African Journal of Biotechnology Vol. 11(31), pp. 8000-8004, 17 April, 2012
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB12.513
ISSN 1684–5315 © 2012 Academic Journals

Full Length Research Paper

Clinical significance of RECK and MMP-9 expression in cutaneous squamous cell carcinoma

Qian Wu, Li-jun Hao*, Jian-yu Han and Li-jun Wang.

Plastic Surgery Center, the First Affiliated Hospital of Harbin Medical University, Harbin 150001, Heilongjiang Province, People's Republic of China.

Accepted 19 March, 2012

Matrix metalloproteinase 9 (MMP-9) is recognized for its ability to promote tumor invasion and metastasis. However, its role and that of a novel regulator of MMP-9, reversion-inducing-cysteine-rich protein with kazal motifs (RECK), have not been explored in cutaneous squamous cell carcinoma (SCC). We investigated expression of MMP-9 and RECK in SCC to determine the clinical significance of their expression and potential uses for diagnostic, prognostic and therapeutic approaches. Immunohistochemistry was used to analyze expression in 36 SCC and 13 healthy skin samples collected at our hospital. RECK expression was detected in 33.3% of SCC samples, significantly fewer than for control samples (84.6%, *P*<0.05). In contrast, MMP-9 was expressed in 77.8% of SCC samples, significantly more than in control samples (30.8%, *P*<0.05). RECK and MMP-9 expression in SCC samples were negatively correlated (*r* = -0.406, *P*<0.05). Furthermore, negative RECK and positive MMP-9 expression in SCC tissues were correlated with differentiation degree and lymph node metastasis (*P*<0.05) but not with gender or age. Thus, low/absent expression of RECK and increased expression of MMP-9 correspond to increased disease severity. These proteins may play opposing roles in pathogenesis of SCC and may serve as future diagnostic or prognostic markers. Therefore RECK should be further investigated for therapeutic potential as an MMP-9 inhibitor.

Key words: Cutaneous squamous cell carcinoma, RECK, MMP-9, immunohistochemistry, clinicopathological parameter.

INTRODUCTION

Skin squamous cell carcinoma (SCC), a malignant tumor derived from keratinocytes in epithelial tissues, accounts for 20% of skin tumors (Bradford, 2009). Invasion and metastasis of SCC result in poor prognosis and even death. For any tumor, invasion and metastasis begin with destruction of the basement membrane and degradation of extracellular matrix (ECM). These actions require (MMP-9) (Ivaska and Heino, 2000; Loukopoulos et al., 2003). MMP-9 and related proteins are required for skin cell proliferation and wound healing (Philips et al., 2011).

*Corresponding author. E-mail: haoljharbin@126.com.

Abbreviations: MMP-9, Matrix metalloproteinase 9; **SCC,** squamous cell carcinoma; **RECK,** reversion-inducing-cysteine-rich protein with kazal motifs.

However, overexpression of MMP-9 has been shown to promote aging and carcinogenesis by destabilizing ECM (Philips et al., 2011).

Interestingly, a recently discovered protein, reversion-inducing-cysteine-rich protein with kazal motifs (RECK) has been reported to act as a regulator of MMPs and appears to inhibit expression of MMP-9, preventing tumor invasion and metastasis (Namwat et al., 2011; Chang et al., 2008). RECK expression has been detected in skin cells (Zibert et al., 2010). Thus, this protein may possess a therapeutic potential for preventing invasion and metastasis of SCC by inhibiting MMP-9 activity.

