ORIGINAL ARTICLE

Correlation between Pokemon and Mouse Double Minute 2 Homolog in the Carcinogenesis of Lung Squamous Cell Carcinoma in Rats

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ABSTRACT

This study was designed to investigate the correlation between proto-oncogene POK erythroid myeloid ontogenetic factor (Pokemon) and mouse double minute 2 homolog (MDM2), an oncogene, in the carcinogenesis of lung squamous cell carcinomas in rats. Protein and messenger ribonucleic acid (mRNA) expressions of Pokemon and MDM2 in different stages of rat lung squamous cell carcinoma were measured using immunochemistry staining and in situ *hybridization assays. Lung squamous cell carcinoma were viewed under the microscope in 60 rats (success rate: 80%) after treatment with carcinogen. Among these rats, 21 had bronchial epithelial hyperplasia, 13 had atypical hyperplasia, 28 had carcinoma* in situ*, 20 had invasive carcinoma and 16 had metastatic carcinoma. There were significant differences in the Pokemon and MDM2 expressions between the control group and the atypical hyperplasia group or squamous cell carcinoma group (*$p < 0.05$*). There were also significant differences of both genes between the non-metastatic carcinoma group and the metastatic carcinoma group (*$p < 0.05$*). Pokemon expression was positively correlated with MDM2 expression (*$r = 0.616$*,* $p = 0.000$*). These findings indicate that Pokemon and MDM2 were highly expressed in rat lungs following carcinogenesis in lung squamous cell carcinoma. The expression of Pokemon and MDM2 may contribute to the genesis and development of lung squamous cell carcinoma in rats.*

Keywords: *In situ* hybridization, lung cancer, lung squamous cell carcinoma, MDM2, Pokemon

Correlación entre el Pokemon y el murino doble minuto 2 homólogo en la carcinogénesis del carcinoma pulmonar de células escamosas en ratas

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RESUMEN

Este estudio fue diseñado para investigar la correlación entre el proto-oncogén factor eritroide mieloide ontogénico de la proteína POK (Pokemon) y el oncogén murino doble minuto 2 (MDM2) en la carcinogénesis del carcinoma pulmonar de células escamosas en ratas. Las expresiones de la proteína y ácido ribonucleico mensajero (ARNm) del Pokemon y el MDM2 en las diferentes etapas del carcinoma pulmonar de células escamosas en ratas fueron medidas

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mediante ensayos de tinción inmunoquímica e hibridación in situ*. Los carcinomas pulmonares de células escamosas fueron vistos bajo el microscopio en 60 ratas (tasa de éxito: 80%) después del tratamiento con el agente carcinógeno. De estas ratas, 21 tuvieron hiperplasia epitelial bronquial, 13 tuvieron hiperplasia atípica, 28 tuvieron carcinoma* in situ*, 20 tuvieron carcinoma invasivo, y 16 tuvieron carcinoma metastásico. Hubo diferencias significativas en las expresiones de Pokemon y MDM2 entre el grupo de control y el grupo de hiperplasia atípica o el grupo de carcinoma de células escamosas (*$p < 0.05$*). También hubo diferencias significativas de ambos genes entre el grupo de carcinoma no metastásico y el grupo de carcinoma metastásico (*$p < 0.05$*). La expresión de Pokemon se correlacionó positivamente con la expresión de MDM2 (*$r = 0.616$, $p = 0.000$*). Estos resultados indican que Pokemon y MDM2 hallaron una alta expresión en los pulmones de ratas luego de la carcinogénesis del carcinoma pulmonar de células escamosas. La expresión de Pokemon y de MDM2 pueden contribuir a la génesis y el desarrollo del carcinoma pulmonar de células escamosas en las ratas.*

Palabras claves: Hibridación *in situ*, cáncer del pulmón, carcinoma pulmonar de células escamosas, MDM2, Pokemon

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INTRODUCTION

The development and metastasis of tumours are a complex process that involves an expression of serial genes. The activation of pro-oncogenes and the inactivation of tumour suppressor genes are important contributory mechanisms underlying tumour progression and metastasis (1). The detection of tumour markers is a rapid, sensitive and non-invasive technique that offers a novel approach for the early diagnosis of the genesis of lung cancer (2). Many genes and microRNAs have been identified as the biomarkers of lung cancer (3–5). Their expression levels in the lung tissues and even in the blood can indicate the genesis and development of certain kinds of lung cancer.

