CXCR4/CXCR7/CXCL12 axis promotes an invasive phenotype in medullary thyroid carcinoma

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Background: Medullary thyroid carcinoma (MTC) is a rare and challenging endocrine malignancy. Once spread, the therapeutic options are limited and the outcome poor. For these patients, the identification of new druggable biological markers is of great importance. Here, we investigated the prognostic and biological role of the C-X-C chemokine receptors type 4 and 7 (CXCR4/7) in MTC.

Methods: Eighty-six MTC and corresponding non-neoplastic thyroid specimens were immunohistochemically stained for CXCR4/7 using tissue microarray technology and expression levels correlated with clinicopathological variables. Medullary thyroid carcinoma cell line TT was treated with recombinant human SDF1α/CXCL12 (rh-SDF1α) and CXCR4 antagonists AMD3100 and WZ811. Changes in cell cycle activation, tumour cell invasiveness as well as changes in mRNA expression levels of genes associated with epithelial–mesenchymal transition (EMT) were investigated.

Results: High CXCR4 expression was associated with large tumour size and metastatic disease. CXCR4 antagonists significantly reduced tumour cell invasiveness, while the treatment with rh-SDF1α stimulated invasive growth, caused cell cycle activation and induced EMT.

Conclusions: The CXCR4/CXCR7/CXCL12 axis plays an important role in MTC. We provide first evidence that the chemokine receptors might serve as potential therapeutic targets in patients with advanced MTC and offer new valuable insight into the underlying molecular machinery of metastatic MTC.

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Received 24 April 2017; revised 13 September 2017; accepted 14 September 2017; published online 7 November 2017

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www.bjcancer.com | DOI:10.1038/bjc.2017.364

British Journal of Cancer (2017) 117, 1837–1845 | doi: 10.1038/bjc.2017.364

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Medullary thyroid carcinoma (MTC) is a rare endocrine malignancy, which accounts for ~5% of all thyroid carcinomas. It derives itself from the calcitonin-secreting, parafollicular C cells of the thyroid gland (Ball, 2009). Unlike other thyroid malignancies, MTC shows a strong hereditary predisposition. Approximately 25% of all cases are associated with an activating germline mutation of the RET oncogene (Donis-Keller et al, 1993; Takahashi, 1995). RET encodes for a receptor tyrosine kinase that influences cell death and differentiation as well as migration and cell cycle progression. Interestingly, RET gain-of-function mutations are also present in the majority of sporadic MTCs (Hu et al, 2014).

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syndromes harbour different extrathyroidal endocrine manifestations such as pheochromocytoma or primary hyperparathyroidism, MTC is the dominant clinical sign, present in almost all the patients (Eng et al, 1996; Leboulleux et al, 2004; Wells et al, 2013).

In addition to CXCR4, SDF1α also binds to CXCR7, a chemokine receptor found to be significantly upregulated in different types of epithelial tumours (Gebauer et al, 2011; Schrevel et al, 2012; Chen et al, 2015). It has been demonstrated that CXCR7 promotes tumour cell survival by inhibiting apoptosis and facilitates colony formation through increased local adhesion properties (Burns et al, 2006). Other studies suggested that CXCR7 functions as a co-receptor to CXCR4, thus enhancing SDF1α-mediated G protein signalling (Llevye et al, 2009; Sun et al, 2010).

The aim of our study was to investigate the stage-dependent expression of CXCR4 and CXCR7 in MTC and to elucidate the functional implications of the CXCR4/CXCR7/CXCL12 axis in MTC.

**MATERIALS AND METHODS**

**Patient selection and clinicopathological data.** All patients who underwent all types of thyroidectomy for histologically confirmed MTC, irrespective of tumour stage and microscopic resection margin, at the Department of Surgery (A), University Hospital Düsseldorf between 1986 and 2003 were retrospectively reviewed and included in this study. Our exclusion criteria comprised incomplete pathological report, missing clinical data, incomplete resection or insufficient tissue material for subsequent analysis. Follow-up data were obtained from our prospectively maintained clinical database. Overall survival was defined as the time in months from the date of surgery until death of any cause or until the last follow-up at which survivors were censored. Patients who died within the first 30 days upon surgery were not included in the survival analysis. Clinical data including sex, age at first diagnosis, serum calcitonin levels prior to surgery, genetic profiles regarding sporadic and inherited MTC as well as the initial tumour stage were retrospectively reviewed. During the time of the study different calcitonin assays had been used. Here we focussed on the numeric calcitonin basal blood levels prior to surgery irrespective of the assay method employed. Medullary thyroid carcinomas were staged according to the 8th edition of the UICC classification (Brierley et al, 2016).

