available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Expression of metastin and a G-protein-coupled receptor (AXOR12) in epithelial ovarian cancer

Kohkichi Hata[a,*], *Dipok Kumar Dhar*[b], *Yoh Watanabe*[c], *Hidekatsu Nakai*[c], *Hiroshi Hoshiai*[c]

[a]*Department of Tumor Biology, Kagawa Prefectural University of Health Sciences, Takamastu 761-0123, Japan*
[b]*Institute of Hepatology, RF & UCL Medical School, London WC1E 6HX, UK*
[c]*Department of Obstetrics and Gynecology, Kinki University of Medicine, Osaka-Sayama 589-8511, Japan*

ARTICLE INFO

Article history:
Received 21 December 2006
Received in revised form
16 February 2007
Accepted 7 March 2007
Available online 17 April 2007

Keywords:
Metastin
AXOR12
Gene expression
Real-time quantitative RT-PCR
In situ hybridisation
Epithelial ovarian cancer
Prognosis

ABSTRACT

Background: Metastin, a product of the *KiSS-1* gene, is a ligand for a G-protein-coupled receptor (AXOR12) and is a strong suppressant of metastasis. The aim of this study was to evaluate whether *metastin* and *AXOR12* gene expressions affect prognosis of patients with epithelial ovarian cancer.

Methods: The expression levels of *metastin*, *AXOR12* and *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene expression were analysed by the real-time quantitative reverse transcription-polymerase chain reaction in 76 epithelial ovarian cancer surgical specimens. Their expression (metastin/GAPDH and AXOR12/GAPDH ratios) was correlated with the clinical findings. Furthermore, cellular distribution of metastin and AXOR12 mRNA was examined by *in situ* hybridisation on tissue sections.

Results: The median and range of mRNA expression for metastin and AXOR12 were 0.047 and 0.01–13.57, and 4.00 and 0.011–135.13, respectively. Patients were dichotomised into two groups having low and high expressions by using the median value as the cutoff. A good agreement was noticed between *metastin* and *AXOR12* gene expression levels (kappa coefficient; 0.74). The presence of residual tumour following resection was negatively associated with *metastin* ($P = 0.0084$) and *AXOR12* ($P = 0.0148$) gene expressions indicating an association of low expression of these genes in more aggressive, and advanced tumours. By univariate Cox regression analysis, the prognosis of the patients with low *AXOR12* gene expression was significantly worse than those with high *AXOR12* gene expression ($P = 0.030$). The combination of *metastin* and *AXOR12* gene expression level was also significantly associated with the prognosis ($P = 0.049$). Transcripts for both metastin and AXOR12 were detected in the epithelial ovarian carcinoma cells.

Conclusions: These results present a new insight into the understanding of the biological behaviour of epithelial ovarian cancer. Metastin/AXOR12 signalling may suppress the invasive phenotype of epithelial ovarian cancer.

1. Introduction

KiSS-1 is a human metastasis suppressor gene,[1] which suppresses metastasis of human melanoma[2] and breast carcinoma[3] without affecting tumourigenicity. Ohtaki and colleagues[4] showed that *Kiss-1* encodes a carboxy-terminally amidated peptide with 54 amino-acid residues, which have been isolated from human placenta as the endogenous ligand

* *Corresponding author:* Tel.: +81 87 870 1578; fax: +81 87 870 1204.
E-mail address: hata@chs.pref.kagawa.jp (K. Hata).
0959-8049/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2007.03.004

of a G-protein-coupled receptor (named AXOR12 or hOT7T175) and is named as metastin. The binding of metastin to its receptor inhibits chemotaxis *in vitro*, enhances the expression and activity of focal adhesion kinase and inhibits the ability of meatstin receptor overexpressing melanoma cells to metastasise *in vivo*.[4] In another model, metastin inhibited chemotaxis, invasion, motility and growth of Chinese hamster ovary (CHO) cells designed to overexpress the metastin receptor, and attenuated pulmonary metastasis of hOT7T175-transfected B16–BL6 melanomas.[5]

Recently, a significant reduction in KiSS-1 or metastin expression has been reported in tumours with high metastatic potential.[6–10] Moreover, reduced KiSS-1 expression became a strong prognostic marker in patients with urinary bladder cancer[9] and gastric carcinoma.[10] These findings may open the possibility of future clinical application of these proteins, KiSS-1, metastin, and AXOR12, for prevention of cancer invasion and metastasis, and thus may improve patient prognosis. These promising results provoked us to evaluate the expression of these genes and their prognostic impact on epithelial ovarian cancer, which is the fourth most common cause of cancer death in women and the most common cause of death in women dying from a gynaecologic tumour.[11]

In this study, we sought to determine mRNA expression of metastin and AXOR12 using the real-time quantitative reverse transcription-polymerase chain reaction (RT- PCR) in cases of epithelial ovarian cancer. The gene expression of metastin and AXOR12 was correlated with clinicopathological parameters and their impact on patient survival was evaluated. Moreover, cellular distribution of metastin and AXOR12 mRNA was examined by *in situ* hybridisation.

