Matrix Metalloproteinase 2 Is A Direct Target of The RAN-GTP Pathway And Mediated The Invasion, Migration And Metastasis of Human Breast Cancer Cells

Mohamed El-Tanani (✉ m.el-tanani@bradford.ac.uk)

University of Bradford Faculty of Life Sciences <https://orcid.org/0000-0002-4735-5445>

Yin-Fai Lee

University of Bradford School of Lifelong Education and Development: University of Bradford

Yusuf Haggag

Tanta University

Mark Sutherland

University of Bradford School of Lifelong Education and Development: University of Bradford

Shu-Dong Zhang

Ulster University - Coleraine Campus: Ulster University

Angela Plat-Higgins

University of Liverpool School of Biomedical Sciences: University of Liverpool Institute of Translational Medicine

Philip S Rudland

University of Liverpool School of Biomedical Sciences: University of Liverpool Institute of Translational Medicine

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Abstract

Background: Ras-related nuclear protein (RAN) causes increases in invasion *in vitro* and is associated with early breast cancer patients deaths *in vivo*. However, the underlying mechanism is unknown.

Methods: Effect of RAN expression on potential targets MMP2, ATF3, CXCR3 was measured by Real-Time PCR / Western blots. Effects of MMP2 and RAN expression on cancer cell lines (e.g. MDA-MB231) was measured by soft-agar, cell adhesion, and invasion assays. Correlation between MMP2 and RAN and patient survival times was examined in breast cancer patients.

Results: Knockdown of RAN led to reduction of MMP2 and its potential regulators ATF3 and CXCR3 in breast cancer cell lines. Knockdown of ATF3 or CXCR3 downregulates MMP2 without affecting RAN, indicating that RAN regulates MMP2 through ATF3 and CXCR3. Both knockdown of RAN and MMP2 reduced cell adhesion, migration and growth in agar invasion whilst overexpression of MMP2 reversed the knockdown of RAN. Moreover, the level of immunoreactive RAN and MMP2 are positively associated with each other and with patient survival times, respectively in breast cancer specimens, suggesting that a high level of RAN may be a pre-requisite for MMP2 overexpression.

Conclusions: Our results suggest that MMP2 expression can stratify progression of breast cancers with a high and low incidence of RAN and both RAN and MMP2 in combination can be used for accurate patient stratification of breast cancer metastasis.

Background

RAN is a small GTPase involved in various cellular processes including nucleocytoplasmic transport, mitotic spindle organization and nuclear envelope formation (1, 2).

Recently, we have shown that when an expression vector for RAN is transfected into benign, non-invasive breast Rama 37 cells, it has the ability to transform their phenotype, producing increased cell invasion *in vitro* and the induction of metastasis in syngeneic rats *in vivo* (3). Silencing RAN using small interfering RNAs reversed the induction of this metastatic phenotype (3). But how RAN induces these changes is unknown. Here we identify a matrix metalloproteinase (MMP), MMP2, and its upstream regulators, activating transcription factor 3 (ATF3) (4–10) and chemokine receptor 3 (CXCR3) (11, 12) that are targets for the RAN-induced increases in metastatic-related properties *in vitro* and show a significant association between RAN, MMP2 and survival time of breast cancer patients *in vivo*.

Worldwide, there are an estimated 1 million cases and 0.5 million deaths from breast cancer (BC) annually but the underlying mechanisms that cause metastasis and death are largely unknown. One molecule RAN is overexpressed in breast and other cancers, and its overexpression is a poor prognostic indicator (13, 14) and is correlated with increased aggressiveness of tumour cells *in vitro* and *in vivo* (3, 15, 16).

Methods And Materials

Transfection and infection

Infections were performed using GeneJuice® (Promega, Southampton, UK). Viral particles were harvested 48 hours post-transfection and were applied to the target cells with 6 µg/ml polybrene supplement for 4 hours. Same amount and same batch of viral particles were used for any comparisons made in present study. Cell lines were designated shRan for short hairpin RNA, shMMP2 for MMP2 and shScr for scrambled, noncoded shRNA (Sigma-Aldrich, Dorset, UK) (17).

Culture conditions

Cells were cultured in normal medium (17) until 24 hours post-infection. Cells were harvested at 72 hours post-infection for mRNA extraction and protein analyses, unless otherwise specified.

Real-Time polymerase chain (RT-PCR)

RNA was extracted using Trizol (Invitrogen, Paisley, UK) and reverse transcription was performed using SuperScript™ III first strand synthesis system (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed according to the

manufacturer's instructions (Applied Biosystem, Foster City, CA) using a Taqman® assay for RAN (Hs01044225_g1), for MMP2 (Hs01548727_m1), for ATF3 (Hs00231069_m1) and for CXCR3 (Hs01847760_s1), Applied Biosystem). Data are the mean of three independent experiments $\pm$ SD. All results were normalized using a housekeeping gene, β -actin.

Western blot

Total protein was extracted using N-Per kit from Pierce to visualise the antibodies (Supplementary Table 4). Data are the mean of three independent experiments $\pm$ SD. All results were normalized using a housekeeping gene, β -actin and quantified using densitometry readings.

