Construction and Expression of Two Cytoplasmic hnRNPK Mutants

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Abstract

Heterogeneous nuclear ribonucleoprotein K (hnRNPK) is a member of the hnRNP family which is a DNA/RNA binding protein shuttling between nucleus and cytoplasm but mainly located in the nuleus in most physiological conditions. Previous studies from our lab and the others have shown that hnRNPK is upregulated and displays abnormal cytoplasmic localization in the lung adrenocarcinoma tissue. The functional significance and its related mechanisms of cytoplasmic hnRNPK in the physiopathology of cancer remains still elusive. In order to address this question, we have constructed two hnRNPK mutated forms lacking respectively the nuclear localization signal (NLS) and amino acids 323-337, and examined their subcellular distribution in lung adenocarcinoma H1299 cells in comparison with wild type hnRNPK. This provides appropriate tools for further studies aiming to assess the functions of the cytoplasmic hnRNPK in the physiopathology of cancer and elucidate the related mechanisms.

Keywords

hnRNPK , NLS, nuclear export, nuclear import, recombinant plasmid.

1. Introduction

hnRNPK (MW 65 KDa) is a 464 amino acid protein possessing the following principle functional domains: a NLS domain (nuclear localization signal, amino acids 21-37), a KNS domain (nuclear shuttling domain, amino acids 338-361)[1,2], three DNA-RNA biding homology domains (KHI1, KHI2, and KHI3), a K-protein-interactive region and a C-terminal protein kinase-binding domain[3]. The NLS domain is a classical bipartite-basic type, which mediates hnRNPK transport from the cytolasm to the nucleus. The KNS domain is a novel signal for nuclear import and nuclear export identified in hnRNPK protein. Phosphorylation of hnRNPK in its KNS domain is important for its nucleocytoplasmic shuttling by promoting the cytoplasmic localization of hnRNPK[2]. Moreover, it has been reported that amino acids 323-337 may provide a non-specific spacer function for KNS domain to become more accessible for the nuclear import machinery[2]. Thus, the deletion of the NLS signal or the amino acids 323-337 of hnRNPK might result in the nuclear-cytoplasmic translocation of this protein.

hnPNPK has been shown to regulate gene expression and signal transduction[4]. Numerous studies demonstrated a close association between hnRNPK and tumors, and an overexpression state of hnRNPK in a number of human cancers, including lung adenocarcinoma[5, 6]. Our recent study showed that hnRNPK is overexpressed in adenocarcinoma cells and furthermore, displays un abnormal cytoplasmic localization. Due to the multiple functions of hnRNPK protein in cancer cells, clarifying the functional significance of this ectopic expression might be important for the comprehensive understanding of hnRNPK function and the mechanisms of the cancerogenesis and development of cancer. To this aim, we have constructed two hnRNPK mutated forms lacking respectively the nuclear localization signal (NLS) and amino acids 323-337, and examined their

subcellular distribution in lung adenocarcinoma H1299 cells in comparison with wild type hnRNPK. The function and related mechanisms of these two mutated hnRNPK forms will be futher characterized in the future studyies.

2. Materials and Methods

2.1 plasmid construction

Flag-hnRNPKΔNLS plasmid (lacking NLS domain) and Flag-hnRNPKΔ2 plasmid (lacking NLS domain and amino acids 323-337) were produced by PCR from Flag-hnRNPKWT plasmid using the following primers:

Primer mean	Sequence
Flag-hnRNPKΔNLS-F	GGAAGATGGTGATGGGATT
Flag-hnRNPKΔNLS-R	GGAAGATGGTGATGGGATT
Flag-hnRNPKΔ2-1F	ATAAAAGAATTCAACACTGATGAGATGGTTGAATTACG
Flag-hnRNPKΔ2-1R	CAGCACTGAAACCGGCCATGAGGTCTCCCCCTC
Flag-hnRNPKΔ2-2F	GACCTCATGGCCGGTTTCAGTGCTGATGAAACTTG
Flag-hnRNPKΔ2-2R	ATAAAACTCGAGTTAGAATCCTTCAACATCTGCATACTG