To determine the roles of both MMP-9 and RECK in SCC, we used immunohistochemistry to detect their expression in 36 SCC tissue samples and 13 normal skin samples. We sought to establish the relationship between RECK and MMP-9 expression as well as the correlation

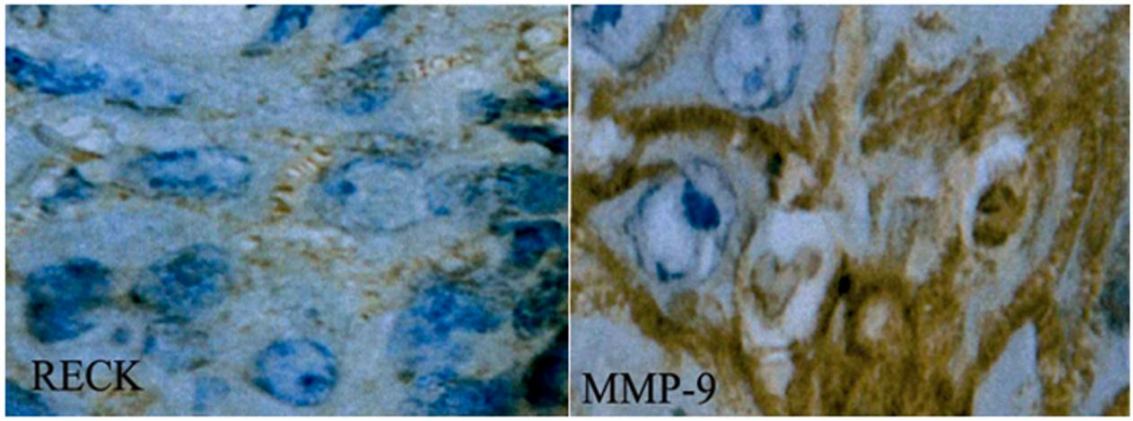

Figure 1. Positive expression of RECK and MMP-9 in SCC tissues (×400). MMP-9, Matrix metalloproteinase 9; SCC, squamous cell carcinoma; RECK, reversion-inducing-cysteine-rich protein with kazal motifs.

of their expression with clinicopathological parameters of SCC to provide novel insight into SCC diagnosis, prognosis and treatment.

MATERIALS AND METHODS

Sample collection

Skin specimens were collected from 36 patients who had been pathologically diagnosed with SCC at the First Affiliated Hospital of Harbin Medical University. Each case had detailed clinical data and did not receive preoperative chemotherapy or radiotherapy. Study population included 15 males and 21 females ranging in age from 42 to 71 years (mean age = 58.1±7.6 years). Cases were classified according to World Health Organization (WHO) pathological criteria, as follows: highly differentiated in 15 cases, moderately differentiated in 12 cases, and poorly differentiated in nine cases; and no lymph node metastasis in 22 cases and metastasis in 14 cases. In addition, 13 normal skin samples were obtained from cutting edge during out-patient surgery for controls.

Immunohistochemistry

Tissues were fixed in neutral formalin, dehydrated and embedded in paraffin by conventional methods. Samples were sectioned (4 μM thickness) and collected on glass slides. Sections were then dewaxed for 5 min with dimethylbenzene, rehydrated through an alcohol gradient, rinsed in distilled water, soaked for 5 min in phosphate buffered saline (PBS) and heated for antigen retrieval. Upon cooling, 3% hydrogen peroxide solution was used to block endogenous peroxidase activity. Slides were sealed with non-specific serum and then placed in a wet box and incubated for 10 min at room temperature. Primary antibodies against MMP-9 or RECK [mouse anti-human monoclonal (MMP-9) or rabbit anti-human polyclonal (RECK) antibodies, Santa Cruz Biotechnology] were added to the wet box prior to overnight incubation at 4°C. Slides were washed with PBS three times before the addition of biotinylated secondary antibodies and incubated for 30 min at room temperature. Finally, the slides were washed with PBS three times prior to addition of streptococcus avidin-peroxidase (SP kit, Fuzhou Maixim Bio-technology Development Co., Ltd) and incubated at 37°C for 30 min. Diaminobenzidine (DAB; Fuzhou Maixim Bio-technology Development Co., Ltd) was used to develop staining. Sections were counterstained with haematoxylin, dehydrated through an ethanol gradient, and sealed with neutral gum. Known positive tissues were used as a positive control, and PBS was used in place of primary antibodies as a negative control.

