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ORIGINAL ARTICLE

Expression and clinical contribution of MRGD mRNA in non-small cell lung cancers

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Summary

Purpose: *MAS-related G protein-coupled receptor, member D (MRGD) has been reported to be involved in tumorigenesis in vivo. However, the clinical role of MRGD in non-small cell lung cancer (NSCLC) remains unclarified. The purpose of the current study was to detect the expression of MRGD mRNA in NSCLC formalin-fixed (FF), paraffin-embedded (PE) tissues and to investigate the clinicopathological significance of the MRGD level in NSCLC patients.*

Methods: *The expression of MRGD mRNA was examined in 125 NSCLC tissue samples together with paired para-noncancerous FF/PE tissues by using real time quantitative PCR (qRT-PCR). Furthermore, the relationship between MRGD level and clinicopathological parameters of NSCLC was analyzed.*

Results: *The average level of MRGD in NSCLC tumor tissues (1.0682±0.6096) was remarkably higher than that in the adjacent non-cancerous lung tissue (0.3994±0.2838, p<0.001). The area under curve (AUC) of receiver operating characteristic curve (ROC) of MRGD mRNA was 0.853 (95% CI: 0.808-0.898, p<0.001). Moreover, the level of MRGD mRNA was found to be correlated to lymph node metastasis (r=0.219, p=0.014), tumor size (r=0.221, p=0.013) and clinical TNM stage (r=0.187, p=0.037). Finally, the survival of patients in high MGRD expression group was 7.94±9.85 months, remarkably shorter than that of the low expression group (20.84±1.19 months, p=0.049).*

Conclusions: *MRGD may be a vital diagnostic and prognostic factor in NSCLC. MRGD possesses the potential to become a new target for the molecular therapy of NSCLC.*

Key words: *MRGD, non-small cell lung cancer, qRT-PCR, survival*

Introduction

Lung cancer is the most lethal malignant tumor worldwide [1,2]. Despite progress in surgery, systemic therapy and radiotherapy, the 5-year survival for all lung cancer patients remains between 15 and 20% [3,4]. Newer therapeutic approaches rely on particular molecular alterations or biomarkers to provide opportunities for a personalized strategy to specific patient populations [5-7]. G protein-coupled receptor (GPCR) superfamily members can activate a number of physiological signaling and play a vital role in the development and function of different organs [8]. Additionally, some GPCRs have been reported to be upregulated in primary and metastatic cells of head and neck squamous cell carcinoma, NSCLC, breast, prostate and gastric carcinomas, melanoma and diffused large B cell lymphoma [9,10]. MRGD is a member of GPCR family encoded by a gene located on human chromosome 11q13.2. MRGD is regarded to be one of the players in pain sensation and/or

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transduction [11]. Furthermore, MRGD could also be involved in tumorigenesis and could become an anticancer drug target [12]. However, only one research group has elucidated the role of MRGD in the tumorigenesis and progression of NSCLC [12] by using in vivo model and a small size of clinical samples (n=33). Moreover, to date, no report has been found to explore the relationship between MRGD expression and the clinicopathological parameters in NSCLC. Thus, the purpose of the current study was to investigate the expression and clinicopathological contribution of MRGD mRNA in NSCLCs with a larger sample size of 125 patients.

Methods

Tissue samples

One hundred and twenty five NSCLC patients (75 males and 50 females) were retrospectively analyzed in the present study. Tissues tested were FF, PE NSCLC tissues and their paired adjacent non-tumorous lung tissues. All patients involved in the study were collected from the First Affiliated Hospital, Guangxi Medical University, from January 2012 to February 2014. The mean age of NSCLC patients was 61.10 years (range 23-90). The study was approved by the Ethics Committee of the First Affiliated Hospital, Guangxi Medical University, China. All participating patients provided written informed consent. Two independent pathologists rechecked all cases without knowing beforehand the diagnosis and clinicopathological information. The clinicopathological information is summarized in Table 1. The EGFR status, including EGFR gene amplification with FISH, EGFR protein expression with IHC and EGFR mutation with qRT-qPCR, was detected as previously reported [13,14].

