

## RESEARCH ARTICLE

# Expression of the Pokemon Proto-oncogene in Nasopharyngeal Carcinoma Cell Lines and Tissues

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### Abstract

To study the differentiated expression of the proto-oncogene Pokemon in nasopharyngeal carcinoma (NPC) cell lines and tissues, mRNA and protein expression levels of CNE1, CNE2, CNE3 and C666-1 were detected separately by reverse transcription polymerase chain reaction (RT-PCR), real-time PCR and Western-blotting. The immortalized nasopharyngeal epithelial cell line NP69 was used as a control. The Pokemon protein expression level in biopsy specimens from chronic rhinitis patients and undifferentiated non keratinizing NPC patients was determined by Western-blotting and arranged from high to low: C666-1>CNE1>CNE2> CNE3>NP69. The Pokemon mRNA expression level was also arranged from high to low: CNE1>CNE2>NP69>C666-1>CNE3. Pokemon expression of NP69 and C666-1 obviously varied from mRNA to protein. The Pokemon protein level of NPC biopsy specimens was obviously higher than in chronic rhinitis. The data suggest that high Pokemon protein expression is closely associated with undifferentiated non-keratinizing NPC and may provide useful information for NPC molecular target therapy.

**Keywords:** Nasopharyngeal carcinoma (NPC) - proto-oncogene - Pokemon - expression - cell lines - tissues

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### Introduction

The proto-oncogene Pokemon is a POZ and Krüppel (POK) erythroid myeloid ontogene, a member of the POK family. Because of containing broad complex, tramtrack, and bric-a-brac/poxvirus and zinc finger (BTB/POZ) domain, Pokemon protein has several important structures, such as zinc finger and actin-binding repeats (Albagli et al., 1995; Collins et al., 2001). It is also called FBI-1 (Pessler et al., 1997), OCZF (Kukita et al., 1999), LRF (Davies et al., 1999), Zbtb7A (Kelly and Daniel, 2006). Pokemon can specially suppress a regulation pathway between ARF and p53, as a result, tumorigenesis will be promoted with down-regulated ARF (Maeda et al., 2005).

Pokemon is aberrantly overexpressed in nasopharyngeal carcinoma (NPC) tissue by reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry (IHC) (Gao et al., 2009). NPC incidence and mortality trends have been high in southern China (Wei et al., 2010; Wei et al., 2010; Huang et al., 2012). It is necessary to study further the connection between Pokemon and tumorigenesis of NPC. According to the World Health Organization (WHO) classification system (1978), non-keratinising carcinoma and undifferentiated carcinoma (types II and III) can be considered together as undifferentiated carcinomas of the nasopharyngeal type (UCNT) (Tao

and Chan, 2007; Yoshizaki et al., 2013), which is also called undifferentiated non keratinizing according to the WHO classification system (2005) (Barnes et al., 2005). The number of UCNT patients is over 95% of NPCs in southern China including Guangxi Province (Tao and Chan, 2007). Therefore, UCNT is used as a appropriate research object to illustrate the connection between Pokemon expression level and UCNT.

### Materials and Methods

#### *Patient selection*

After obtaining patient approval, 10 chronic rhinitis and 10 NPC fresh tissues were obtained by biopsy. The tissues were confirmed by pathology from the formalin-fixed paraffin wax-embedded samples. The pathological type of NPC was UCNT. This study was approved by the ethics committee of The People's Hospital of Guangxi Zhuang Autonomous Region.

#### *Cell lines and culture*

The human NPC epithelial cell lines CNE1, CNE2, CNE3, C666-1 and immortalized nasopharyngeal epithelial cell line NP69 were preserved in Research Center of Medical Sciences, The People's Hospital of Guangxi Zhuang Autonomous Region (Nanning, China). CNE1, CNE2, CNE3 and C666-1 were grown in RIPM

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1640 with 10% FBS (Gibco, Carlsbad, USA). NP69 was grown in Keratinocyte-SFM (Gibco) with 5% Bovine Pituitary Extract and Recombinant Epidermal Growth Factor (Gibco).

#### RNA extraction and quality test

Total RNA of the cell lines was extracted by TRIZOL (Invitrogen, Carlsbad, USA). RNA amount was detected by Nanodrop 2000 (Thermo Fisher Scientific Inc, Waltham, USA). 4 µl total RNA was analyzed by 1.2% agarose gel electrophoresis with 0.6 mol/L formaldehyde (Robert, 2008). The gel was photographed by Bio Imaging System Gene Genius (Syngene, Cambridge, United Kingdom). An equal amount (4 µg) of total RNA was synthesized as a first-strand cDNA using the RevertAidTM First Strand cDNA Synthesis Kit (Fermentas, Pittsburgh, USA).