2. Patients and methods

2.1. Patients

Patients with epithelial ovarian cancer treated between January 1990 and December 2005 at the Kinki University Hospital, Osaka-Sayama, Japan, were included in this study. Eligible patients had a histological diagnosis of primary epithelial ovarian cancer, and were suitable for adequate surgical staging. Patients were excluded from this study when surgically resected specimens were not available, had undergone any kind of preoperative therapy, had cancers other than ovarian cancer or had severe complications. All research was conducted with patients' informed consent to have their tissue banked for future unspecified studies. The present study conformed with the ethical standards of the Helsinki declaration of World Medical Association. The median age of the 76 eligible patients was 56 years (range, 31–84 years). Twenty-one of them were premenopausal. Patients were staged according to the 1987 criteria recommended by FIGO.[12] There were 34 stage I patients, 3 stage II patients, 35 stage III patients and 4 stage IV patients. The staging system defined by FIGO, as described elsewhere[13,14], assumes that an adequate staging operation has been performed. Tumours were classified histologically according to the World Health Organization (WHO) criteria[15] as serous ($n = 39$), mucinous ($n = 18$), endometrioid ($n = 10$), clear cell ($n = 8$) and transitional cell ($n = 1$). The tumours were classified histologically as either having well differentiated ($n = 46$) or being moderately differentiated ($n = 14$), or poorly differentiated ($n = 7$).[16] The number of poorly differentiated tumours is smaller than that of well differentiated tumours. This seems to be unusual compared to European series. However, this is a typical population in Japanese ovarian cancers.[13,14,17]

The surveillance for recurrent disease usually consisted of physical examination, Papanicolaou smear and serology with tumour marker examination (e.g. CA 125, CA 19-9, carcinoembryonic antigen, sialyl Tn) every month for the first year, every 2 months for the second and third years, and every 3 months for the fourth and fifth years. After 5 years, the patients were examined semiannually. A chest radiograph and CT scan or sonogram were obtained every 6 months for 5 years after surgery and every year thereafter, and if necessary MRI was performed. Recurrent disease was confirmed either pathologically or radiographically or serologically. Follow-up information was obtained from medical record, letter or telephone contact with patients, and information from referring physician. Survival data were available for all patients (median follow-up 36.5 months, range 4–196 months). Of these, 73 patients received platinum and/or paclitaxel-based chemotherapy. Two patients with stage Ia tumours of endometrioid adenocarcinoma and mucinous cystadenocarcinoma, and one with stage IV tumour of serous cystadenocarcinoma had no further treatment after surgery.

2.2. Tissue specimen and RNA preparation

Fresh surgical specimens from all patients were obtained. A dissecting microscope was used to avoid any contamination of cancerous tissue with non-cancerous tissue material. The tissue samples were stored at −80 °C for subsequent quantification of mRNA expression.

2.3. RNA preparation and real-time quantitative RT-PCR procedure

Total RNA was isolated from frozen tissues using a commercially available extraction method (Isogen; Nippon Gene Inc., Tokyo, Japan).

Complementary DNA (cDNA) was prepared by random priming from 1000 ng of total RNA using a First-Strand cDNA Synthesis kit (Pharmacia-LKB, Uppsala, Sweden). We performed real-time quantitative PCR using the TaqMan system (Applied Biosystems). The expression levels of each gene (*metastin* and *AXOR12*) and internal reference glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) were measured by multiplex PCR using TaqMan probes labelled with 6-carboxyfluorescein (FAM) or VIC, respectively. The primers and TaqMan probes were designed using Primer Express v 2.0 software (Applied Biosystems). The sequences of each primer and TaqMan probe (forward primer, reverse primer, TaqMan probe) were *metastin*, 5′-GCAGGTCCTTCTCCCGCT-3′, 5′-GCCAGATCCCCGCACC-3′, 5′-CACCAGCACCGCGCCCTG-3′; *AXOR12*, 5′-TGGCACCCACGCAGCTA-3′, 5′- AGTTGCTGTAGGACATGCAGTGA-3′, 5′-CCGCCTACGCGCTTAAGACCTGG-3′. We purchased the Pre-Developed TaqMan Assay Reagents, GAPDH primer/probe set from Applied Biosystems. Real-time PCR

amplification and product detection was performed using an ABI PRISM 7300 Sequence Detection System (Applied Biosystems) as recommended by the manufacturer. The simultaneous measurement of each gene-FAM and GAPDH-VIC permitted normalisation of the amount of cDNA added per sample. The quantity of cDNA for each experimental gene was normalised to the quantity of GAPDH cDNA in each sample. Relative expression was determined by using the ΔΔCt (threshold cycle) method according to the manufacturer's protocol (User Bulletin #2). Each assay included a standard curve sample in duplicate, a no-template control and a cDNA sample from the tumour specimen in triplicate. All samples with a coefficient of variance higher than 10% were retested. Furthermore, the sequences of PCR products were analysed and they were identical to the sequence of each gene.

2.4. *In situ hybridisation*

To localise metastin and AXOR12 mRNA, *in situ* hybridisation technique was employed. Parraffin-embedded sections were used for *in situ* hybridisation. Serial sections were used from each patient for sense and antisense probe. A digoxigenin-labelled sense and antisense RNA probe was transcribed by T3 and T7 RNA polymerase, respectively, with a DIG RNA labelling kit according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). Hybridisation and the immunohistochemical steps were done as we described previously.[10,18] Placental tissue served as a positive control.