The PrimeSTAR® HS DNA Polymerase (TAKARA, Japan) was used for PCR system:

Component	Amount
Template plasmid	≤100 ng
Primer forward	1 μL
Primer reverse	1 μL
5 × PrimeSTAR Buffer	10 μL
2.5 μM dNTP Mixtures	4 μL
PrimeSTAR HS DNA Polymerase	1 μL
ddH_2O	up to 50 μL

Then, PCR reactions were performed according the following procedure. The annealing temperatures of hnRNPKΔNLS, hnRNPKΔ2-1 and hnRNPKΔ2-2 are 55℃, 50℃ and 55℃, respectively:

Temperature	Time	Cycle
95℃	2 min	1
95℃	30 sec	
50-60℃	30 sec	30
68℃	2 min / kb + 1 min	

Next, we performed 1% agarose gel electrophoresis and extracted PCR product by TIANgel midi purification kit (TIANGEN, China). Then hnRNPKΔ2 fragment was generated by PCR from the PCR product of hnRNPKΔ2-1 fragment and hnRNPKΔ2-2 fragment as templates. The reaction procedure is as same as above, except that the annealing temperature of the procedure is 57℃:

Component	Amount
hnRNPK△2-1	(100 ng × 855 kb) / (384 kb × C_1)
hnRNPK△2-2	(100 ng × 384 kb) / (855 kb × C_2)
Flag-hnRNPK△2-1F	1 μL
Flag-hnRNPK△2-2R	1 μL
5 × PrimeSTAR Buffer	10 μL
2.5 μM dNTP Mixtures	4 μL
PrimeSTAR HS DNA Polymerase	1 μL
ddH2O	up to 50 μL

C_1: the concentration of hnRNPK△2-1 fragment; C_2: the concentration of hnRNPK△2-2R fragment. These PCR products and vector were double-digested according following reaction system, respectively. Then place tube in water of 37℃ for 4 hrs:

Component	Amount
Vector or insert	1 μg
10 × H Buffer	2 μL
*Eco*R I	1 μL
Xho I	1 μL
ddH_2O	up to 20 μL

Then the double-digested product was ligated by DNA blinting kit (TAKARA, Japan). The reaction system incubate overnight at room temperature:

Component	Amount
Double-digested Vector	100 ng / C_{vector}
Double-digested Insert	(500 ng × 4300 kb) / (1300 kb × C_{insert})
10 × Ligation Reacton Buffer	2 μL
T4 DNA Ligase	1 μL
ddH2O	up to 25 μL

Next, we transformed recombinant plasmid into DH5α: Thaw 100 μL competent bacteria on ice; Add 10 μL plasmids to the bacteria; Mix gently by pipetting up and down the tube 4-5 times to mix the plasmids and bacteria; Place the mixture on ice for 30 min; Heat shock at 42℃ for 30 min; Add 900 μL of room temperature media to the tube; Place tube at 37℃ for 60 min, shake vigorously (250 rpm); Warm selection plates to 37℃; Spread 100 μL of the mixture onto the plates; Incubate overnight at 37℃.

Finally, we identified positive clones by colony PCR, and sent the positive clones to Shanghai Health Company for sequencing.

2.2 cell culture and immunofluorescence (IF) assay

Lung adenocarcinoma H1299 cells (Institute of life science Chinese Academy of Sciences, Shanghai, China) were maintained in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand island, NY) with 10 % fetal bovine serum (PAN-Biotech, Germany), in a humidified atmosphere containing 5 % CO_2 at 37 ℃ as described previously [7, 8].

H1299 cells were transfected with Flag-hnRNPKWT plasmid, Flag-hnRNPK△NLS plasmid and Flag-hnRNPK△2 plasmid, respectively. IF was performed as described previously [9, 10]. The primary Flag antibody (1: 800 dilution) and the Alexa Fluor 488 conjugated secondary antibodies (ZSGB-BIO, China) were used for detection. DAPI staining was used to determine the morphology of cell nuclei. The imaging experiments were performed on laser scanning confocal microscopes (LSM700, Zeiss, Jena, Germany) equipped with a Zeiss Plan-Neofluar 40× / 1.3 NA Oil Dic objective as described previously [9, 10].