RECK and MMP-9 are visualized as brownish-yellow granules in cytoplasm. To score staining, 10 high-power fields were analyzed per sample and samples were classified by intensity as follows: samples with no visible staining received a 0, pale yellow staining received a 1, yellow staining received a 2, and brownish-yellow received a 3. Additionally, the percentage of positively-staining cells was determined from the total number of tumor cells, and samples were assigned scores as follows: ≤5% of tumor cells positive was assigned a 0, 6 to 25% positive was assigned a 1, 26 to 50% positive was assigned a 2, 51 to 75% was assigned a 3, and ≥76% positive was assigned a 4. Total scores for each case represent the sum of scores from staining intensity and from the percentage of positively-stained cancer cells, with total scores of 0 represented as (-), total scores of one to two represented as (+), total scores of three to five represented as (++), and total scores of six to seven represented as (+++).

Statistical methods

SPSS17.0 statistical software was used for statistical analysis. χ^2 test was used to compare expression of RECK and MMP-9 among groups and Spearman rank correlation was used to analyze correlation between RECK and MMP-9 expression. Analyses were two-sided, with alpha level of 0.05 and $P<0.05$ considered statistically significant.

RESULTS

RECK and MMP-9 expression in SCC

RECK and MMP-9 were both detected in normal and SCC skin samples as brownish-yellow granules in the cytoplasm (Figure 1). RECK protein was detected in 12/36 cases (33.3%; Table 1) of SCC, a significantly lower proportion than for normal skin samples (11/13, 84.6%; χ^2 =10.172, $P<0.05$). In contrast, MMP-9 was expressed in 28/36 cases (77.8%; Table 2) of SCC, compared to just 4/13 (30.8%) normal skin samples. This

Table 1. Expression of RECK in SCC and normal skin samples [*n* (%)].

Skin sample	*n*	-	+	++	+++
SCC	36	24 (66.7)	3 (8.3)	5 (13.9)	4 (11.1)
Normal	13	2 (15.4)	3 (23.1)	4 (30.8)	4 (30.8)
Total	49	26 (53.1)	6 (12.2)	9 (18.4)	8 (16.3)

χ^2 =10.172, *p*=0.017. SCC, squamous cell carcinoma.

Table 2. Expression of MMP-9 in SCC and normal skin samples [*n* (%)].

Skin sample	*n*	-	+	++	+++
SCC	36	8 (22.2)	10 (27.8)	11 (30.6)	7 (19.4)
Normal	13	9 (69.2)	4 (30.8)	0	0
Total	49	17 (34.7)	14 (28.6)	11 (22.4)	7 (14.3)

χ^2 =12.613, *p*=0.006. SCC, squamous cell carcinoma; *n,* number of samples.

Table 3. Correlation between RECK and MMP-9 expression in SCC samples.

RECK	*n*	MMP-9 [*n* (%)]			
		-	+	++	+++
-	24	3 (12.5)	5 (20.8)	10 (41.7)	6 (25.0)
+	3	3 (100.0)	0	0	0
++	5	1 (20.0)	2 (40.0)	1 (20.0)	1 (20.0)
+++	4	1 (25.0)	3 (75.0)	0	0
Total	36	8 (22.2)	10 (27.8)	11 (30.6)	7 (19.4)

r = -0.406, *p*=0.014. MMP-9, Matrix metalloproteinase 9; RECK, reversion-inducing-cysteine-rich protein with kazal motifs. *n,* number of samples.

difference in MMP-9 expression was also statistically significant (χ^2=12.613, *P*<0.05). Further, RECK expression and MMP-9 expression in SCC tissues were negatively correlated (*r* = -0.406, *P*<0.05; Table 3).

RECK and MMP-9 expression and clinicopathological parameters

To determine whether the altered expression of RECK and MMP-9 in SCC samples are associated with disease severity, we assessed expression in comparison with various clinicopathological parameters. RECK (Table 4) and MMP-9 (Table 5) expression in SCC tissues was correlated with degree of differentiation and lymph node metastasis (both *P*<0.05), but not with gender or age. For RECK, samples with low or absent expression tended to have higher differentiation degree and/or metastasis. In contrast, for MMP-9, samples with higher expression had higher differentiation degree and/or metastasis.