Quantitative real-time RT-PCR

RNA isolation and normalization were performed as previously described [13,15,16]. All mRNAs were assayed using Gene Expression Assays, in accordance with the manufacturer's instructions provided by Applied Biosystems, Waltham, MA, USA. All reverse transcription (RT) reactions were run in an ABI Prism7900 Real Time PCR System (Applied Biosystems), including no-template controls and RT minus controls. The probe and primer sequences were:
MRGD probe: TGTGTGCCACCATGCCTGGCTAATT,
MRGD FWD: GCTCACTACAACCTCAATGTGCC,
MRGD REV: GCCACATAGCAAGATCTCATCTCTAC.

Samples were normalized to β-actin for gene expression normalization (Applied Biosystems, confidential sequences). Gene expression levels were calculated using the 7900 Real Time Sequence Detection System software (Applied Biosystems). All real-time PCR reactions were performed in triplicate. Relative expression of MRGD mRNA was calculated using the comparative 2-Cq method ($Cq = Cq_{MRGD} - Cq_{\beta\text{-actin}}$).

Statistics

SPSS 20.0 (Munich, Germany) was used to conduct the statistical analyses. Results were representative of three independent experiments unless otherwise stated. Results were presented as mean±standard deviation (SD). Student's paired or unpaired t-test was used to examine the significance between paired or unpaired groups. One-way analysis of variance (ANOVA) test was accomplished to examine the significance between groups of various differentiation grading and pathological type. Spearman's correlation test was performed for the association between MRGD level and other parameters. ROC was employed to identify the diagnostic value. Correlation between MRGD and prognosis of NSCLC patients was evaluated by Kaplan-Meier method with log-rank test. Statistical significance was set at $p<0.05$ level.

Results

The data demonstrated that the mean level of MRGD in NSCLC tumor tissues (1.0682±0.6096) was significantly higher as compared to that in the adjacent non-cancerous lung tissue (0.3994±0.2838, $p<0.001$, Figure 1, Table 1). Besides, ROC curve was used to evaluate the diagnostic value of MRGD mRNA in lung cancer. The AUC of MRGD mRNA was 0.853 (95% CI: 0.808-0.898, $p<0.001$, Figure 2).

To study the potential contribution of MRGD in the progression of NSCLC, we further explored the possible correlations between MRGD expres-

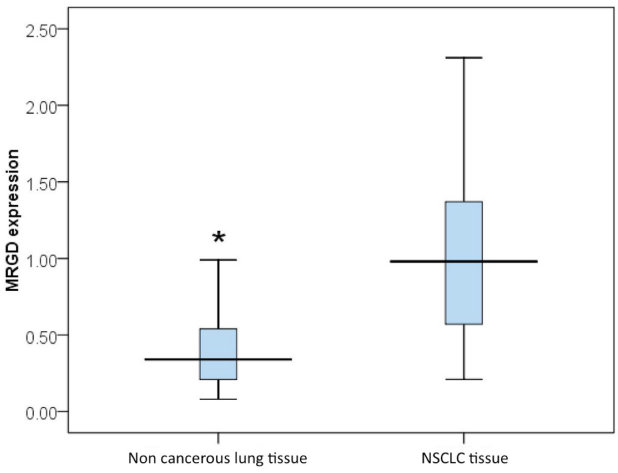

Figure 1. MRGD mRNA expression in NSCLC and non-cancerous lung tissues. Quantitative real-time PCR was performed to detect the expression of MRGD in non-small cell lung cancer (NSCLC) tissue and adjacent non-cancerous lung tissue. *p<0.05.