POK erythroid myeloid ontogenetic factor (Pokemon) is one of the members of POK protein family, which is encoded by Zbtb7 gene (6). A recent study demonstrated that Pokemon was a pro-oncogene that enables cancer cells to resist ageing and death, and participated in regulating the expression and activities of other pro-oncogenes and oncogenes (6). Pokemon was unregulated following lymphomagenesis (6). The following studies demonstrated that Pokemon was expressed in lung cancer tissues and may be relevant to the genesis of lung cancer (7, 8). Mouse double minute 2 homolog (MDM2) is a protein that is encoded by the MDM2 gene. It is a negative regulator of the p53 tumour suppressor. The expression and activation of MDM2 inhibit p53 transcriptional activities and its functions, and in turn p53 also negatively regulates MDM2 expression and activities, which form negative feedback loops (9, 10). The expression of MDM2 has also been related to the development of lung cancer (11).

Although Pokemon and MDM2 have been viewed as the biomarkers of lung cancer, their expression and correlations in different stages of lung squamous cell carcinoma have not been elucidated. In this study, we investigated Pokemon and MDM2 expression and correlations following the development of lung squamous cell carcinoma in rats.

SUBJECTS AND METHODS

Animals

Ninety male and female Wistar rats in the ratio 1:1 were purchased from the Experimental Animal Centre of Henan Province, China. Methylcholanthrene (442 388) and diethyl nitrosamine (73 861) were obtained from the Shanghai Sigma Company (Shanghai, China). Lung squamous cell carcinoma rat models were established as previously described (12). The rats were randomly divided into two groups: tumour group (75 animals) and control group (15 animals).

The rats in the tumour group were treated with carcinogen and those in the control group were treated with iodized oil. On days 30, 60, 90, 120 and 180 following treatment with the drug, the rats (15 animals in the tumour group and 3 in the control group) were sacrificed and the tissues from the perfusion sites were collected. Based on the pathological grades, the rats in the tumour group were divided into the following groups: atypical

hyperplasia, squamous cell carcinoma, non-metastatic carcinoma and metastatic carcinoma.

In situ hybridization

The Pokemon and MDM2 *in situ* hybridization kit, the rabbit antimouse polyclonal antibody kit, BCIP/NBT colour kit and DAB chromogenic kit were purchased from Wuhan Boster Company. The tissues were dehydrated, immersed in pre-hybridization solution and made into slices according to standard protocols. After the inactivation of the endogenous enzymes, the slices were incubated with freshly diluted 3% citric acid pepsin for 20 minutes and subsequently, the slices were incubated in pre-hybridization solution at 38ºC for four hours, followed by incubation in hybridization solution at 40ºC for 15 hours. After washing with phosphate buffer saline (PBS), the slices were incubated in mouse anti-digoxin for 120 minutes, and then treated with streptomycin affinity peroxidase complex (SABC) and biotin peroxidase for five minutes at room temperature. After washing, the slices were re-stained with haematoxylin and then mounted.

Immunochemistry staining

The Pokemon and MDM2 immunochemistry kits, as well as the rabbit anti-mouse polyclonal antibody kit, were purchased from Wuhan Boster Company. Tissue slices were dewaxed, boiled into the citrate buffer for 20 minutes, allowed to cool naturally for more than 20 minutes, and then incubated in 3% H_2O_2 at 37ºC for 10 minutes, in order to block and inactivate the endogenous peroxidase. Then the slices were incubated in rabbit anti-mouse polyclonal antibody (1:100) at 4°C overnight. After washing three times with PBS, the slices were incubated in secondary antibody and then horseradish peroxidase. Subsequently, the slices were stained with DAB solution and re-stained with haematoxylin.

Semi-quantitative score analysis

The Pokemon and MDM2 messenger ribonucleic acid (mRNA) expressions were analysed by calculating the blue/purple particles in the cytoplasm as previously reported (13). More than 100 cells and at least five high-resolution fields in each slice were analysed. The average percentage of positive staining was calculated based on the numbers of positive cells *versus* the total cells in the slices. The grades were as follows: 0–5% – 0 point, 6–25% – 1 point, 26–50% – 2 points, 51–75% – 3 points, and >75% – 4 points. The staining degrees were quantified based on the staining colours and intensity. The uncoloured staining was viewed as zero point; the purple/blue positive staining was viewed as one point; the brown/blue positive staining was viewed as two points; and the deep blue/purple staining was viewed as three points. The final score results were: positive staining grades plus staining degrees: 0–1 was negative, 2–3 was weakly positive, 4–5 was moderately positive, and 6–7 was strongly positive.

Statistical analysis

The data were presented as mean ± standard deviation and analysed using the χ^2 test. The statistical difference between the two groups was analysed using the q test. The correlation analysis between Pokemon and MDM2 was performed using Pearson correlation analysis. All the data were statistically analysed by the SPSS 17.0 software.