DISCUSSION

The *RECK* gene was initially isolated as a novel transforming and suppressor gene from a cDNA expression library transfected into v-Ki-ras-transformed NIH3T3 cells by Takahashi et al. (1998). Highly conserved in *Drosophila*, mice, and humans, *RECK* mRNAs were detected in many normal human tissues and cell lines (Eisenberg et al., 2002). However, Takahashi et al. (1998) reported that RECK was not expressed in cancer cell lines or transformed cell lines. Thus, RECK is typically expressed only in normal cells, not tumor cells (Mori et al., 2007); this feature may allow RECK to be used as a novel diagnostic indicator with high sensitivity and specificity. Indeed, RECK has been explored as a diagnostic marker in cancers other than SCC. Moreover, RECK expression is lower in human breast (Span et al., 2003) and gastric (Song et al., 2006) cancers than in normal tissues. Our findings of reduced RECK expression in SCC samples support this previous work.

Additionally, the apparent tumor-suppressing activity of RECK holds some promise as a potential therapy for cancer. Studies have shown that invasion and metastasis capabilities are inhibited in cell lines transfected with RECK (Petruzzelli et al., 1998). Restoring RECK expression in cells otherwise not expressing the protein results in inhibition of tumor progression. Human studies

Table 4. Correlation between expression of RECK and clinicopathological parameters in SCC samples [*n* (%)].

Parameter	*n*	-	+	++	+++	χ^2	*P*
Gender							
Male	15	10 (66.7)	2 (13.3)	3 (20.0)	0	4.320	0.229
Female	21	14 (66.7)	1 (4.8)	2 (9.5)	4 (19.0)		
Age (years)							
<60	16	11 (68.8)	2 (12.5)	3 (18.8)	0	4.309	0.230
≥60	20	13 (65.0)	1 (5.0)	2 (10.0)	4 (20.0)		
Pathological grade							
High	15	5 (33.3)	2 (20.0)	3 (20.0)	4 (26.7)		
Middle	12	11 (91.7)	0	1 (8.3)	0	14.8112	0.022
Low	9	8 (88.9)	0	1 (11.1)	0		
Lymph node metastasis							
No	22	11 (50.0)	2 (9.1)	5 (22.7)	4 (18.2)	8.123	0.044
Yes	14	13 (92.9)	1 (7.1)	0	0		

RECK, reversion-inducing-cysteine-rich protein with kazal motifs; SCC, squamous cell carcinoma.

Table 5. Correlation between expression of MMP-9 and clinicopathological parameters in SCC samples [*n* (%)].

Parameter	*n*	-	+	++	+++	χ^2	*P*
Gender							
Male	15	5 (33.3)	2 (20.0)	4 (26.7)	3 (20.0)	2.120	0.548
Female	21	3 (14.3)	7 (33.3)	7 (33.3)	4 (19.0)		
Age (years)							
<60	16	3 (18.8)	3 (18.8)	6 (37.5)	4 (25.0)	1.913	0.591
≥60	20	5 (25.0)	7 (35.0)	5 (25.0)	3 (15.0)		
Pathological grade							
High	15	8 (53.3)	7 (46.7)	0	0		
Middle	12	0	3 (25.0)	8 (66.7)	1 (8.3)	39.387	0.001
Low	9	0	0	3 (33.3)	6 (66.7)		
Lymph node metastasis							
No	22	6 (27.3)	10 (45.5)	5 (22.7)	1 (4.5)	14.887	0.002
Yes	14	2 (14.3)	0	6 (42.9)	6 (42.9)		

MMP-9, Matrix metalloproteinase 9; SCC, squamous cell carcinoma.