Table 1. Relationship between MRGD mRNA and clinicopathological characteristics of NSCLC patients ($\bar{x}$±s)

Characteristics		*n*	*Expression of MRGD mRNA*		
			2^{-cq}	*t*	*p value*
Tissue	NSCLC	125	1.0682±0.6096	10.67[A]	<0.001
	Adjacent non-cancerous lung	125	0.3994±0.2838		
Age (years)	≥60	68	1.1122±0.6168	0.882	0.380
	<60	57	1.0156±0.6020		
Gender	Male	75	1.1091±0.5813	0.797	0.427
	Female	50	1.0198±0.6511		
Grade of differentiation	Good	17	0.9941±0.7390	*F*= 0.881[B]	0.417
	Moderate	78	1.0356±0.5520		
	Poor	30	1.1947±0.6751		
Pathological types	Adenocarcinoma	101	1.0329±0.6356	*F*= 0.880[B]	0.417
	Squamous cell carcinoma	23	1.2174±0.4781		
	Large cell carcinoma	1	1.2000±0.0000		
Smoking	Yes	30	1.0460±0.5320	0.699	0.487
	No	38	0.9589±0.4918		
Lymph node metastasis	Yes	69	1.2010±0.6334	2.777	0.006
	No	56	0.9045±0.5406		
Vascular infiltration	Yes	35	1.1943±0.6609	1.449	0.150
	No	90	1.0191±0.585		
Tumor diameter (cm)	≥3	65	1.1935±0.5895	2.441	0.016
	<3	60	0.9323±0.6066		
Clinical TNM stage	I & II	54	0.9261±0.5515	2.312	0.022
	III & IV	71	1.1762±0.6329		
EGFR amplification	Yes	18	0.9228±0.5035	0.716	0.484
	No	39	1.1136±0.5087		
EGFR protein expression	Low	40	1.0988±0.5036	0.444	0.663
	High	17	0.9465±0.5258		
EGFR mutation	Wild type	44	1.0707±0.5119	0.220	0.829
	Mutation [C]	13	0.9946±0.5217		

A: paired t-test. B: ANOVA test. C: EGFR mutation including in-frame deletions (exon 19) and point mutations resulting in a substitution of arginine for leucine at codon 858 (L858R, exon 21)

sion and clinicopathological characteristics in NSCLC tissues. Significantly higher level of MRGD mRNA was found in NSCLC patients with lymph node metastasis (1.2010±0.6334) compared with those without such metastasis (0.9045±0.5406, p=0.006, Figure 3A). The relative expression of MRGD mRNA in patients with tumor larger than 3 cm (1.1935±0.5895) was remarkably higher compared to that with tumor smaller than 3cm (0.9323±0.6066, p=0.016, Figure 3B). Furthermore, the relative expression of MRGD mRNA in advanced stage III and IV (1.1762±0.6329), was noticeably upregulated compared with that in early stage I and II (0.9261±0.5515, p=0.022, Figure 3C, Table 1). Simultaneously, additional Spearman's correlation test indicated an accordant correlation between MRGD mRNA expression and the following clinicopathological parameters: lymph node metastasis (r=0.219, p=0.014), tumor size (r=0.221, p=0.013) and clinical TNM stage (r=0.187, p=0.037). However, no correlation was found between MGRD and age, gender, grade of differentiation, pathological type, smoking or vascular infiltration. Neither was MGRD related to any of the EGFR statuses, including EGFR gene amplification, EGFR protein expression or EGFR mutation.

Among the 57 patients with complete information of follow up, 29 cases had high MGRD level (higher than the median level of 0.980) and 28

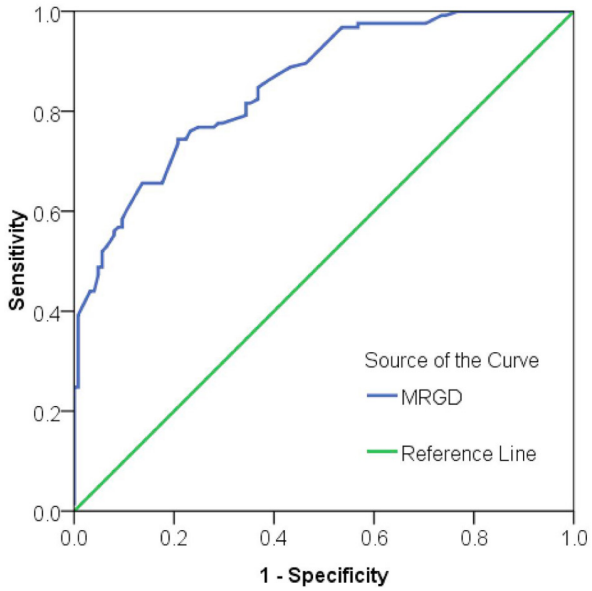

Figure 2. Diagnostic value of MRGD in NSCLC. ROC curve of MRGD level in NSCLC. The area under the curve (AUC) of MRGD was 0.853 (95% CI: 0.808-0.898, p<0.001).