#### Reverse transcription polymerase chain reaction (RT-PCR) and sequencing

The following primer sequences were used for amplification of Pokemon cDNA (NM\_015898.2): forward, 5'-ATCCTGAGTGGGCTGAACGAG-3' and reverse, 5'-GCGCTGACGAAGTCGATCT-3', the sequence length was 188 bp. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference control (NM\_002046.4): forward, 5'-CCATCTTCCAGGAGCGAGA-3', reverse, 5'-GGTCATGAGTCCTTCCACGAT-3', the sequence length was 305 bp. The primer sequences were designed by Oligo 6.0. The reaction conditions consisted of 12 µl 2xTaq PCR Mix (Tiangen, Beijing, China), 0.5 µl template, 0.5 µl forward primer, 0.5 µl reverse primer and 11.5 µl ddH<sub>2</sub>O. The reaction program consisted of an initial denaturation step at 95 °C for 10min, denaturation at 94 °C for 35 sec, annealing at 66 °C for 35sec, extension at 72 °C for 35 sec for 32 cycles and a final extension at 72 °C for 10min. The sequences were amplified using S1000 Thermal Cycler PCR (Bio-Rad, Hercules, USA). Gray value of electrophoretic bands was analyzed by Gene Tools.

Purified Pokemon PCR products were analyzed by the 3730 automatic DNA sequencer (ABI, Carlsbad, USA).

#### Real-time PCR

The Pokemon (NM\_015898.2) primers: forward, 5'-ATCCTGAGTGGGCTGAACGAG-3' and 5'-GCGCTGACGAAGTCGATCT-3'. GAPDH was used as an internal reference control. GAPDH (NM\_002046.4) forward, 5'-CCATCTTCCAGGAGCGAGA-3', reverse, 5'-GGTCATGAGTCCTTCCACGAT-3'. The reaction conditions consisted of Super Real PreMix 10 µl and 50xROX 0.4 µl (Tiangen), 0.5 µl template, 0.5 µl forward primer, 0.5 µl reverse primer and 7.4 µl ddH<sub>2</sub>O. The reaction program consisted of an initial denaturation step at 95 °C for 15min, denaturation at 95 °C for 10 sec and annealing at 60 °C for 32sec for 40 cycles, dissociation stage at 95 °C for 15 sec, 60 °C for 1 min, 95 °C for 15 sec, 60 °C for 15 sec. 7500 Real-time PCR system (ABI) was used for the experiment. Similar results were obtained in three independent experiments. Pokemon RNA expression level was calculated by Delta-delta Ct, a high result

meaned Pokemon RNA expression level was high (Livak and Schmittgen, 2001).

#### Western-blot

The proteins (20 µg) were subjected to 10% SDS polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, USA) by Mini-Protean System (Bio-Rad). The membranes were incubated for 1 h at room temperature (RT) in blocking buffer (5% skim milk in TBS-T) and then incubated with the appropriate antibodies (1:500 dilution, Pokemon antibody ab70208 from Abcam, Cambridge, United Kingdom; 1:1200 dilution, β-actin antibody from Beyotime, Shanghai, China) overnight at 4 °C. After washing with TBST-T, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbit (1:8000 dilution, ZSGB-BIO, Beijing, China) and anti-mouse (1:12000 dilution, Beyotime) for 2 h at 37 °C. Detection was performed using BeyoECL Plus (Beyotime). Similar results were obtained in three independent experiments. Gray value of bands was analyzed by Image J.

#### Statistical analysis

Data analysis of Real-time PCR and western-blot was performed by Sigma Plot 8.0. Statistical comparisons amount the data were performed by t test (SPSS.13.0). *P* values less than 0.05 was considered to be statistically significant.

## Results

#### Quality test of total RNA from the cell lines

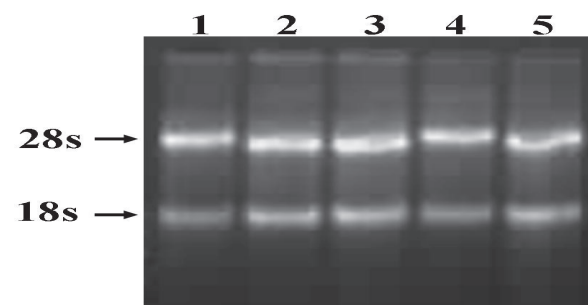
Total RNA of the cell lines was not degraded and not polluted by DNA or protein (Table 1, Figure 1). A260/A280 of all RNA value was between 1.90 and 2.00.