corroborate these findings. Van der Jaqt et al. (2006) in a study of 63 surgically-resected colorectal cancer specimens, reported reduced RECK mRNA expression in tumors compared to normal tissues. In addition, expression was not correlated with tumor size, lymph node metastasis, or distant metastasis. Masui et al. (2003) while studying 50 surgically-resected pancreatic cancer specimens, reported that RECK protein expression was lower than in adjacent normal tissues. Additionally, the invasiveness of tumors with positive expression was weaker compared to those not expressing RECK. In fact, patients with high RECK expression in their tumor samples had significantly better prognosis and long-term survival compared to those without RECK expression. Our results reveal that RECK expression in skin SCC samples correlate with tissue differentiation and lymph node metastasis, specifically, as in pancreatic cancer, those samples with low or absent RECK expression had increased disease severity. These findings indicate that RECK is suppressed during the

development and progression of skin SCC.

MMP-9 has been recognized as a tumor promoter (thanks to its ability to degrade type IV collagen-a critical component of ECM and basement membranes (Newby, 2005)) to promote endothelial cell migration and tumor angiogenesis, and to regulate cell adhesion during tumor cell metastasis (Sato et al., 1993; O'Grady et al., 2007). Consistent with findings in other tumor types, we found that MMP-9 expression is significantly higher in skin SCC than in normal skin samples. To our knowledge, this is the first report of MMP-9 expression in skin SCC. Additionally, MMP-9 expression in skin SCC correlates with tumor differentiation and lymph node metastasis. Thus, the overexpression of MMP-9 in skin SCC appears to promote the development and progression of SCC. This information may allow MMP-9 to be developed into a diagnostic and prognostic marker of SCC.

Importantly, research has shown that RECK negatively regulates MMP-9, both *in vitro* and *in vivo*, inhibiting MMP-9 secretion and activation and subsequently, reducing lung cancer cell metastasis (Takagi et al., 2009; Chang et al., 2008, Buhmeida et al., 2009). Indeed, our results show that RECK and MMP-9 expressions are negatively correlated in skin SCC, consistent with results observed in nasopharyngeal carcinoma studies (Li and Deng, 2010). These results suggest that reduced RECK expression allows MMP-9 to be overexpressed, leading to disease progression. Therefore, developing RECK as a therapeutic molecule to inhibit MMP-9 and reduce tumor differentiation and metastasis represents an important avenue for future research.

In summary, reduced RECK expression and increased MMP-9 expression in skin SCC promotes the progression and metastasis of SCC. Thus, RECK and MMP-9 should be explored for use as diagnostic and prognostic markers of skin SCC. RECK also warrants investigation as a potential therapeutic molecule targeting MMP-9.

REFERENCES

Bradford PT (2009). Skin cancer in skin of color. Dermatol. Nurs. 21(4):170-177.

Buhmeida A, Bendardaf R, Hilska M, Collan Y, Laato M, Syrjänen S, Syrjänen K, Pyrhönen S (2009). Prognostic significance of matrix metalloproteinase-9 (MMP-9) in stage II colorectal carcinoma. J. Gastrointest. Cancer, 40(3-4): 91-97.

Chang CK, Hung WC, Chang HC (2008). The Kazal motifs of RECK protein inhibit MMP-9 secretion and activity and reduce metastasis of lung cancer cells *in vitro* and *in vivo.* J. Cell. Mol. Med. 12(6B): 2781-2789.

Eisenberg I, Hochner H, Sadeh M, Argov Z, Mitrani-Rosenbaum S (2002). Establishment of the genomic structure and identification of thirteen single-nucleotide polymorphisms in the human RECK gene. Cytogenet. Genome Res. 97(1-2): 58-61.

Ivaska J, Heino J (2000). Adhesion receptors and cell invasion: mechanisms of integrin-guided degradation of extracellular matrix. Cell. Mol. Life. Sci. 57(1): 16-24.

Li R, Deng Y (2010). Expression of RECK, RAGE and MMP-9 in nasopharyngeal carcinoma and its significance. Lin Chung, Er Bi Yan, Hou Tou, Jing Wai Ke, Za Zhi, 24(18): 823-827.