2.5. *Statistical analysis*

Kappa statistic was used as a measure of agreement between *metastin* and *AXOR12* gene expression. The kappa coefficient values of up to 0.40 were considered to indicate poor agreement; values between 0.41 and 0.75, moderate to good agreement; and values greater than 0.75, excellent agreement.[19] Mann–Whitney *U* test and Kruskal–Wallis one-way analysis of variance by ranks were used as appropriate for the evaluation of differences between end-points. The Cox proportional hazards model was used in survival analysis. Maximum likelihood parameter estimates and likelihood ratio statistics (LRS) in the Cox proportional hazards models were obtained with the use of a statistical package, EPICURE.[20] Kaplan–Meier curves were compared by the univariate Cox regression analysis. All *P* values presented were two-sided. A *P* value of less than 0.05 was considered significant.

3. Results

3.1. *Each gene expression and clinicopathological features*

The median and range of mRNA expression were 0.047 and 0.01–13.57, and 4.00 and 0.011–135.13 for metastin and AXOR12, respectively. The patients were divided into low or

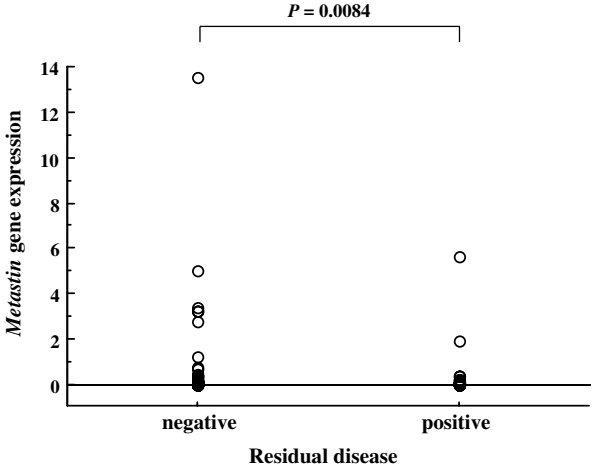

Fig. 1A – *Metastin* gene expression in patients with negative and positive residual disease.

Table 1 – Clinicopathological features and gene expression

Clinicopathological features	Metastin gene expression median (range)	*P*-value	AXOR12 gene expression median (range)	*P*-value
Age at the time of diagnosis		0.0058		0.1069
<56 (*n* = 36)	0.119 (0.001–5.689)		5.683 (0.054–32.309)	
⩾56 (*n* = 40)	0.021 (0.001–13.566)		3.205 (0.011–36.116)	
FIGO stage		0.0539		0.1279
I–II (*n* = 37)	0.071 (0.001–13.566)		4.529 (0.133–33.588)	
III–IV (*n* = 39)	0.021 (0.001–5.689)		3.056 (0.011–36.116)	
Residual disease		0.0084		0.0148
Negative (*n* = 43)	0.097 (0.001–13.566)		4.853 (0.133–36.116)	
Positive (*n* = 33)	0.010 (0.001–5.689)		2.560 (0.011–25.007)	
Histological subtype		0.0832		0.071
Serous (*n* = 39)	0.014 (0.001–3.424)		1.651 (0.033–36.116)	
Mucinous (*n* = 18)	0.160 (0.001–5.689)		5.585 (0.011–23.375)	
Endometrioid (*n* = 10)	0.073 (0.001–3.256)		10.341 (0.054–33.588)	
Clear cell (*n* = 8)	0.139 (0.003–13.566)		11.413 (0.133–23.869)	
Histological grade		0.2289		0.1575
Well differentiated (*n* = 46)	0.054 (0.001–5.689)		4.258 (0.011–33.588)	
Moderately differentiated (*n* = 14)	0.009 (0.001–1.963)		3.414 (0.033–36.116)	
Poorly differentiated (*n* = 7)	0.003 (0.001–0.049)		1.856 (0.054–4.441)	
Unclassified (*n* = 9)	0.133 (0.003–13.566)		6.838 (0.133–23.869)	

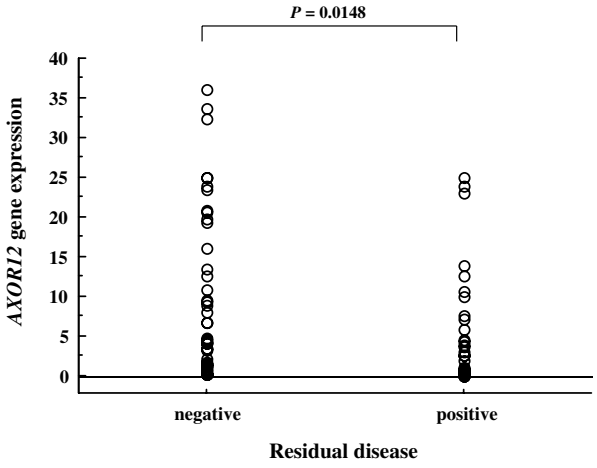

Fig. 1B – A G-protein-coupled receptor (AXOR12) gene expression in patients with negative and positive residual disease.

