-catenin expression in lung cancer cells.

NKD1 knockdown resulted in a significant reduced expression of NKD1. Meanwhile, Dishevelled-1 and β-catenin protein expression was dramatically up-regulated (P<0.05). Note that Dishevelled-3 expression showed no change in NKD1 knockdown cells (P>0.05).
Figure 6. NKD1 knockdown enhanced the invasive ability of lung cancer cells, while co-transfection with NKD1 siRNA and anti-Dishevelled-1 antibody reduced the invasive ability of NKD1 knockdown cells.

The number of cells invading the lower surface of the filter is shown. *P < 0.05.
Figure 7. NKD1 knockdown could enhance the transcriptional activity of MMP-7 gene (P<0.05). However, when the NKD1 knockdown cells were treated with Dishevelled-1 antibody, MMP-7 mRNA level was decreased significantly (P<0.05).
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Table 2. Relationship between NKD1 expression and the clinicopathological factors in patients with NSCLC

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SCC, squamous cell carcinoma; AC, Adenocarcinoma; pTNM staging, pathologic TNM staging.
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<td>Lymph node metastasis</td>
<td>0.002</td>
<td>0.421</td>
<td>0.265</td>
<td>0.726</td>
</tr>
<tr>
<td>Reduced NKD1 expression</td>
<td>0.017</td>
<td>1.864</td>
<td>1.143</td>
<td>2.975</td>
</tr>
</tbody>
</table>
Figure 1

(A) Western blot analysis showing the expression of NKD1 and β-Actin proteins in normal (N1, N2, N3) and tumor (T1, T2, T3) tissues. The bar graph on the right indicates the relative expression of NKD1 protein in normal and tumor tissues.

(B) Western blot analysis showing the expression of NKD1 and β-Actin proteins in HBE, BE1, and LH7 cell lines. The bar graph on the right indicates the relative expression of NKD1 protein in these cell lines.
Figure 2