RESULTS

Pathological changes of lung squamous cell carcinoma in rats

Normal bronchi, invasive carcinoma and low differentiated squamous carcinoma can be seen in the rats' lungs following HE staining (Figs. 1A and B). In some slices, different stages of carcinogenesis such as bronchial epithelial hyperplasia, squamous metaplasia, dysplasia, carcinoma *in situ*, invasion and poorly differentiated carcinoma coexisted in the same fields (Fig. 1C). Lung squamous cancer, lymph node metastasis and pleural invasion simultaneously appeared in the rats' lungs on days 120 and 180 (data not shown). Sixty of the 75 rats suffered from squamous cell carcinoma of the lung, including 21 cases with bronchial epithelial hyperplasia, 13 cases with atypical hyperplasia, 28 cases with carcinoma *in situ*, 20 cases with invasive carcinoma and 16 cases with metastatic carcinoma. Of note, multiple phases coexisted in the same rat cancer lesions.

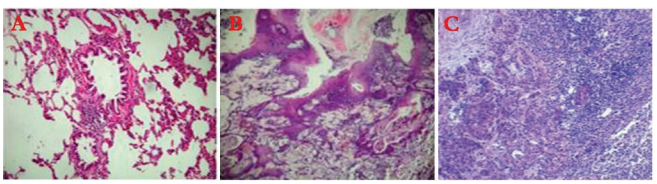

Fig. 1: HE staining shows different stages of lung carcinoma in rats. A: normal bronchus (× 200); B: invasive carcinoma (× 200); C: low differentiated squamous carcinoma.

Pokemon expressions in different stages of carcinoma tissues

Pokemon mRNA was mainly localized in the cytoplasm with scattered or diffused distribution (Figs. 2A and B), but proteins were mainly located in the nucleus (Figs. 2C and D).

Semi-quantitative score analysis indicated that the score of Pokemon mRNA in the normal bronchial epithelium (control group) was 1.25 ± 1.03, while the scores in the hyperplasia, atypical hyperplasia and squamous cell carcinoma groups were 2.11 ± 0.79, 2.88 ± 1.10 and 4.17 ± 1.05, respectively. The variance among all the groups was $F = 31.454$ ($p < 0.05$). The differences among the control group and atypical hyperplasia group or squamous cell carcinoma group were statistically significant ($p < 0.05$), while there was no significant difference between the control group and the hyperplasia group, nor between the hyperplasia group and the atypical hyperplasia group ($p > 0.05$). Overall, the Pokemon expressions in the control group were lower than in all the carcinogenesis groups. The score of Pokemon mRNA expression in the metastatic carcinoma group was also significantly higher than in the non-metastatic carcinoma group ($p < 0.05$, Table 1).

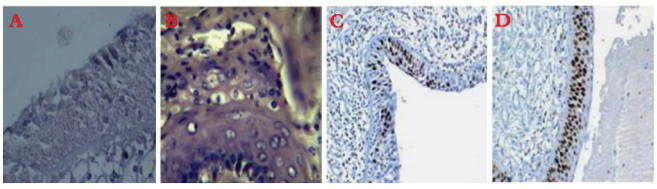

Fig. 2: *In situ* hybridization assays show Pokemon mRNA and protein expressions in the rat lung tissues. A and B: Pokemon mRNA expression (× 400); C and D: Pokemon protein expression.

MDM2 expressions in different stages of carcinoma tissues

Mouse double minute 2 homolog mRNA and protein were localized in the cell nucleus with a scattered or diffused distribution (Figs. 3A–D).

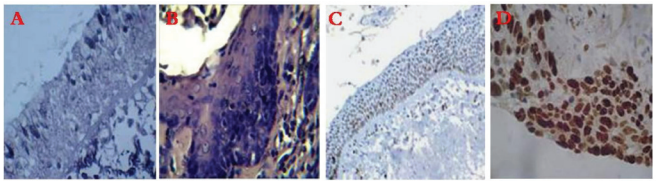

Fig. 3: *In situ* hybridization assays show MDM2 mRNA and protein expressions in the rat lung tissues. A and B: MDM2 mRNA expression (× 400); C and D: MDM2 protein expression (× 400).