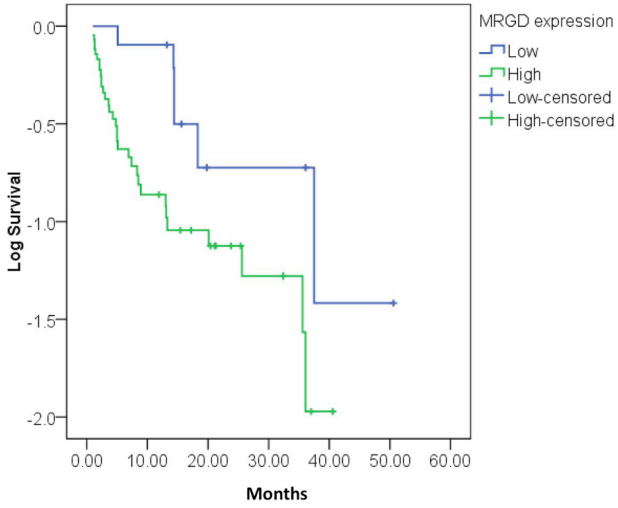

Figure 4. Kaplan-Meyer curves of MRGD expression in NSCLC. Patients with high MRGD expression had a significantly poorer prognosis than those with low expression (p=0.049).

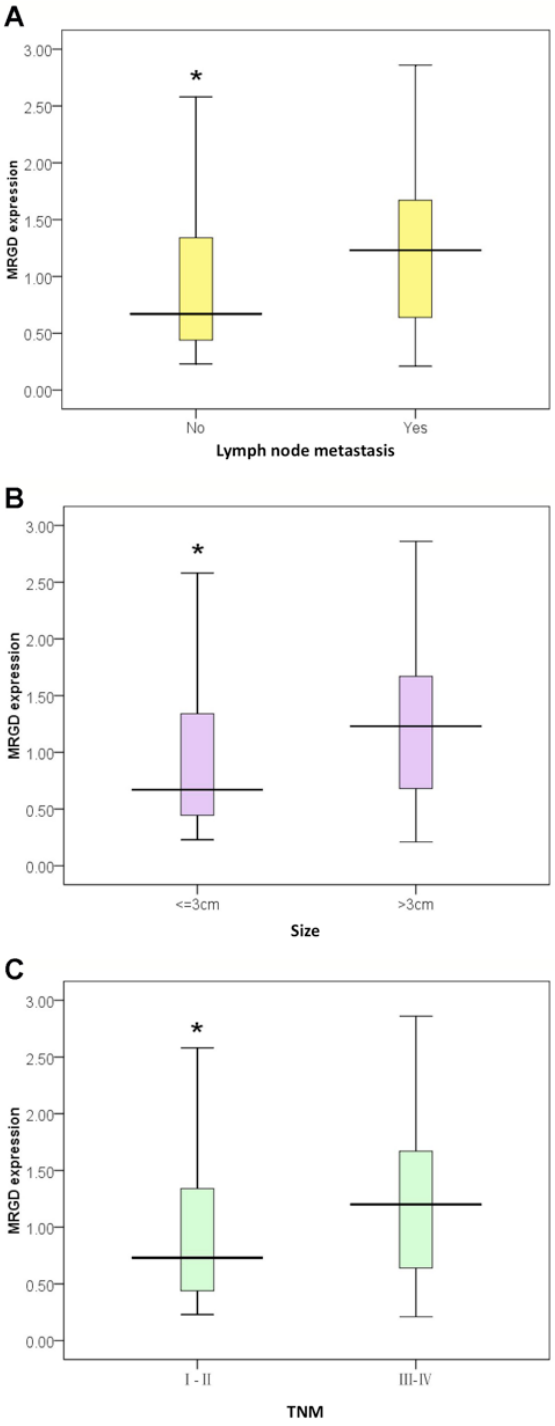

Figure 3. Relationship between MRGD mRNA expression and some clinicopathological features in NSCLC. A: lymph node metastasis; B: tumor size; C: clinical TNM stages. * p<0.05.

cases had low MGRD level. The survival of high MGRD expression group was 7.94±9.85 months, apparently shorter than that of the low expression group (20.84±1.19 months, p=0.049, Figure 4).