Cell adhesion assay

For the cell adhesion assay, 40000 cells/well in normal medium were seeded in a 96-well plate coated with fibronectin and allowed to settle for 30 min. Suspended cells were removed by washing 4 times with PBS. Adhered cells were fixed and stained with crystal violet. The excess dye was washed out and the retained dye was extracted. The absorbance at 595nm was measured in a microplate reader. Data are the mean of three independent experiments $\pm$ SD.

Boyden chamber migration and invasion assays

Migration and invasion assays were performed as previously described (18). Briefly, 5000 and 50000 cells in serum-free conditions were seeded into the upper Boyden chamber (Millipore) on top of the membrane with or without a Matrigel coating, respectively, for migration and invasion assays. The cells were allowed to migrate/invade towards the underside for 24 hours with 10 ng/ml HGF as a chemoattractant. Cells on the underside of the membrane were fixed and stained with crystal violet solution. Data are the mean of three independent experiments $\pm$ SD.

Soft agar assay

Soft agar assay was performed as previously described (19). 5000 suspension cells in normal medium containing 0.35% (w/v) low-melting-point agarose were overlaid onto a solidified normal medium containing 0.7% (w/v) low-melting-point agarose. Cells were incubated at 37°C with 5% (v/v) carbon dioxide for 2 to 3 weeks. Colonies were visualized by staining with crystal violet and counted. Data are the mean of three independent experiments $\pm$ SD.

Patients and specimens. A retrospective study was undertaken using samples of 181 primary tumours from unselected breast cancer patients as described previously (de Silva Rudland *et al.*, 2011; Rudland *et al.*, 2010). Briefly, patients received no adjuvant therapy including hormonal therapy and only patients with operable breast cancer (T1-4, N0-1) were included. Patient follow-up times ranged from 14.5 to 19.4 years (mean 16.4 $\pm$ 0.1 years) with a mean $\pm$ SE survival time of 9.0 $\pm$ 0.5 years. Ethical approval was obtained from NRES Committee North West REC Ref 12/NW/0778, Protocol no. UoL000889, IRAS no 107845. Samples were preserved in neutral buffered formalin and embedded in paraffin wax as described previously (Rudland *et al.*, 2000).

IHC staining. Histological sections cut at 4 μ m were mounted on slides, treated with 0.05% v/v H₂O₂ in methanol to inhibit endogenous peroxidase (Rudland *et al.*, 2000) and incubated with the relevant primary and horseradish peroxidase labelled antibodies/polymers in kits (DAB) (Dako Ltd, Ely, UK), as described previously (de Silva *et al.*, 2011; Ismail *et al.*, 2017). Positive staining corresponded to an oxidised brown precipitate of diaminobenzidine (DAB). Slides were finally mounted in Glycergel mounting medium (Dako). Blocked antibodies prepared by mixing 1mg/ml of the relevant blocking peptide/protein abolished this staining. Appropriate immune serum also yielded no staining. Western blots of breast cell lines verified the specificity of all antibodies used by yielding the appropriately-sized molecular weight bands on SDS – polyacrylamide gels.

IHC scoring analysis. IHC-stained sections were analysed and scored by two independent observers using light microscopy according to the percentage of stained carcinoma cells from 2 well separated sections of each specimen, 10 fields per section at 200x magnification and a minimum of 200 cells per field, as described previously (de Silva Rudland *et al.*, 2011; Ismail *et al.*, 2017). Staining for all proteins had already been separated into two categorical groups, a negative and positive group with a cut-off of either 1% or 5% of carcinoma cells staining, according to which cut-off yielded the more significant difference and greater relative risks: 1% cut-offs for RAN, cMyc, Ki67, CK5/6 and 5% cut-offs for cMet, MMP2, ER α , c-erbB-2, PgR (de Silva Rudland *et al.*, 2011; Yuen *et al.*, 2012; 2013; 2016; Ismail *et al.*, 2017). The association of staining for each protein separately in this set of patients was calculated from life tables constructed from survival data using Kaplan Meier plots and analysed by Wilcoxon (Gehan) statistics (Rudland *et al.*, 2000). Patients who died from

causes other than cancer were censored. Unadjusted relative risk (RR) for survival with 95% confidence interval (95% CI) was calculated using Cox's univariate analysis (Rudland *et al.*, 2010). Association of IHC staining for RAN or MMP2 with other tumour variables was assessed by cross-tabulations using Fishers Exact test (2-sided) using either 1% or 5% cut-offs. For multiple comparisons the resultant *P* values were corrected by the Holm-Bonferroni formulae of $I-(I-P)^n$, where *n* is the number of tumour variables. Binary Logistic Regression was used for calculation of the relative independent association (RA) of staining for one protein with the remaining proteins in the group. To determine if the association of patient survival with RAN, MMP2 etc. was significant within a group of proteins, Cox's multivariate analyses were performed on 181 patients, incomplete data arose mainly from lack of sampling (de Silva Rudland *et al.*, 2011). Data analysis was performed using Excel (Microsoft, Redmond, WA), and SPSS version 22 (SPSS, Chicago, IL). The sensitivity and specificity, positive predictive response (PPR) and negative predictive response (NPP) were calculated and compared between 1% and 5% of RAN and MMP-2 cut-offs, respectively.