#### RT-PCR and sequencing of the cell lines

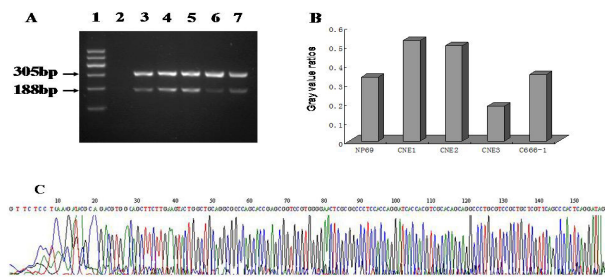
The electrophoretic bands indicated that CNE3

**Table 1. The Concentration of Total RNA of the Cell Lines**

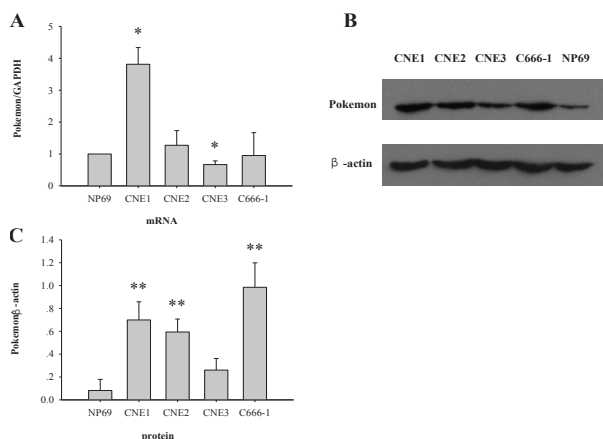
Sample	A260	A280	A260/A280	Concentration (ng/µl)
NP69	10.679	5.499	1.94	427.2
CNE1	17.408	8.973	1.94	696.3
CNE2	18.704	9.546	1.96	748.2
CNE3	38.573	20.124	1.92	1542.9
C666-1	12.176	6.412	1.9	487



**Figure 1. Gel electrophoresis of Total RNA of the Cell Lines.** Lanes 1-5 represented NP69, CNE1, CNE2, CNE3 and C666-1, respectively. Two bands represented 28s and 18s respectively in every lane



**Figure 2. RT-PCR and Sequencing of the Cell Lines.** (A) Lanes 1-6 represented DNA Marker, negative control, NP69, CNE1, CNE2, CNE3, C666-1, respectively. The product length of the Pokemon PCR product was 188bp, the product length of the GAPDH PCR product was 305bp. (B) The bar charts represented gray value ratios of Pokemon and GAPDH mRNA. (C) The products of Pokemon PCR were sequenced



**Figure 3. Real-time PCR and Western-blot of the Cell Lines.** (A) The data of NP69 was used as a control, delta-delta Ct calculated mRNA expression level of Pokemon in other cell lines. (B) The bands represented the proteins expression level of Pokemon and  $\beta$ -actin of the cell lines. (C) The bar charts represented ratios of Pokemon and  $\beta$ -actin protein. All data represented the mean of three independent experiments  $\pm$  SD. Statistical significance was tested using Independent-sample t test with  $*P<0.05$  and  $**P<0.01$

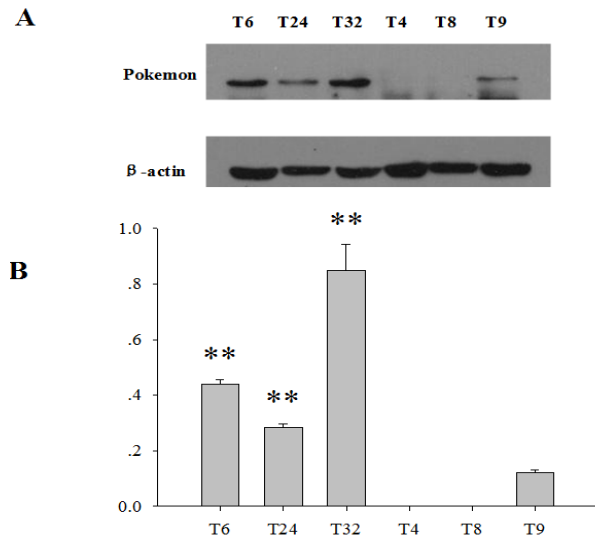
Pokemon band was weakest (Figure 2A). The gray value ratios of Pokemon and GAPDH indicated: CNE1>CNE2>NP69 >C666-1>CNE3 (Figure 2B). PCR sequences of the cell lines were matched with the Pokemon, according to the NCBI blast database (Figure 2C).