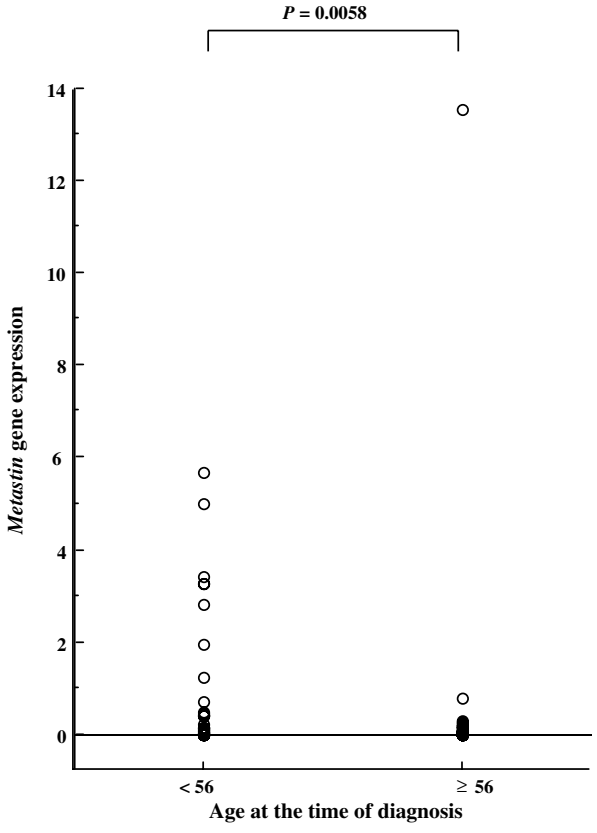

Fig. 1C – *Metastin* gene expression according to the age at diagnosis (<56 versus ⩾56).

high groups for *metastin* and AXOR12 gene expressions using the median value as the cutoff, respectively. A good agreement was noted between *metastin* and AXOR12 gene expression levels (kappa coefficient; 0.74). The values of *metastatin* and AXOR12 gene expressions in ovarian cancers are classified according to patients' age at diagnosis, stage of disease, presence or absence of residual tumour mass after initial surgery, histological subtype and grade (Table 1). The presence of residual tumour was negatively associated with *metastin* ($P = 0.0084$) (Fig. 1A) and AXOR12 ($P = 0.0148$) (Fig. 1B) gene expressions, respectively. The patients' age at diagnosis was significantly associated with *metastin* gene expression ($P = 0.0058$) (Fig. 1C).

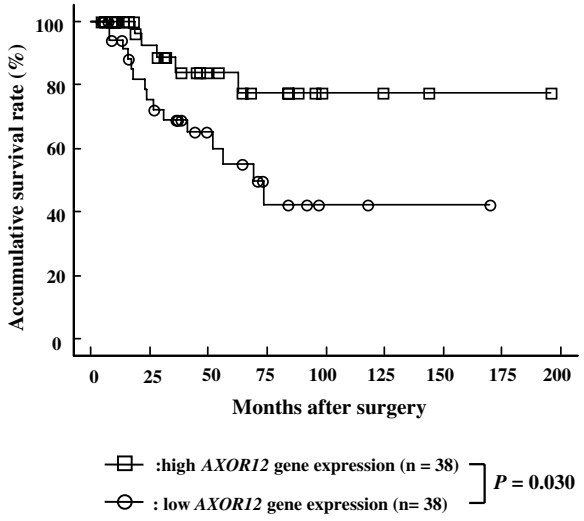

Fig. 2A – Comparison of survivals between groups with a high G-protein-coupled receptor (AXOR12) gene expression and low AXOR12 gene expression according to univariate Cox regression analysis.

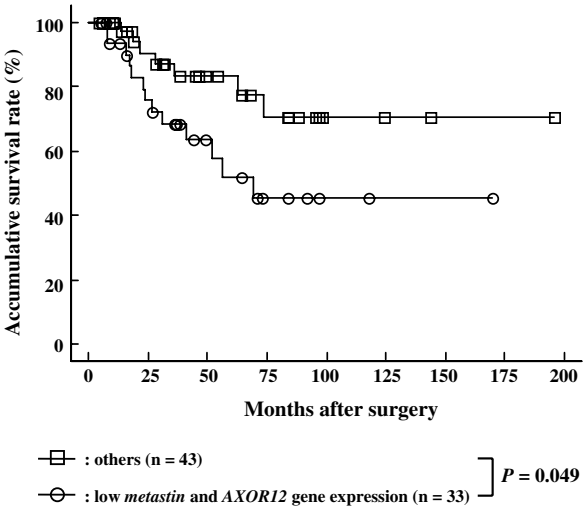

Fig. 2B – Comparison of survivals between groups with both low *metastin* and a G-protein-coupled receptor (AXOR12) gene expression, and others according to univariate Cox regression analysis.

3.2. *Each gene expression and prognosis*

As shown in Fig. 2A, we found the prognosis of the patients with low AXOR12 gene expression to be significantly worse than that of those with high AXOR12 gene expression by univariate Cox regression analysis ($P = 0.030$). *Metastin* gene

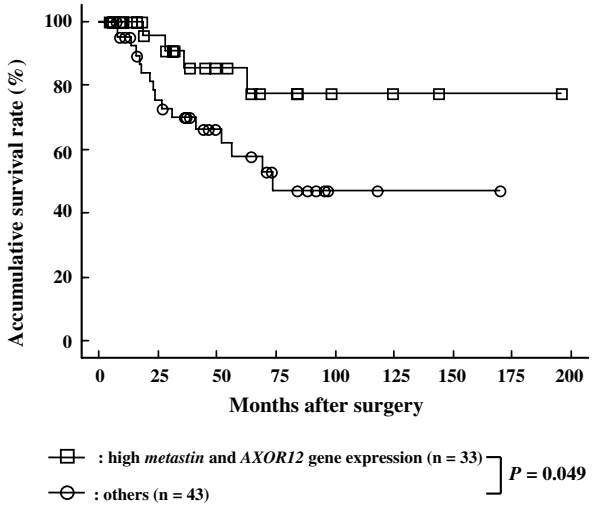

Fig. 2C – Comparison of survivals between groups with both high *metastin* and *a G-protein-coupled receptor (AXOR12)* gene expression, and others according to univariate Cox regression analysis.