3. Results

3.1 design of plasmid diagram

First, we retrieved the sequence of NLS domain and amino acids 323-337 of hnRNPK from reference[2]. Then we designed primers and plasmid diagram using Primer Premier 5 and SnapGene software (Fig. 1).

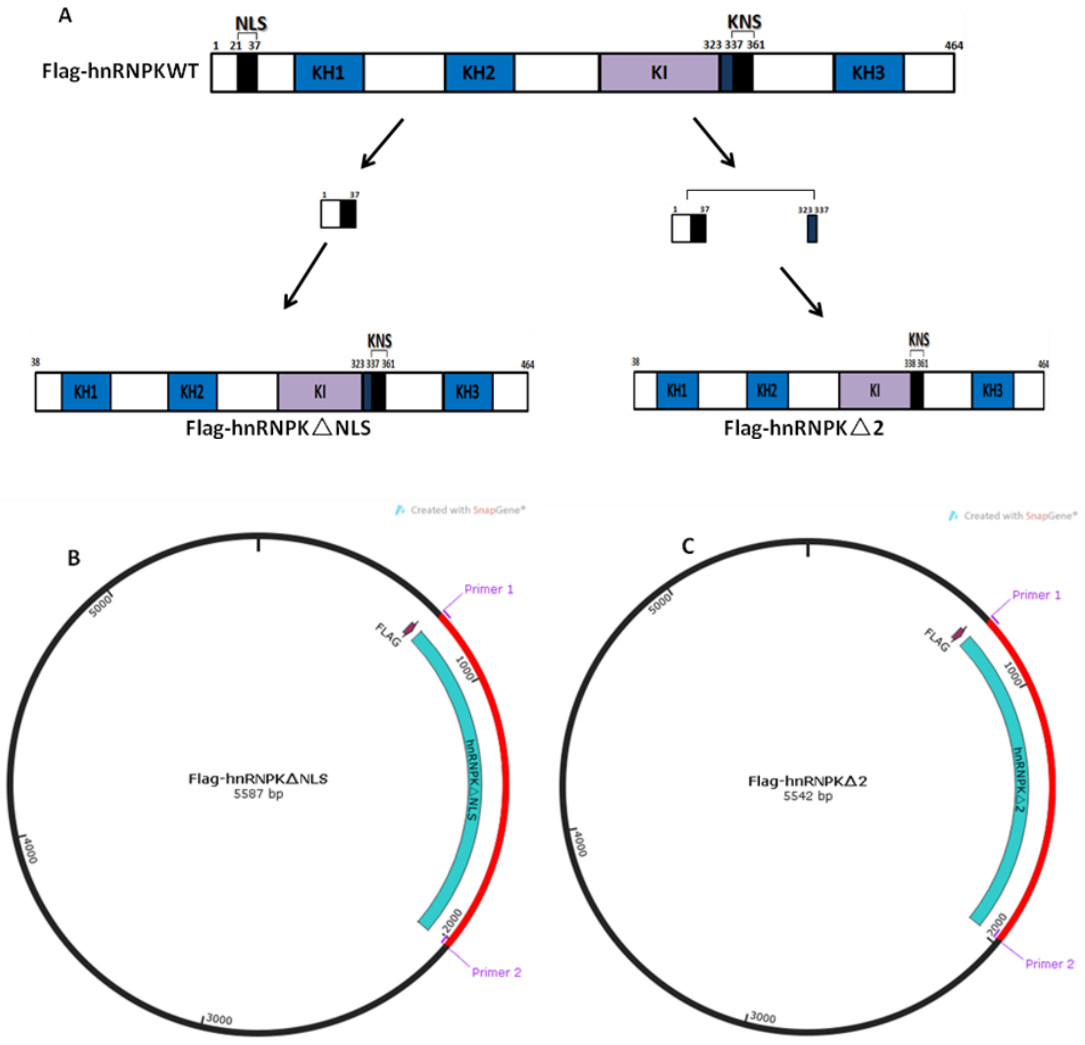

Fig. 1 Design of plasmid diagram. (A) The experimental schemes illustration of recombinant plasmid of hnRNPK. (B, C) The plasmid diagram of Flag-hnRNPK△NLS and Flag-hnRNPK△2, respectively.