Statistical Analysis

Statistical analysis was performed using SPSS 19.0 software (IBM, Armonk, NY). Differences between groups in *in vitro* experiments were tested by Student's *t* test (two groups) and analysis of variance (ANOVA) with post hoc Games–Howell. Differences in expression levels between groups/ samples in the human specimens were analyzed by Chi², Fisher exact test, or Mann–Whitney *U* tests, where applicable. The association between the expression level and patient survival was recorded by Kaplan–Meier plots and compared by Wilcoxon–Gehan tests. A *P* value of less than 0.05 was considered statistically significant. All statistical tests were two-sided.

Results

Knockdown of RAN results in the downregulation of MMP2 in cancer cell lines

Previously, we have shown that knockdown of RAN using potent shRNA results in apoptosis and changes of cell properties including cell adhesion, migration and invasion (18, 19). Using these shRNAs, we now have found that silencing RAN by shRNA specific for RAN in the breast cancer cell line MDA-MB231 resulted in down-regulation of RAN and MMP2 mRNA and protein. MDA-MB231 cells were infected by scrambled shRNA (shScr) or RAN-shRNA (shRAN) which generated MDA-MB231-shScr or MDA-MB231-shRAN, respectively. MDA-MB231-shRAN cells displayed decreased mRNA levels of RAN and MMP2 by approximately 5 and 3 folds, respectively, compared to MDA-MB231-shScr controls (Fig. 1A & B; *P* < 0.001). Immunoblot analysis demonstrated that both RAN and MMP2 proteins were reduced in MDA-MB 231-shRAN cells compared to scrambled MDA-MB231-shScr cells by similar folds to their mRNAs of 5.5 and 2.7-fold, respectively (Fig. 1C; *P* < 0.0001).

Reduction of MMP2 expression by RAN knockdown reduces cell adhesion and invasion

In a previous study MDA-MB231-shRAN cells resulted in a significant decrease in cell adhesion and colony formation compared to MDA-MB231-shScr control cells (17–19). In this study MDA-MB231-shMMP2 cells produced a significant decrease in MMP2 by 2.7 fold (Fig. 2A) and in cell adhesion by 52% and in colony formation in agar by 68% compared to MDA-MB231-shScr control cells (Fig. 2B & 2C, *P* < 0.05).

Immunoblots demonstrated that overexpression of MMP2 increased MMP protein by 3.9 fold without affecting levels of RAN in MDA-MB231 vector control cells (PBabe + shscr) (Fig. 2C; *P* < 0.0001). This elevated level of vector-produced MMP2 was maintained in MDA-MB231 shRAN cells in which RAN was reduced by 3.3-fold (*P* = 0.0001). When RAN levels were reduced in MDA-MB231-shRAN cells, there was a significant decrease in cell adhesion by nearly 5-fold (Fig. 2D, *P* < 0.01) and in invasion by 4-fold compared to scrambled shRNA transfected cells (Fig. 2E, *P* < 0.01). When MMP2 was overexpressed in MDA-MB231-shRAN cells, there was no significant change in cell adhesion (Fig. 2D, *P* > 0.05) and in cell invasion (*P* > 0.05) compared to control shScr cells.

We then further investigated the importance of MMP2 in two more breast cancer cell lines, MCF-7 and T47D was investigated. Knockdown of MMP2 in these two breast cancer cell lines (Fig. 3A and C, *P* < 0.0001) resulted in a statistically significant decrease in their ability to produce MMP2 by 3.9-fold and by 2-fold, respectively, and to form colonies in soft agar by 6.6 and 7.1 fold, respectively (Fig. 3B and D, *P* < 0.05). These results suggest that expression of MMP2 is important for these breast cancer cell lines to maintain their tumorigenic properties *in vitro*. RAN knockdown reduced the number of colonies growing in soft agar for MCF-7-shRAN cells compared

to vector alone shScr controls by nearly 40% (Fig. 3E, $P < 0.0001$). When the MCF-7-shRan cells were overexpressing MMP2 (Fig. 3E), there was no significant change in cell adhesion and in the number of colonies in soft agar compared to the vector alone controls (Fig. 3F, $p > 0.5$). This result showed that knockdown of RAN led to reduced colony formation, but this reduction could be overcome when the levels of MMP2 were raised. Collectively, our results suggest that RAN precedes MMP2 in a signalling pathway which controls, in part, the invasive properties of breast cancer cells.

Mechanism Of Regulation Of Mmp2 By Ran

We have investigated whether silencing of RAN can lead to down-regulation of MMP2 upstream regulatory genes of ATF3 (20) and/or CXCR3 (21) in RAN knocked-down MDA-MB231. MDA-MB231-shRAN cells showed that levels of ATF3 and CXCR3 mRNAs decreased significantly by 5.5 folds compared to those in the MDA-MB231-shScr control cell line (Fig. 4A, $P < 0.001$). Immunoblots also demonstrated that silencing RAN led to reduced levels of ATF3, CXCR3 and MMP2 proteins by 3.0, 3.0, and 3.9-folds, respectively, in MDA-MB231-shRAN cells compared to scrambled MDA-MB 231-shScr cells (Fig. 4B; $P < 0.0001$). When CXCR3 was silenced in MDA-MB231-shCXCR3 cells, CXCR3 mRNA decreased by nearly 7-fold ($P < 0.05$), but there was no significant change in ATF3 mRNA ($P > 0.05$) (Fig. 4C). Similarly, when CXCR3 was silenced in MDA-MB231-shCXCR3 cells, the MMP2 protein decreased by 3.0-fold (Fig. 4D, $p < 0.01$), but there was no significant change in ATF3 protein levels (Fig. 4D, $p > 0.05$). When ATF3 was silenced in MDA-MB231-shATF3 cells by about 7-fold, there was a significant decrease in both MMP2 and CXCR3 mRNA levels by 60% and 67%, respectively (Fig. 4E). Similarly silencing ATF3 led to reduced levels of ATF3, CXCR3 and MMP2 proteins by 2.8, 3.0, and 2.4-folds, respectively, in MDA-MB231-shATF3 cells compared to scrambled MDA-MB231-shScr cells (Fig. 4F; $P < 0.0001$). These results suggest that RAN probably reduced MMP2, at least in part, by reducing firstly ATF3 and then CXCR3.