The score on MDM2 expression in the normal bronchial epithelium (control group) was 1.21 ± 0.74, while the scores in the hyperplasia, atypical hyperplasia and squamous carcinoma groups were 2.09 ± 0.76, 3.03 ± 1.00 and 4.17 ± 1.18, respectively. The variance among all the groups was $F = 30.669$ ($p < 0.05$). The difference between the control group and the squamous cell carcinoma group was statistically significant ($p < 0.05$), while the differences between the normal control group and the hyperplasia group, and between the hyperplasia group and the atypical hyperplasia group were not significant ($p > 0.05$). Mouse double minute 2 homolog expression in the control group was also lower than in all the carcinogenesis groups. The difference between the metastatic cancer and non-metastatic cancer groups was statistically significant ($p < 0.05$, Table 1).

Table 1: Scores of Pokemon and MDM2 mRNA expression in normal and different stages of tumour tissues ($x \pm s$)

Groups	Pokemon mRNA	MDM2 mRNA
Normal control	1.25 ± 1.03	1.01 ± 0.74
Hyperplasia	2.11 ± 0.79	2.09 ± 0.76
Atypical hyperplasia	2.68 ± 1.10*	3.03 ± 1.00*
Squamous carcinoma	4.17 ± 1.05*	4.17 ± 1.18*
Non-metastatic carcinoma	3.64 ± 1.11#	3.79 ± 1.15#
Metastatic carcinoma	4.53 ± 0.86	4.59 ± 0.93

*$p < 0.05$; ^^$p < 0.05$; ^$p < 0.05$ *vs* normal control group; #$p < 0.05$ *vs* metastatic carcinoma group.

Correlation between mRNA expressions of Pokemon and MDM2 in rat lung squamous cell carcinoma

The ratio of MDM2 mRNA positive/Pokemon mRNA positive in the lung tissues was 86.84%, while the ratio of MDM2 mRNA negative/Pokemon mRNA negative was 24.24%, indicating a positive correlation between these two genes ($r = 0.616$, $\chi^2 = 41.362$, $p = 0.000$, Table 2).

Table 2: Correlation of Pokemon and MDM2 mRNA expressions in lung tissues

Pokemon mRNA		MDM2 mRNA	
	n	+	–
+	76	66	10
–	33	8	25
Total	109	74	35

$r = 0.616$; $\chi^2 = 41.362$; $p = 0.000$

DISCUSSION

Pokemon gene is located in the third subzone of the first zone in the short arm of the human chromosome 19 (19P13.3). The gene contains two exons and two introns,

and encodes a 155 amino acid protein with a highly conserved protein-protein interaction domain (BTB domain) in the N-terminal and zinc finger structure in the C-terminal (14). Guo *et al* (15) reported that Pokemon mRNA level was much higher in bladder cancer tissues than in normal tissues. Other studies also indicated a a high expression of Pokemon in lung cancers (7, 8). These studies suggest a possibility to diagnose cancers by detecting Pokemon gene expression (16).

To investigate the expression pattern of Pokemon gene following the development of lung cancer, we established a lung squamous cell carcinoma rat model and analysed Pokemon expression using *in situ* hybridization in lung squamous cell carcinoma and adjacent tissues. The results revealed a significant difference in Pokemon expression between the control group and the atypical hyperplasia or squamous cell carcinoma group ($p < 0.05$), accompanied by a gradually increased expression trend following the progression of lung cancer. The pathological score of Pokemon expression in the metastatic carcinoma tissues was higher than in the non-metastatic tissues. These results were consistent with previous reports by Zhao *et al* which showed a high expression of Pokemon at gene and transcription levels in human non-small cell lung cancer, but low or no expression in normal and adjacent tissues (7).

Mouse double minute 2 homolog is an oncogene identified in BALB/c mice cell lines and located in the 12q13–14 chromosome region. Under physiological conditions, MDM2 is induced by wild-type P53 and promotes the degradation of P53 (17). Our results showed that MDM2 expression was higher in the atypical hyperplasia and the squamous cell carcinoma groups than in the control group. Of note was that MDM2 expression was gradually increased following the progression of lung cancer in the rats. Like Pokemon, the pathological score on MDM2 was also higher in the metastatic carcinoma tissues than in the non-metastatic tissues. These results indicate that the expression of MDM2 gene is involved in the development and progression of squamous cell carcinoma in rats.

We also analysed the correlation between Pokemon and MDM2 mRNA expressions in squamous cell carcinomas using *in situ* hybridization. The results demonstrated a positive correlation between the expressions of these two genes ($r = 0.616$, $p = 0.000$). This suggested that MDM2 and Pokemon expressions were both mutually reinforced in the development of lung squamous cell carcinoma. Our findings indicated that Pokemon and MDM2 were involved in the development and metastasis of lung squamous cell carcinoma, and simultaneous intervention strategies for both genes may be a better strategy to prevent and treat lung cancer.

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