3.2 construction of the nuclear export recombinant plasmid of hnRNPK

Secondly, the two mutated hnRNPK forms were constructed and cloned into Flag expressing vector by PCR using Flag-hnRNPKWT plasmid as template, as described in materials and methods. 1% agarose gel electrophoresis was then performed to verify the result of PCR. As shown in Fig. 2, 8 of the 10 verified clones for the hnRNPKΔNLS construct, and 6 of the 9 verified clones for the hnRNPKΔ2 constructs are positive clones.

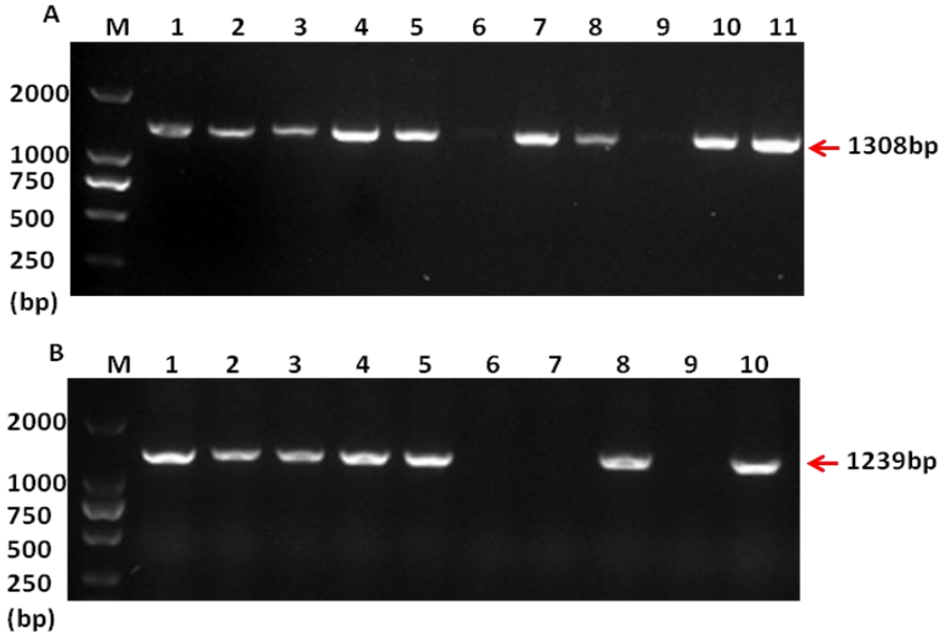

Fig. 2 Verification of construction of hnRNPK mutants by colony PCR. (A, B) M: 2000 bp DNA marker (TAKARA, Japan); 1: the PCR product of hnRNPKΔNLS fragment and hnRNPKΔ2 fragment, respectively; 2-11 (A) and 2-10 (B): the PCR product of positive clones (red arrows).

3.3 Analysis of the subcellular distribution of hnRNPK△NLS and hnRNPKΔ2 mutants

Subsequently, we used IF technique to examine the subcellular localization of the hnRNPK mutants constructed in comparison with wild type hnRNPK as described in materials and methods. As shown in Fig. 3, Flag-hnRNPKΔNLS and Flag-hnRNPKΔ2 are localized evenly in the nucleus and the cytoplasm, whereas Flag-hnRNPKWT is localized exclusively or mainly in the nucleus. Comparatively, the Δ2 deletion has a less impact than the ΔNLS deletion on the subcellular localization of hnRNPK (Fig. 3D).