Association of RAN-related molecules with patient survival times in human breast cancer

Next, we investigated the relationship in human breast cancer between RAN and other potential prognostic molecules/markers with patient demise probably as a result of metastasis. To simplify subsequent statistical analyses, the individual carcinoma cell staining groups for each molecule were separated into two categorical groups using previously-determined cut-offs of 1% or 5% to separate those which were the most significantly different in patient survival times (Materials & Methods). The largest significant differences in relative risk (RR) in this group of 181 patients were as follows:

RAN ($\chi^2 = 35.4$, RR = 14.9), cMet ($\chi^2 = 32.9$, RR = 10.7), cMyc ($\chi^2 = 40.3$, RR = 9.5), MMP2 ($\chi^2 = 64.8$, RR = 7.7) and CK5/6 ($\chi^2 = 43.3$, RR = 5.6) (SupplementaryTable 1). Stainings for ER α ($\chi^2 = 1.24$, RR = 0.81), c-erbB-2 ($\chi^2 = 1.93$, RR = 1.33) and Ki67 ($\chi^2 = 1.6$, RR = 1.3) were not significantly different in this group of patients, although they were significant in a larger patient group (de Silva Rudland et al, 2011). By comparison this group of patients either with or without tumour involvement of lymph nodes showed a significant RR of 2.3 ($\chi^2 = 14.64$, 1df, $P < 0.001$), those groupings based on tumour size and histological grade were not significantly different (SupplementaryTable 1).

Table 1
Probability of independent association of staining for RAN and other molecular markers

Test ^a variable	Other ^b variables	Coeff β^c	SE of β^c	χ^2^d	P ^e	RA ^f	95% CI ^f
RAN	cMet	1.221	0.536	5.185	0.023	3.39	1.19–9.69
	cMyc	0.677	0.521	1.687	0.194	1.97	0.71–5.47
	MMP2	1.099	0.628	3.064	0.080	3.00	0.88–10.27
	Ki67	0.117	0.493	0.056	0.813	1.12	0.43–2.96
cMet	RAN	1.228	0.525	5.462	0.019	3.41	1.22–9.56
	cMyc	0.866	0.498	3.018	0.082	2.38	0.90–6.31
	MMP2	2.067	0.566	13.327	< 0.001	7.9	2.60–23.96
	Ki67	0.488	0.484	1.015	0.314	1.63	0.63–4.21
cMyc	RAN	0.697	0.515	1.833	0.176	2.01	0.73–5.50
	cMet	0.852	0.504	2.856	0.091	2.34	0.87–6.30
	MMP2	1.644	0.550	8.923	0.003	5.18	1.76–15.22
	Ki67	0.079	0.469	0.029	0.866	1.08	0.43–2.72
MMP2	RAN	0.982	0.627	2.451	0.117	2.67	0.78–9.13
	cMet	2.047	0.571	12.869	< 0.001	7.74	2.53–23.70
	cMyc	1.587	0.553	8.238	0.004	4.89	1.65–14.46
	Ki67	0.305	0.468	0.424	0.515	1.36	0.54–3.40
^a Principle IHC-staining variable for probability of association with other tumour variables using cut-offs defined in Tables 1,2.							
^b Sets of other IHC-staining variables were included in binary Logistic Regression Analysis using cut-offs defined in Tables 1,2 to separate positive and negative staining groups.							
^c Value of coefficient β (Coeff β) with its standard error (SE) in binary Logistic Regression Analysis (Materials and Methods).							
^d Logistic Regression statistic χ^2 .							
^e Probability of association with test variable from Logistic Regression statistic χ^2 in each case.							
^f Relative Association (RA) and 95% confidence interval (95%CI) from binary Logistic Regression Analysis.							

Association of RAN and target molecules in primary breast tumours

Results of IHC staining in primary tumours for RAN and its relationship with that of other molecular tumour markers showed that RAN was very significantly associated with c-Met ($P = 6.6 \times 10^{-5}$), cMyc ($P = 4.4 \times 10^{-5}$), MMP2 ($P = 5.7 \times 10^{-6}$), and CK5/6 ($P = 5.5 \times 10^{-5}$) but not at all with Ki67, ER α , c-erbB-2, tumour size and histological grade ($P \geq 0.94$) and only of possible borderline significance with TRNBC (P uncorrected = 0.06) and involved lymph nodes alone (P uncorrected = 0.037). The most significant association of staining for RAN was with that for MMP2 ($P = 5.7 \times 10^{-6}$) (Table 2). When staining for MMP2 was tested for its relationship with staining for the other tumour variables, it was strongly significantly associated with the same variables as RAN: cMet ($P = 6.4 \times 10^{-9}$), cMyc ($P = 1.8 \times 10^{-7}$), CK5/6 ($P = 7.5 \times 10^{-7}$), as well as with RAN itself ($P = 5.7 \times 10^{-6}$). The other tumour variables were not significantly associated with MMP2 ($P \geq 0.35$) (SupplementaryTable 2).