Table 3 – The results of multivariate Cox regression analysis

Variables	Hazard ratio	95% confidence interval	P-value
FIGO stage			
I–II (n = 37)	Referent		
III–IV (n = 39)	12.08	2.39–61.16	0.003
Histological grade			
Others (n = 69)	Referent		
Poorly (n = 7)	3.08	1.13–8.41	0.028
AXOR12 gene expression ratio			
High (n = 38)	Referent		
Low (n = 38)	0.35	0.03–4.92	0.439
Combination of metastin and AXOR12 gene expression ratio			
Others (n = 43)	Referent		
Both low (n = 33)	1.44	0.27–7.64	0.668
Both high (n = 33)	Referent		
Others (n = 43)	5.26	0.50–55.21	0.167

expression itself had no impact on patient survival, however, combination of *metastin* and *AXOR12* gene expression had significant impact on patient prognosis (Figs. 2B and 2C). Moreover, FIGO stage (stages III–IV; $P = 0.001$), residual disease (positive; $P = 0.0004$) and histological grade (poorly; $P = 0.0005$) were found to be significantly associated with a poor prognosis in univariate Cox regression analysis (Table 2). Older age at the time of diagnosis and serous tumours type are generally thought to be more aggressive.[21] However, no significant association for these variables could be found in this study (Table 2). Multivariate Cox regression analysis revealed that FIGO stage (III–IV; $P = 0.003$) and histological grade (poorly; $P = 0.028$) are the independent prognostic factors in this series (Table 3).

3.3. *In situ* hybridisation

Transcripts for *metastin* and *AXOR12* were detected in the syncytiotrophoblasts of placental tissue as positive controls, respectively (Fig. 3). Transcripts for *metastin* and *AXOR12* were

Table 2 – The results of univariate Cox regression analysis

Variables	Hazard ratio	95% Confidence interval	P-value
Age at the time of diagnosis value	1.02	0.98–1.06	0.244
FIGO stage			
I–II (n = 37)	Referent		
III–IV (n = 39)	11.50	2.64–50.01	0.001
Residual disease			
Negative (n = 43)	Referent		
Positive (n = 33)	14.34	3.31–62.14	0.0004
Histological subtype			
Others (n = 37)	Referent		
Serous (n = 39)	1.10	0.45–2.69	0.838
Histological grade			
Others (n = 69)	Referent		
Poorly (n = 7)	5.29	2.07–13.49	0.0005
Metastin gene expression ratio			
High (n = 38)	Referent		
Low (n = 38)	2.32	0.89–6.05	0.084
AXOR12 gene expression ratio			
High (n = 38)	Referent		
Low (n = 38)	3.06	1.11–8.43	0.030
Combination of metastin and AXOR12 gene expression level			
Others (n = 43)	Referent		
Both low (n = 33)	2.52	1.004–6.33	0.049
Both high (n = 33)	Referent		
Others (n = 43)	3.00	1.004–8.99	0.049