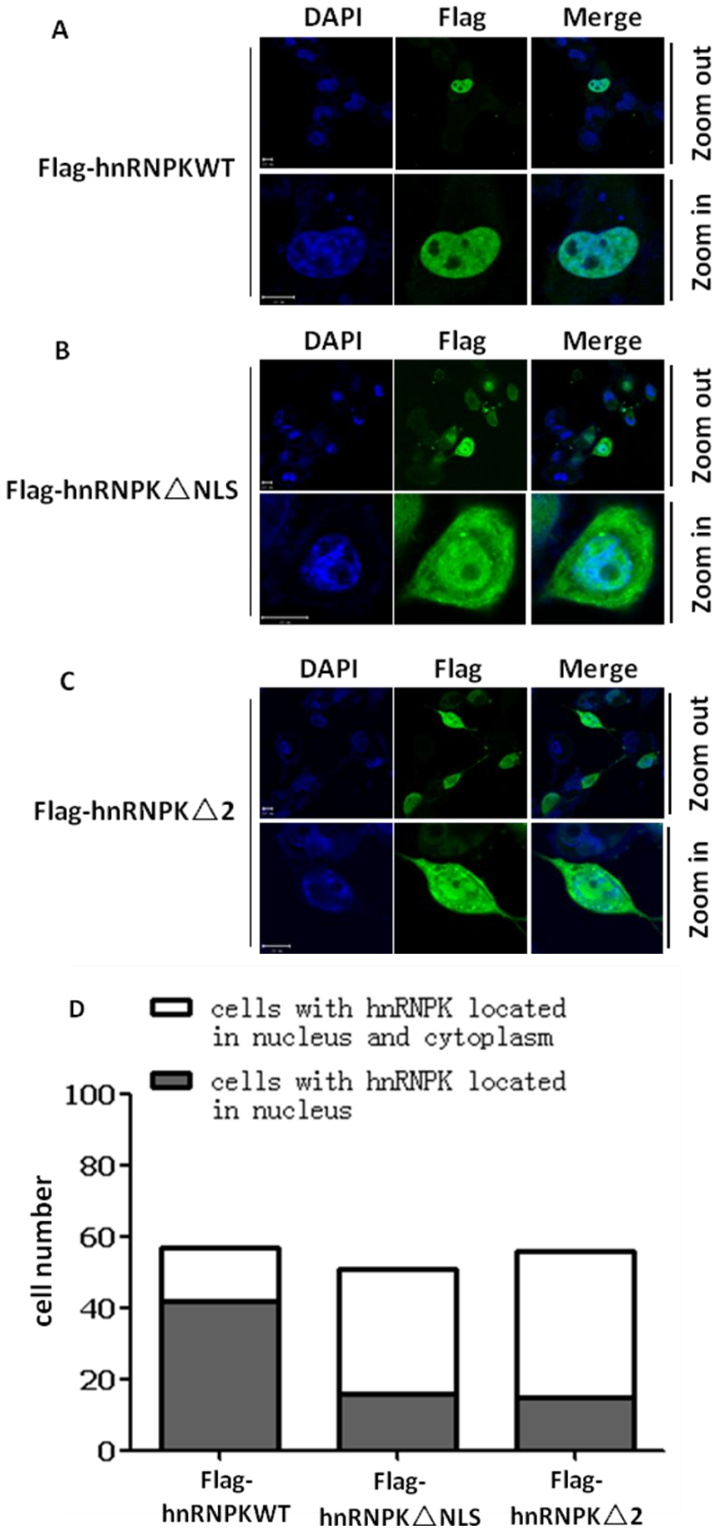

Fig. 3 Subcellular localization of the transfected Flag-hnRNPKWT (A), Flag-hnRNPKΔNLS (B), and Flag-hnRNPKΔ2 (C) by immunofluorescence. Bar: 10 μm. (D) Quantification of cells with various subcellular distribution of the indicated hnRNPKWT and mutant constructs.

4. Conclusion

In this study, we found that NLS domain of hnRNPK is important for the regulation of subcellular localization of hnRNPK, while the amino acids 323-337 seemly to play a less important role. However, the generalization of such conclusion could be imprudent since under different physiological conditions, the involvement of these two targeting signals could be different. In the upcoming studies, Flag-hnRNPKΔNLS and Flag-hnRNPKΔ2 plasmids will be useful tools for the studies aiming to elucidate the functional relevance of the cytoplasmic localization of hnRNPK in adenocarcinoma cells.

Acknowledgements

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