Table 2
Summary of results for Cox's proportional hazards for cancer-related deaths

Tumour variable ^a	Coeff β^b	SE of β^b	χ^2^c	P ^d	RR ^e	95% CI ^e
Set A						
RAN	1.305	0.622	4.398	0.036	3.69	1.09–12.49
cMet	1.153	0.503	5.257	0.022	3.17	1.18–8.49
cMyc	1.246	0.445	7.827	0.005	3.48	1.45–8.32
MMP2	1.127	0.310	13.225	< 0.001	3.10	1.68–5.67
Set B						
RAN	2.025	0.594	11.600	0.001	7.57	2.36–24.28
cMet	1.985	0.470	17.879	< 0.001	7.28	2.90–18.27
Set C						
RAN	2.286	0.592	14.918	< 0.001	9.84	3.08–31.39
cMyc	1.873	0.427	19.252	< 0.001	6.51	2.82–15.02
Set D						
RAN	2.056	0.600	11.727	0.001	7.82	2.41–25.36
MMP2	1.642	0.260	40.023	< 0.001	5.17	3.11–8.59
^a In Set A comparisons were made between duration of survival time of patients with tumours stained for Ran, cMet, cMyc and MMP2; overall $\chi^2 = 96.21$, 4df, $P < 0.001$. In Set B comparisons between patients with tumours stained for Ran and cMet; overall $\chi^2 = 52.4$, 2df, $P < 0.001$. In Set C comparisons between patients with tumours stained for RAN and cMyc; overall $\chi^2 = 60.6$, 2df, $P < 0.001$. In Set D comparisons between patients with tumours stained for RAN and MMP2; overall $\chi^2 = 96.5$, 2df, $P < 0.001$. IHC cut-offs as described in Tables 1,2.						
^b Value of β coefficient (= $\log_e RR$) and standard error (SE) in Cox's multiple regression analysis (Materials and Methods).						
^c Cox's statistic χ^2 .						

When staining for RAN was tested for its relative probability of association (RA) with that of its potential target molecules cMet, cMyc, MMP2 and Ki67 using binary logistic regression, the RAs with cMet and MMP2 were the strongest (RA = 3.0 to 3.4), but that with Ki67 was not significant (RA = 1.12, $P = 0.81$) (Table 1). Moreover, when staining for c-Met was analysed, it also showed the strongest associations with that for RAN (RA = 3.41, $P = 0.019$) and for MMP2 (7.9, $P < 0.001$), but the strongest association for cMyc was with MMP2 and that for MMP2 was with cMet (RA = 7.7) (Table 1), suggesting a closer association between these 3 molecules than with RAN itself.

Association Of Ran, Mmp2 And Patient Survival

When staining for RAN, cMet, cMyc and MMP2 were tested together for independent association with patient survival times using Cox's multivariate analysis, they all showed some significant degree of independence ($P \leq 0.036$) with similar relative risks (RR) for patient demise of 3.1 to 3.7 fold (Table 2). These RRs were considerably less than the 7 to 15-fold decreases obtained in univariate analyses (SupplementaryTable 1). When analysed in binary combinations of staining for RAN with that for cMet, for cMyc or for MMP2, the RR for patient demise was suppressed from 14.9 to 7.6–7.8 fold for RAN with cMet or with MMP, but only to 9.8 fold with cMyc (Fig. 5, SupplementaryTable 3). The effects of staining for RAN and for MMP2 on RR were synergistic, increasing from 17.1 and 23.1, respectively, to 82.1 or, in terms of patients surviving, from 64% and 60%, respectively, to only 6% surviving after nearly 20 years (Fig. 5). Moreover, there was a significant decrease in patient survival times for the doubly stained RAN + MMP2 + over the single-stained RAN + MMP2-group ($P < 0.001$), but not over the RAN-MMP2 + group ($P = 0.21$) (SupplementaryTable 3).

The sensitivity, specificity, positive predictive response (PPR) and negative predictive response (NPR) of IHC scoring for RAN, MMP2 and RAN with MMP2 are presented in Table 3. The NPR is shown to be the best performing for the two biomarkers RAN and MMP2 taken together than when one of them is used alone for the prediction of patients alive (Table 3).