#### Real-time PCR and Western-blot of the cell lines

The mRNA expression level of Pokemon clearly indicated: CNE1>CNE2>NP69>C666-1>CNE3 (Figure 3A). However, the NP69 Pokemon protein bands was weakest. The result was different with mRNA expression of NP69, which was higher than C666-1 and CNE3. Pokemon expressions of CNE1 and C666-1 were probably highest (Figure 3B). The Pokemon protein expression level clearly indicated: C666-1>CNE1>CNE2>CNE3>NP69 (Figure 3C).

#### Western-blot of chronic rhinitis and UCNT

Subsequently, 3 chronic rhinitis and 3 NPC biopsy specimens were selected randomly in 10 chronic rhinitis and 10 NPC biopsy specimens. As a result, the protein



**Figure 4. Western-blot of Chronic Rhinitis and UCNT.**

(A) The bands represented the proteins expression level of Pokemon and  $\beta$ -actin of chronic rhinitis and UCNT. (B) The data of chronic rhinitis T9 was used as a control. The bar charts represented ratios of Pokemon and  $\beta$ -actin protein. All data represented the mean of three independent experiments  $\pm$  SD. Statistical significance was tested using Independent-sample t test with  $*P<0.05$  and  $**P<0.01$

bands indicated that Pokemon protein expression was commonly high in UCNT (T6, T24 and T32). however, Pokemon was not expressed in chronic rhinitis (T4 and T8), there was a little protein expression in T9 (Figure 4A). The Pokemon protein expression level of UCNT was further high than Pokemon protein expression of chronic rhinitis (Figure 4B).

## Discussion

Our group firstly used the NPC cell lines from different tissue types to study Pokemon mRNA and protein expression. CNE1 came from a well differentiated squamous carcinoma (Department of Virology, 1978). CNE2 came from a poorly differentiated squamous carcinoma (Gu et al., 1983). CNE3 came from an UCNT with primitive adenoid structure, then transformed to a poorly differentiated adenocarcinoma (Liu et al., 2013). C666-1 came from an undifferentiated cell lines (Cheung et al., 1999). The results showed that different Pokemon protein level was expressed in different cell lines. Pokemon was highest in C666-1 and lowest in CNE3, there was no significant difference between well differentiated and poorly differentiated squamous carcinoma. Furthermore, immortalized nasopharyngeal epithelial cell NP69 was used as a normal control because of its non-tumorigenic characteristic (Tsao et al., 2002). As a result, Pokemon protein expression of the four NPC cell lines was higher than NP69 Pokemon protein. Selecting UCNT and chronic rhinitis biopsy specimens separately as the experimental group and control group, we found that Pokemon protein of NPC tissues was obviously higher than Pokemon of control group. The result was in conformity with IHC result of NPC tissues, which was highly expressed in 86 NPC tissues (77.9%) and higher than expression in 30 nasopharyngitis tissues (23.3%) (Gao et al., 2009).



The different results illustrate that the regulation mechanisms including Pokemon exist possibly some difference through comparing Pokemon mRNA and protein expression levels in the cell lines. Pokemon mRNA expression level of CNE1 was highest, C666-1 result was only higher than CNE3 result, NP69 result was surprisingly higher than C666-1 result. However, Pokemon protein expression level of C666-1 was highest, NP69 result was lowest. It is well known that transcription of Pokemon is repressed in some degree by directly binding competition of Sp1 and p53, and interacted by corepressors such as mSin3A, NCoR, SMRT (Choi et al., 2009). Therefore, NP69 Pokemon protein expression being repressed in the maximal degree illustrates conversely that Pokemon protein probably plays an important role in the carcinogenesis of NPC through repressing tumor suppressor genes and promoting oncogenes. The speculation is consistent with some studies which Pokemon participates in various regulation mechanisms (Lin et al., 2012; Yang et al., 2012; Jin et al., 2013; Zhang et al., 2013; Zhu et al., 2013).

In view of continuous presence of Epstein-Barr Virus (EBV) in C666-1 (Cheung et al., 1999; Liu et al., 2013), the EBV oncogenes probably participate in the regulation mechanisms of Pokemon, even promote Pokemon protein expression and in coordination with elevating tumorigenicity of EBV products, which play an pathogenic role in the development of NPC (Yip et al., 2010; Yoshizaki et al., 2013). According to WHO classification system standard (2005), the pathological type of C666-1 is UCNT. Because of close relationship between UCNT and EBV (Klein et al., 1974; Andersson-Anvret et al., 1979; Wei et al., 1996; Han et al., 2012), the study data will be useful for clinical diagnosis and therapy of UCNT if C666-1 is used for further study.

In a summary, the study confirms further the association between Pokemon and NPC. Pokemon probably will become a newly molecular target therapy of UCNT. Basing on the study, we are observing the changes of biological characteristics of the NPC cell lines after interrupting Pokemon mRNA expression of by short hair RNA (shRNA) (data not shown).

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