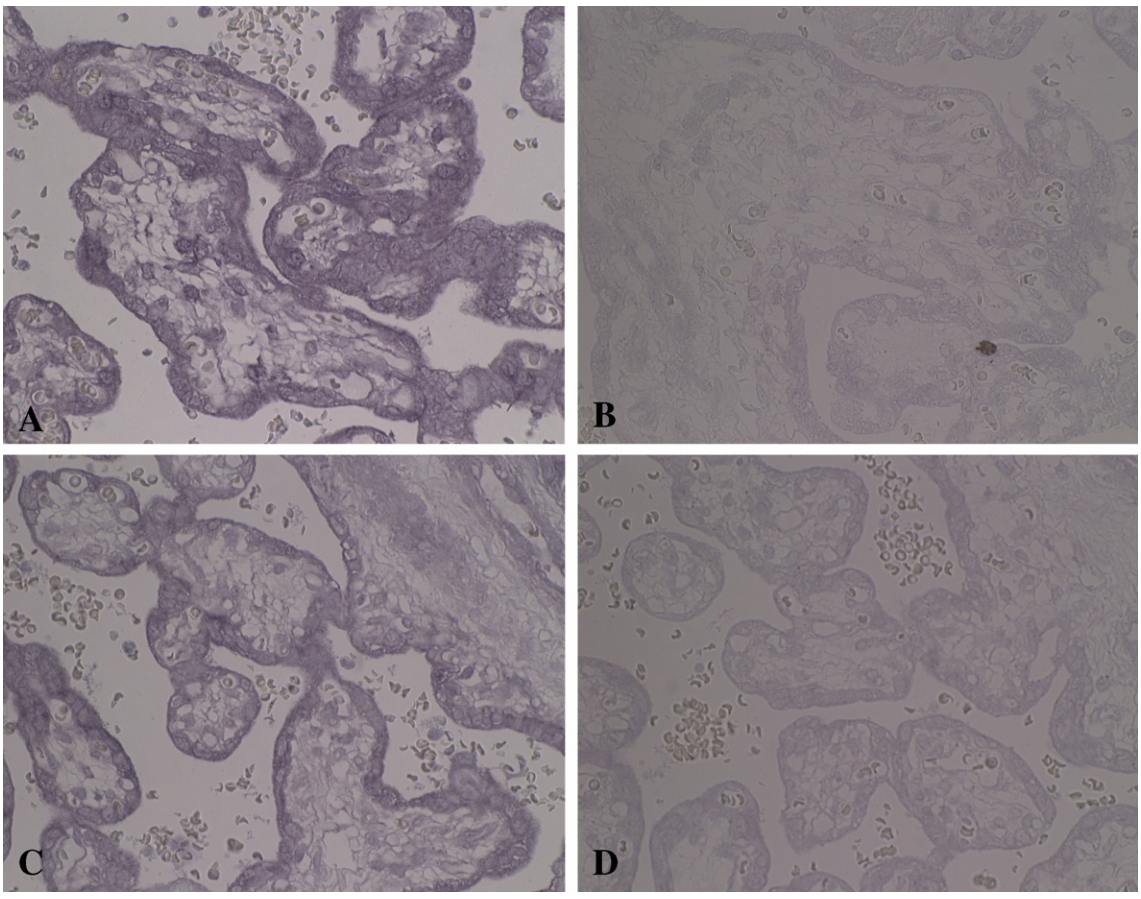

Fig. 3 – *In situ* hybridisation analysis of metastin and a G-protein-coupled receptor (AXOR12) expression in a section of the placenta. Metastin (A, original ×400) and AXOR12 (C, original ×400) mRNA-expressing cells are found in the syncytiotrophoblasts, respectively. In metastin (B, original ×400) and AXOR12 (D, original ×400) sense-control hybridisation, only background colour with no distinction is observed, respectively.

observed in the epithelial ovarian carcinoma cells, respectively (Fig. 4).

4. Discussion

The predicted KiSS-1 proteins consist of 145 amino acids, with a secretory signal sequence located at the N-terminus, suggesting that KiSS-1 functions as a secretory protein.[22] However, the full-length KiSS-1 protein has not been detected in a secreted form. Instead, three truncated fragments of KiSS-1 occur naturally in human placenta and are termed as metastin (54 amino acids), kisspeptin-14 (14 amino acids) and kisspeptin-13 (13 amino acids).[4] Furthermore, metastin was identified as a ligand for an orphan G-protein-coupled receptor, designated as AXOR12.[23] Jiang and colleagues[24] reported the differential expression of KiSS-1 and AXOR12 in human ovarian cancer cell lines. SKOV3 cells expressed AXOR12, but lacked the expression of KiSS-1. They established KiSS-1 infected SKOV3 cell line, and found that KiSS-1 expression inhibited the migration of SKOV3 cells and reduced colony formation of SKOV3 cells without affecting cell proliferation.[24] These results suggest that KiSS-1 serves as a metastasis suppressor for ovarian cancer. In this study, we evaluated the expression level of *metastin* and *AXOR12* genes in epithelial ovarian cancer, and a good agreement was noted between *metastin* and *AXOR12* gene expression levels (kappa coefficient; 0.74). Moreover, high *AXOR12* gene expression and high expression of both *metastin* and *AXOR12* genes significantly were associated with the improved patient prognosis in this study. Metastin/AXOR12 signalling might suppress the tumour aggressive phenotype in epithelial ovarian cancer. Similar results have been reported in melanoma,[6] thyroid cancer,[7] oesophageal carcinoma,[8] urinary bladder cancer[9] and gastric carcinoma.[10]

More recently, Martin and colleagues[25] noted that KiSS-1 expression is increased in human breast cancer, particularly in patients with aggressive tumours and with mortality. Also, it has been reported that Kiss-1 promotes metastasis in a human breast cancer cell line in an *in vitro* study. These results are in direct contrast to a number of previous studies[6–10] and show that KiSS-1 plays a role beyond the initial metastasis repressor in breast cancer. Ikeguchi and colleagues[26] examined the clinical importance of KiSS-1 and its receptor gene expression in hepatocellular carcinoma. They evaluated 60 surgically resected carcinomas using real-time quantitative RT-PCR and found that there was no loss of KiSS-1 in carcinomas compared to non-cancerous cirrhotic livers. Conversely, they found a high expression of the receptor in