Table 3
Sensitivity, Specificity, NPR and PPR for Ran and MMP-2 (IHC assay)

Breast Cancer Sub-type	IHC Assay	IHC Staining score at diagnosis of BC	Patients Alive ¹		Patients died from cancer ²		Total Patients		Sensitivity (true + ve rate)	Selectivity (true -ve rate)	PPR ³	NPR ⁴
			No.	% Alive	No.	% Died	No.	% Total	%	%	%	%
All	RAN (Nuc)	< 1% (-ve)	42	44.7	3	3.4	45	24.9		44.7		93.3
All	RAN (Nuc)	2 to 5% (+ve)	52	55.3	84	96.6	113	75.1	96.6		74.3	
All	RAN (Nuc)	Total	94	100	87	100	181	100				
All	MMP2	< 2% (-ve)	83	88.3	21	24.1	104	57.5		88.3		79.8
All	MMP2	2 to 5% (+ve)	11	11.7	66	75.9	77	42.3	75.9		91.7	
All	MMP2	Total	94	100	87	100	181	100				
All	RAN(Nuc)/MMP2	-ve/-ve	39	41.0	1	1.1	40	22.1		41.0		97.5
All	RAN(Nuc)/MMP2	All others	47	50.0	22	25.3	69	38.1				
All	RAN(Nuc)/MMP2	+ve/+ve	8	8.5	64	73.6	72	39.8	98.9		88.8	
All	RAN(Nuc)/MMP2	Total	94	100	87	100	181	100				
Patients monitored up to 20 years from diagnosis and free from radiotherapy, chemotherapy and hormone therapy, ² cases of death by other causes have been excluded, ³ PPR =positive percentage response, ⁴ NPR= negative percentage response.												

Discussion

Previously, we have shown that knockdown of RAN by shRNA results in reduction of *in vitro* cell biological properties including cell adhesion, colony formation and cell invasion (17–19) as well as *in vivo* metastasis (3). Here RAN knockdown in breast cancer cells reduces MMP2 mRNA and protein levels, probably via ATF3 and CXCR3 which in turn results in a significant reduction in cell adhesion and colony formation in breast cancer cell lines. However, overexpression of MMP2 in RAN knocked-down breast cancer cells results in overcoming RAN silencing and this led to increases in cell adhesion and cell invasion. The fact that transfection of pBabe MMP2 overcomes the knockdown effect of RAN on the levels of MMP2 and consequent biological effects is probably due to the natural promoter being different from that of pBabe.

In this study, knockdown of CXCR3 results in reduction of mRNA / protein levels of MMP2 with no changes in ATF3 expression. However, silencing of AFT3 caused a reduction in both MMP2 and CXCR3 mRNA expression. Thus, it is probable that the RAN/MMP2 pathway is connected in the order of RAN → ATF3 → CXCR3 → MMP2. It has been established that CXCR3 is suppressed in cardiomyocytes and macrophages from ATF3-knockout mice and is positively regulated by ATF3 through an ATF3 transcriptional response element found in its proximal promoter (22). In another study, knockdown of ATF3 using siRNA reduced the expression of MMP2 and inhibited the growth of U373MG cells grown *in vivo* xenografts in nude mice (8). It has also been shown that CXCR3 promotes gastric cancer cell migration and invasion by upregulating MMP2 expression (21). Our results are consistent with these reports.

Although previous publications (17–19) and work in this paper have established a causal relationship between RAN, cMet, cMyc, MMP2 and properties related to metastasis, these studies have been undertaken in cell line models of breast cancer. By using IHC staining of primary breast cancers we have also previously shown that RAN (18), cMet (17), and cMyc (19) are on their own

significantly associated with patient demise from metastatic breast cancer. Now we have shown that increased staining for RAN is very significantly associated with staining for proteins in the cell-signalling pathway containing cMet, cMyc, and MMP2 that is linked to increases in the metastatic properties of cultured cells. Since increased IHC staining is related to increased levels of protein in the carcinoma cells (18), protein levels have also therefore increased by similar levels of at least 5–10 folds between tumours whose patients are at low and those who are at high risk of dying from metastatic disease. These fold increases in cellular levels are sufficient to cause the increases in metastatic properties observed in stably transfected cells in culture as outlined above. There is no significant association of staining for RAN with that for Ki67, consistent with little increase in cell proliferation being observed in RAN transfected cells. The fact that staining for RAN, cMet, cMyc and MMP2 are all very significantly associated with that for CK5/6, but not with that for ER α or c-erbB-2 (SupplementaryTable 2) suggests that these proteins occur mainly in the Basal Cell Type of breast cancers. This subgroup of breast cancers overlaps considerably with the triple receptor negative breast cancer (TRNBC) group (de Silva et al, 2011) and hence may explain the observed borderline association of staining for RAN with the TRNBC subgroup alone (SupplementaryTable 2). The fact that there is a stronger relative association (RA) between staining for MMP2, cMet and cMyc than with that for RAN (Table 1) suggests that the increase in MMP2 in tumours is not solely due to an increase in RAN, but may arise via other signalling mechanisms.

Multiple longitudinal comparisons of survival times with RAN, cMet, cMyc and MMP2 in multivariate analyses showed that all 4 were independently significantly associated with patient survival times, but that either together with RAN or in binary combinations of each one with RAN, RAN's RR of death was partially confounded by these other proteins (Fig. 5, Table 2). These results suggest that all 4 proteins lie on a signalling pathway which increases the RR of patient death from metastatic disease. The partial nature of the confounding of RR for RAN by these other proteins suggests that other pathways not involving RAN are also involved in causing patient death. That the decline in RR for RAN with either Met or MMP2 at nearly 50% (49% or 48%, respectively) was larger than the decline in RR for Met or MMP2 in binary combinations with RAN at nearly 1/3 (32% for both) (Table 2) suggests that Met and MMP2 are more proximal members than RAN in the pathway leading to patient death. This suggestion is further supported by results in Fig. 5. Thus when staining data for MMP2 is added to that for RAN in the primary tumours, there is a significant decrease, but when staining data for RAN is added to that for MMP2, there is no significant decrease in patient survival times. In addition, adding MMP2 to RAN increase the NPR to 98%. Practically when staining data for MMP2 is included with that for RAN, the RR for patient death is increased from the original 14.9 to 82.1 fold.