Discussion

GPCRs belong to a superfamily of cell surface signaling proteins that have a crucial role in many physiological functions and in various diseases, including the progress of tumor and cancer metastasis [9,10]. MRGD, a member of GPCRs, has also been reported to be involved in tumorigenesis [12]. To our knowledge, only Nishimura et al. [12] have elucidated the function of MRGD in cancers *in vitro* and *in vivo*. They found that up-regulation of MRGD in NIH3T3 cells could in-

duce focus formation and multi-cellular spheroid formation, and stimulate tumors in nude mice. Additionally, from clinical cancer tissues, higher expression of MRGD in several lung cancers was found by immunohistochemistry as well as qRT-PCR. When compared to the lung normal portion, the mean MRGD mRNA expression in the lung cancer portion exceeded the amount by 3 times. Furthermore, out of 33 matched lung samples, the MRGD expression in 12 cancers exceeded 3 times the amount in the paired normal lung. The lung paired samples showed the highest frequency (36%) for higher MRGD expression in the cancer portion than that of the normal portion with the criteria exceeding 3 times the amount. However, the sample size was extremely small (n=33) in the study of Nishimura et al. [12]. In the current study, we examined the MRGD mRNA expression in 125 cases of NSCLC samples and their paired non-cancerous lung tissues. We found consistent overexpression of MRGD in NSCLC tissues with the report of Nishimura et al. [12]. The relevant expression of MRGD mRNA was 1.0682 in NSCLC tissues, 2.67 times higher than that in the non-cancerous lung tissues (0.3994). Furthermore, ROC demonstrated that MRGD had an extremely strong diagnostic value for NSCLC with an AUC of 0.853. Nishimura et al. [12] also revealed that the possible mechanism of overexpression of MRGD in NIH3T3 could be related to the induction of the loss of contact inhibition and anchorage-independent growth. Combining the current finding and the literature [12], the possible impact of the oncogenic function of MRGD in NSCLC and the potential role of MRGD in the carcinogenesis of NSCLC is strongly indicated.

Concerning the relationship between MRGD expression and clinicopathological parameters, no studies have been available so far. We primarily revealed that MRGD mRNA expression was related to the deterioration of NSCLC. Firstly, MRGD mRNA was upregulated in the NSCLC tissues with lymph node metastasis and with larger tumor size, as compared to their corresponding groups. Secondly, MRGD mRNA level was positively correlated with the progression of the disease. MRGD mRNA increased when NSCLC developed to the late stage. Finally, patients with high MGRD level tended to have shorter overall survival than those with low level. This indicates that MRGD overexpression closely correlates with factors of an unfavorable prognosis in NSCLC.

The results of our study revealed a noticeable relationship between MRGD and metastasis, tumor growth and prognosis of NSCLC. Therefore, it may be worth examining MRGD expression clinically for the estimate of metastasis and deterioration of NSCLC. EGFR-based therapy has been widely applied in the treatment of NSCLC with variable success. Thus, we were also interested in the relationship between MRGD expression and the EGFR statuses. However, no correlation was found between MGRD mRNA level and all EGFR statuses, including EGFR amplification, EGFR protein expression or EGFR sensitive mutations to tyrosine kinase inhibitors' therapies.

In conclusion, with previous information, the present findings strongly suggest that MRGD may be an important diagnostic and prognostic factor in NSCLC. Current drugs that target GPCRs are directed towards only a few GPCR members. Thus, huge efforts are presently ongoing to develop novel GPCR-based drugs, particularly for cancer . Our findings may identify MRGD as a new target for the molecular therapy of NSCLC.

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