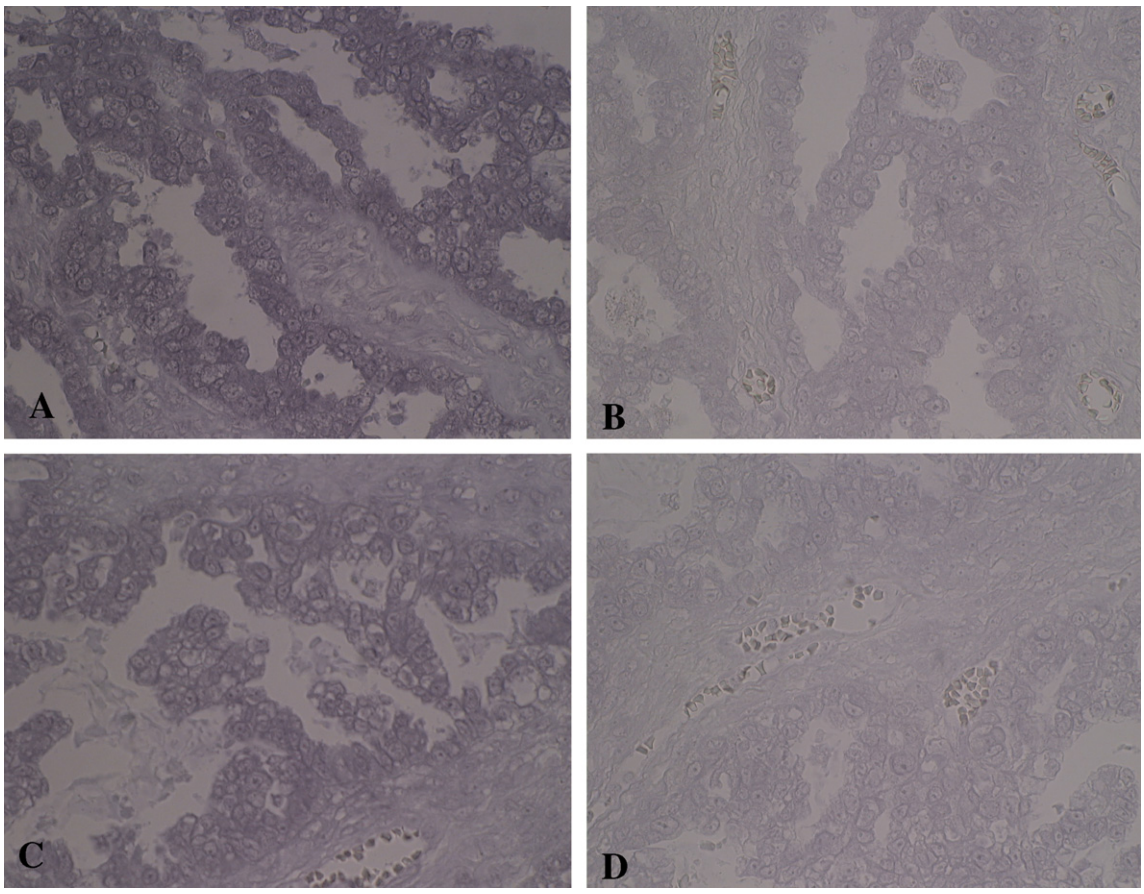

Fig. 4 – Localisation of analysis of *metastin* and a G-protein-coupled receptor (*AXOR12*) in a section of clear cell carcinoma by *in situ* hybridisation. Metastin (A, original ×400) and AXOR12 (C, original ×400) mRNA-expressing cells are found in the epithelial ovarian carcinoma cells, respectively. In metastin (B, original ×400) and AXOR12 (D, original ×400) sense-control hybridisation, only background colour with no distinction is observed, respectively.

the carcinomas. There was over-expression of KiSS-1 and its receptor in six tumours of advanced stage and those patients had poor survival. These authors concluded that over-expression of KiSS-1 and its receptor was frequently observed and correlated with disease progression.[26] It appears that although KiSS-1 may be a possible metastasis suppressor in melanoma,[6] thyroid cancer,[7] oesophageal carcinoma,[8] urinary bladder cancer,[9] gastric carcinoma,[10] and epithelial ovarian cancer, however, this is not always true as it was found in breast and hepatocellular cancers. Further research is necessary before the true role and effect of Metastin/AXOR12 signalling in each tumour can be elucidated.

KiSS-1 peptides, such as metastin and its receptors were highly expressed in placenta,[4,23] and metastin was isolated from human placental extracts.[4] Metastins were detected in plasma, and their concentrations dramatically increased under certain physiological conditions, such as pregnancy. Histochemical studies detected metastin mRNA in human placenta and immunoreactivity in the syncytiotrophoblasts.[27] These data may indicate that metastin is a novel placenta-derived hormone in humans. There are striking similarities between the behaviour of invasive placental cells and the invasive cancer cells.[28,29] Like tumour cells, cytotrophoblastic cells migrate through and invade the uterine wall at the time of implantation. Unlike tumour invasion, this unique interaction between genetically dissimilar trophoblasts and uterine cells is closely regulated and is limited both temporally and spatially by mechanisms that are largely unknown. Considering the localisation of mRNA for metastin and AXOR12 in syncytiotrophoblasts confirmed in the present study and a dramatic elevation of plasma metastin concentration in the first trimester of pregnancy,[27] it is possible that metastin/AXOR12 signalling may be involved in the negative regulation of trophoblast invasion because unlike the tumour cells trophoblasts never metastasise to distant location. In this study, transcripts for metastin and AXOR12 were detected in the epithelial ovarian carcinoma cells, respectively, by *in situ* hybridisation analysis. Similarly, it might be possible that high expression of both *metastin* and *AXOR12* genes in epithelial ovarian cancer cells, as detected in this study, is responsible for the inhibition of cellular invasion and metastasis; however, this speculation is still putative.

The real-time quantitative RT-PCR method we used for the determination of *metastin* and *AXOR12* gene expressions is convenient because it does not require radioisotopes or relatively large amounts of tumour tissues, and is reliable and accurate. Even biopsy samples could be used for an accurate evaluation of *metastin* and *AXOR12* gene expressions. The

real-time quantitative RT-PCR detection method of these genes might serve as a tool to diagnose the high-risk group of patients with epithelial ovarian cancer who might have worse prognosis. Also the expression pattern of these genes may provide a new insight to understand the biology of epithelial ovarian cancer. Further investigation is necessary in a large number of epithelial ovarian cancer patients before the findings of the present study would be considered for clinical application.

Conflict of interest statement

None declared.