Conclusion

In this article, we have established in cell line models of breast cancer a direct relationship between Ran and MMP-2 and properties related to metastasis. This inclusion facilitates a more accurate prognosis and further identifies a subgroup of patients that could benefit more from chemotherapy and from therapy directed against both proteins than against either one alone.

Abbreviations

MMP2: Matrix metalloproteinase 2, BC:Breast Cancer, ATF3:Activating Transcription Factor 3, CXCR3:C-X-C Motif Chemokine Receptor 3,

Mohamed El-Tanani :M El-T, Philip S Rudland:P.S.R, Angela Plat-Higgins:A.P-H, Yin-Fai Lee:Y-F.L, Yusuf Haggag:Y.H, Mark Sutherland:M.S., Shu-Dong Zhang: S-D.Z

Declarations

Ethics approval and consent to participate: Human materials and data were collected in an anonymised fashion under the legally-binding, UK Human Tissue Act supervised by National Research Ethical Committee (NREC) and approved by North West REC (Ref 12/NW/0778), protocol number UOL000889, IRAS number 107845 to Prf. P.S. Rudland.

Consent for publication

The authors consent for publication

Availability of data and material

The authors confirm availability of data and material

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Authors' information

Affiliations

Pharmacological and Diagnostic Research Centre, Al-Ahliyya Amman University, Faculty of Pharmacy, Amman, Jordan.

Institute of Cancer Therapeutics, Faculty of Life Sciences, University of Bradford, Bradford, UK.

Mohamed El-Tanan

School of Pharmacy and Medical Sciences, University of Bradford, Bradford, UK.

Yin-Fai Lee

Department of Pharmaceutical Technology, Faculty of Pharmacy, Tanta University, Tanta, Egypt.

Yusuf Haggag

School of Chemistry and Biomedical Sciences, University of Bradford, Bradford, UK.

Mark Sutherland

Northern Ireland Centre for Stratified Medicine, Biomedical Sciences, University of Ulster, UK.

Shu-Dong Zhang

Institute of Systems, Molecular and Integrative Biology, University of Liverpool, Liverpool, UK.

Angela Plat-Higgins⁵, Philip S Rudland⁵

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Figures

Figure 1A

Figure 1C

Figure 1B

Figure 1

Knockdown of RAN downregulates MMP2 in MDA-MB231 cell line. MDA-MB231 breast cancer cell line was transfected with either shScr or shRAN, yielding shScr, or shRAN1, 2 or 3 cell lines relative mRNA level was obtained from Real-Time PCR analysis for (A) RAN and (B) MMP2 and normalized to that of β -actin. (C) Western blot for RAN, MMP2 and β -actin in breast cancer cell lines. Results are mean of three independent experiments $\pm$ SD. Major hybridizing bands are shown in kilobases (kb). The average fold increase for three different experiments for RAN is lane 1=1 and lane 2= 0.36 ± 0.06 and for MMP2 is lane 1=1 and lane 2= 0.37 ± 0.1 The images shown are representative of the three experiments. The average fold decrease for three different experiments for RAN is 5.5 ± 0.6 and for MMP2 is 2.7 ± 0.3 compared to control shScr infected cells in C.

Figure 2A

Figure 2B

Figure 2C

Figure 2C

Figure 2D

Figure 2E

Figure 2

Effect of MMP2 on in vitro biological properties associated with cancer progression MDA-MB231 breast cancer cells infected with either control shScr or shMMP2 and assayed for (A) adhesion or (B) colony formation in agar. The cell adhesion was normalized to that in shScr cells. Numbers of colonies are shown directly. Results are mean of three independent experiments $\pm$ SD. (C) Western blots for MDA-MB231-shScr control and MDA-MB231-shRAN cells, which had been transfected with a control expression vector alone (pBabe) or with an equivalent MMP2 expression vector. The average fold changes for three different experiments for MMP2 is lane 1=1, lane 2=1.02 $\pm$ 0.2 and lane 3=0.9 $\pm$ 0.3 and for RAN is lane 1=1, lane 2=1.02 $\pm$ 0.4, lane 3=0.34 $\pm$ 0.2. The images shown are representative of the three experiments. (D) cell adhesion assay and (E) cell invasion assays were conducted as described in Materials and Methods and show mean $\pm$ SD for three independent experiments. Major hybridizing bands are shown in kilobases (kb). The average fold decrease for three different experiments for MMP2 is 3.3 $\pm$ 0.4 compared to control shScr infected cells (Figure 2C).