Loukopoulos P, Mungall BA, Straw RC, Thornton JR, Robinson WF (2003). Matrix metalloproteinase-2 and -9 involvement in canine tumors. Vet. Pathol. 40(4): 382-394.

Masui T, Doi R, Koshiba T, Fujimoto K, Tsuji S, Nakajima S, Koizumi M, Toyoda E, Tulachan S, Ito D, Kami K, Mori T, Wada M, Noda M, Imamura M (2003). RECK expression in pancreatic cancer: its correlation with lower invasiveness and better prognosis. Clin. Cancer Res. 9(5): 1779-1784.

Mori T, Moriuchi R, Okazaki E, Yamada K, Katamine S (2007). Tgat oncoprotein functions as a inhibitor of RECK by association of the unique C-terminal region. Biochem. Biophys. Res. Commun. 355(4): 937-943.

Namwat N, Puetkasichonpasutha J, Loilome W, Yongvanit P, Techasen A, Puapairoj A, Sripa B, Tassaneeyakul W, Khuntikeo N, Wongkham S (2011). Downregulation of reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) is associated with enhanced expression of matrix metalloproteinases and cholangiocarcinoma metastases. J. Gastroenterol. 46(5): 664-675

Newby AC (2005). Dual role of matrix metalloproteinases (matrixins) in intimal thickening and atherosclerotic plaque rupture. Physiol. Rev. 85(1): 1-31.

O'Grady A, Dunne C, O'Kelly P, Murphy GM, Leader M, Kay E (2007). Differential expression of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 in non-melanoma skin cancer: implications for tumour progression. Histopathology, 51(6): 793-804.

Philips N, Auler S, Hugo R, Gonzalez S (2011). Beneficial regulation of matrix metalloproteinases for skin health. Enzyme Res. 2011: 427285.

Takahashi C, Sheng Z, Horan TP, Kitayama H, Maki M, Hitomi K, Kitaura Y, Takai S, Sasahara RM, Horimoto A, Ikawa Y, Ratzkin BJ, Arakawa T, Noda M (1998). Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc. Natl. Acad. Sci. USA. 95(22): 13221-13226.

Petruzzelli GJ, Benefield J, Yong S (1998). Mechanism of lymph node metastases: current concepts. Otolaryngol. Clin. North Am. 31(4): 585-599.

Sato H, Kita M, Seiki M (1993). v-Src activates the expression of 92-kDa type IV collagenase gene through the AP-1 site and the GT box homologous to retinoblastoma control elements. A mechanism regulating gene expression independent of that by inflammatory cytokines. J. Biol. Chem. 268(31): 23460-23468.

Song SY, Son HJ, Nam E, Rhee JC, Park C (2006). Expression of reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) as a prognostic indicator in gastric cancer. Eur. J. Cancer, 42(1): 101-108.

Span PN, Sweep CG, Manders P, Beex LV, Leppert D, Lindberg RL (2003). Matrix metalloproteinase inhibitor reversion-inducing cysteine-rich protein with Kazal motifs: a prognostic marker for good clinical outcome in human breast carcinoma. Cancer, 97(11): 2710-2715.

Takagi S, Simizu S, Osada H (2009). RECK negatively regulates matrix metalloproteinase-9 transcription. Cancer Res. 69(4): 1502-1508.

van der Jagt MF, Sweep FC, Waas ET, Hendriks T, Ruers TJ, Merry AH, Wobbes T, Span PN (2006). Correlation of reversion-inducing cysteine-rich protein with kazal motifs (RECK) and extracellular matrix metalloproteinase inducer (EMMPRIN), with MMP-2, MMP-9, and survival in colorectal cancer. Cancer Lett. 237(2): 289-297.

Zibert JR, Løvendorf MB, Litman T, Olsen J, Kaczkowski B, Skov L (2010). MicroRNAs and potential target interactions in psoriasis. J. Dermatol. Sci. 58(3): 177-185.