REFERENCES

1. Lee JH, Miele ME, Hicks DJ, et al. Kiss-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst* 1996;**88**:1731–7.
2. Lee JH, Welch DR. Identification of highly expressed genes in metastasis-suppressed chromosome 6/human malignant melanoma hybrid cells using subtractive hybridization and differential display. *Int J Cancer* 1997;**71**:1035–44.
3. Lee JH, Welch DR. Suppression of metastasis in human breast carcinoma MDA-MB-435 cells after transfection with the metastasis suppressor gene, KiSS-1. *Cancer Res* 1997;**57**:2384–7.
4. Ohtaki T, Shintani Y, Honda S, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* 2001;**411**:613–7.
5. Hori A, Honda S, Asada M, et al. Metastin suppresses the motility and growth of CHO cells transfected with its receptor. *Biochem Biophys Res Commun* 2001;**286**:958–63.
6. Shirasaki F, Takata M, Hatta N, Takehara K. Loss of expression of the metastasis suppressor gene KiSS1 during melanoma progression and its association with LOH of chromosome 6q16.3-q23. *Cancer Res* 2001;**61**:7422–5.
7. Ringel MD, Hardy E, Bernet VJ, et al. Metastin receptor is overexpressed in papillary thyroid cancer and activates MAP kinase in thyroid cancer cells. *J Clin Endocrinol Metab* 2002;**87**:2399–402.
8. Ikeguchi M, Yamaguchi K, Kaibara N. Clinical significance of the loss of Kiss-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in esophageal squamous cell carcinoma. *Clin Cancer Res* 2004;**10**:379–83.
9. Sanchez-Carbayo M, Capodieci P, Cordon-Cardo C. Tumor suppressor role of KiSS-1 in bladder cancer: loss of KiSS-1 expression is associated with bladder cancer progression and clinical outcome. *Am J Pathol* 2003;**162**:609–17.
10. Dhar DK, Naora H, Kubota H, et al. Downregulation of Kiss-1 expression is responsible for tumour invasion and worse prognosis in gastric carcinoma. *Int J Cancer* 2004;**111**:868–72.
11. Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. *CA Cancer J Clin* 2005;**55**:10–30.
12. International Federation of Gynecology and Obstetrics (FIGO). Changes in definitions of clinical staging for carcinoma of the cervix and ovary. Am J Obstet Gynecol 1987;156:263–4.
13. Hata K, Kamikawa T, Arao S, et al. Expression of the thymidine phosphorylase gene in epithelial ovarian cancer. *Br J Cancer* 1999;**79**:1848–54.
14. Hata K, Fujiwaki R, Nakayama K, Miyazaki K. Expression of endostatin gene in epithelial ovarian cancer. *Clin Cancer Res* 2001;**7**:2405–9.
15. Serov SF, Scully RE, Sobin LH. *International histological classification of tumours, no. 9: histological typing of ovarian tumours*. Geneva: World Health Organization; 1973.
16. Arao S, Suwa H, Mandai M, et al. Expression of multidrug resistance gene and localization of P-glycoprotein in human primary ovarian cancer. *Cancer Res* 1994;**54**:1355–9.
17. Hata K, Nakayama K, Fujiwaki R, Katabuchi H, Okamura H, Miyazaki K. Expression of the angiopoietin-1, angiopoietin-2, Tie2, and vascular endothelial growth factor gene in epithelial ovarian cancer. *Gynecol Oncol* 2004;**93**:215–22.
18. Nagasue N, Dhar DK, Yamanoi A, et al. Production and release of endothelin-1 from the gut and spleen in portal hypertension due to cirrhosis. *Hepatology* 2000;**31**:1107–14.
19. Landis JR, Koch GG. The measurement of observer agreement for categorical data. *Biometrics* 1977;**33**:159–74.
20. Preston DL, Lubin JH, Pierce DA. *EPICURE: risk regression and data analysis software*. Seattle: HiroSoft International Corporation; 1990.
21. Beale PJ, Friedlander ML. Prognostic variables in ovarian cancer. In: Lawton FG, Neijt JP, Swenerton KD, editors. *Epithelial cancer of the ovary*. London: BMJ Publishing Group; 1995. p. 96–111.
22. Harms JF, Welch DR, Miele ME. KISS1 metastasis suppression and emergent pathways. *Clin Exp Metast* 2003;**20**:11–8.
23. Muir AI, Chamberlain L, Elshourbagy NA, et al. AXOR12, a novel human G protein-coupled receptor, activated by the peptide KiSS-1. *J Biol Chem* 2001;**276**:28969–75.
24. Jiang Y, Berk M, Singh LS, et al. KiSS1 suppresses metastasis in human ovarian cancer via inhibition of protein kinase C alpha. *Clin Exp Metast* 2005;**22**:369–76.
25. Martin TA, Watkins G, Jiang WG. KiSS-1 expression in human breast cancer. *Clin Exp Metast* 2005;**22**:503–11.
26. Ikeguchi M, Hirooka Y, Kaibara N. Quantitative reverse transcriptase polymerase chain reaction analysis for KiSS-1 and orphan G-protein-coupled receptor (hOT7T175) gene expression in hepatocelluar carcinoma. *J Cancer Res Clin Oncol* 2003;**129**:531–5.
27. Horikoshi Y, Matsumoto H, Takatsu Y, et al. Dramatic elevation of plasma metastin concentrations in human pregnancy: metastin as a novel placenta-derived hormone in humans. *J Clin Endocrinol Metab* 2003;**88**:914–9.
28. Murray MJ, Lessey BA. Embryo implantation and tumor metastasis: common pathways of invasion and angiogenesis. *Semin Reprod Endocrinol* 1999;**17**:275–90.
29. Bischof P, Meisser A, Campana A. Paracrine and autocrine regulators of trophoblast invasion – a review. *Placenta* 2000;**21**:S55–60.