Figure 3A

Figure 3B

Figure 3C

Figure 3D

Figure 3E

Figure 3F

Figure 3

Knockdown of MMP2 reduced growth in agar in MCF-7 and T47D breast cancer cell lines. (A, C) MCF-7 or (B, D) T47D breast cancer lines were infected with either shScr and shMMP2 and (A, B) Western blotted or (C, D) mean $\pm$ SD of numbers of colonies growing in soft agar from three experiments. (A, B) Western blot of representative experiment with mean $\pm$ SD for three independent experiments. Major hybridizing bands are shown in kilobases (kb). The average fold decrease for three different experiments for MMP2 is 3.9 ± 0.4 , 2.0 ± 0.2 and for RAN 2.9 ± 0.2 compared to control shScr infected cells (Figure 3A, B & E, respectively). (E, F), MCF-7, shScr control and MCF-7 shRAN cells were transfected with pbabe or equivalent MMP expression vector and Western blotted. The average fold changes for three different experiments for MMP2 is lane 1=1 and lane 2= 0.25 ± 0.06 (A), for MMP2 is lane 1=1 and lane 2= 0.61 ± 0.04 (B) and (E) for MMP2 is lane 1=1, lane 2= 7.5 ± 0.14 and lane 3= 8.1 ± 1.5 , for RAN is lane 1=1, lane 2= 1.0 ± 0.03 and lane 3= 0.42 ± 0.08 . The images shown are representative of the three experiments. (F) Assayed for colony growth in soft agar. Results are the mean $\pm$ SD for three independent experiments.

Figure 4 A

Figure 4C

Figure 4E

Figure 4 B

Figure 4D

Figure 4F

Figure 4

Mechanism of dysregulation of MMP2 in breast cancer cells. MDA-MB231 breast cancer cells were infected with either shScr or (A, B) shRAN; or (C, D), shCXCR3; or (E, F) shATF3 and then analyzed for (A) mRNAs for ATF3 and CXCR3; (B) Western blotted for RAN, ATF3, CXCR3, MMP2, β -actin. The average fold change for three different experiments for RAN is lane 1=1 and lane 2=0.22 $\pm$ 0.09, for ATF3 is lane 1=1, lane 2=0.51 $\pm$ 0.1, for CXCR3 is lane 1=1 and lane 2= 0.30 $\pm$ 0.07 and for MMP2 is lane 1=1 and lane 2=0.25 $\pm$ 0.05 (4B). (C) Analyzed for mRNAs for ATF3 and CXCR3; or (D) Western blotted for CXCR3, ATF3, MMP2 and β -actin. The average fold change for three different experiments for CXCR3 is lane 1=1 and lane 2=0.36 $\pm$ 0.05, for ATF3 is lane 1=1, lane 2=0.87 $\pm$ 0.04 and for MMP2 is lane 1=1 and lane 2= 0.32 $\pm$ 0.04. (E) Analyzed for mRNAs for ATF3, CXCR3 or MMP2. The level of mRNAs in A, C, E are shown as mean $\pm$ SD for three independent experiments relative to β -actin mRNA and then normalized to the control shScr values. The level of protein in (B,D) is shown for representative RAN, ATF3, CXCR3, MMP2 and β -actin in breast cancer cell lines. Results are mean of three independent experiments $\pm$ SD. Major hybridizing bands are shown in kilobases (kb). The average fold change for three different experiments for ATF3 is lane 1=1 and lane 2=0.4 $\pm$ 0.3, for CXCR3 is lane 1=1, lane 2=0.29 $\pm$ 0.06 and for MMP2 is lane 1=1 and lane 2= 0.44 $\pm$ 0.06. In F the average fold decrease for three different experiments for RAN is 4.5 $\pm$ 0.5, for ATF3 is 3.0 $\pm$ 0.4, for CXCR3 is 3.5 $\pm$ 0.4 and for MMP2 is 3.9 $\pm$ 0.3 compared to control shScr infected cells.

Figure 5

Association of IHC staining for RAN and for MMP2 with overall time of patient survival. Cumulative proportion of surviving patients as a fraction of the total for each year after presentation with carcinomas classified as negatively stained for RAN (-) and MMP2 (-) (set a solid line), positively stained for RAN (+) and negatively stained for MMP2 (-) (set b dotted line), negatively stained for RAN (-) and positively stained for MMP2 (+) (set c dashed line), and positively stained for both RAN (+) and MMP2 (+) (set d dashed and dotted line). Numbers of patients entering each year are shown below. In a median survival (ms) >228 months, final cumulative survival (fcs) 0.97 with 39 censored observations (8 dead of other causes); in b ms >216 months, fcs 0.6 with 44 censored observations (19 dead of other causes); in c ms >216 months, fcs 0.60, with 3 censored observations (1 dead of other causes) and in d ms 46.2 months, fcs 0.06, with 8 censored observations (4 dead of other causes). The 4 curves are highly significantly different (Wilcoxon Gehan statistic $\chi^2 = 75.405$, 3 df, $P < 0.001$). For a vs b Wilcoxon $\chi^2 = 14.02$, 1 df, $P < 0.001$, Cox's univariate RR = 17.11 (95% CI, 2.30-127.6); a vs c $\chi^2 = 10.583$, $P < 0.001$, RR = 23.10 (2.09-255.0); b vs c $\chi^2 = 0.407$, $P = 0.52$, RR = 1.35 (0.32-5.78); b vs d $\chi^2 = 33.09$, $P < 0.001$, RR = 4.8 (2.88-7.99); c vs d $\chi^2 = 1.569$, $P = 0.21$, RR = 3.56 (0.87-14.61); a vs d $\chi^2 = 59.64$, $P < 0.001$, RR = 82.11 (11.34-594.6).

Supplementary Files

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