The study was carried out in accordance to Good Clinical Practice, the Declaration of Helsinki and local rules as well as regulations of the country. Strict anonymity of all patients’ data were established and maintained throughout the study. An approval from the institutional ethics committee of the Medical Faculty, Heinrich Heine University Düsseldorf was obtained (reference number: 3821).

**Tissue microarray and immunohistochemistry.** All formalin-fixed paraffin-embedded tissue samples were provided from the Institute of Pathology, University Hospital Düsseldorf. The construction of the tissue microarrays, immunohistochemistry and analysis of protein expression using the immunoreactivity score (IRS) reported by Remmele (Remmele et al, 1986) were performed as described previously (Werner et al, 2016). The staining intensity of the different samples was reviewed by two independent investigators (TW and CF) in a blinded manner.

For immunohistochemical staining the following primary antibodies were used: mouse monoclonal anti-CXCR4 (1:100 dilution; Abcam, Cambridge, UK), rabbit polyclonal anti-CXCR7 (1:200 dilution; GeneTex, Irvine, CA, USA).

| Isotype controls were performed using mouse IgG1k (MOPC- 21; 1:50 dilution; Abcam) or rabbit immunoglobulin fraction (Code X0903; 1:1000 dilution; Dako, Glostrup, Denmark). CXCR4-expressing tonsil tissue and CXCR7-expressing pancreatic adenocarcinoma served as positive controls. The prognostic power of CXCR4 and CXCR7 was assessed according to the RReporting
**Cell culture and reagents.** The human MTC cell line TT was purchased from the American Type Culture Collection (ATCC, Middlesex, UK; CRL-18033). The cell line was cultivated in F-12K medium (Gibco Thermo Fisher, Waltham, MA, USA), supplemented with 10% foetal bovine serum. Cell line authenticity was confirmed as described previously (Krieg et al., 2014). Recombinant human (rh) SDF1α was purchased from PeproTech (Rocky Hill, NJ, USA). Specific CXCR4 antagonist plerixafor (AMD3100) and WZ811 were obtained from Selleck Chemicals (Houston, TX, USA).

**Invasion assay.** TT cells were treated with different concentrations of rh-SDF1α, AMD3100, WZ811 or vehicle control at equimolar concentrations for 24 h in serum-starved culture medium. Afterwards, cells were harvested and seeded on BD Matrigel invasion chambers (BD Biosciences, Heidelberg, Germany) at a density of 5 x 10^4 cells ml^-1 in F-12K medium supplemented with 0.25% bovine serum albumin. Conditioned Dulbecco’s Modified Eagle Medium from CXCCL2-expressing NIH 3T3 fibroblasts (Burger and Kipps, 2006; Lagergren et al., 2007) was used as chemoattractant and added to the lower chamber. After 24 h of incubation, cells were fixed with methanol and stained with 4',6-diamidino-2-phenylindol (DAPI; 200 ng ml^-1). 4',6-diamidino-2-phenylindol-positive cells were counted in five visual fields of three separate membranes under a fluorescence microscope at x200 magnification (Zeiss Axiosplan 2, Carl Zeiss, Göttingen, Germany).

**FACS cell cycle analysis.** TT cells were treated with different concentrations of rh-SDF1α, AMD3100, WZ811 or vehicle control at equimolar concentrations for 48 h. For subsequent cell cycle analysis, TT cells were harvested and washed in cold PBS and resuspended in 80% ethanol. After incubation for 2 h, cells were washed in PBS and incubated with RNase A (100 μg ml^-1) and propidium iodide (PI; 50 μg ml^-1) for 30 min at 37°C. Finally, cells were analysed by fluorescence-activated cell sorting (FACS) using a BD FACS Canto II (BD Biosciences, San Jose, CA, USA).

**BrdU proliferation assay.** Cell proliferation was assessed in 96-well culture plates with cells plated at a concentration of 4 x 10^3 per well under serum-starved culture conditions. After 24 h, cells were incubated for 48 h with rh-SDF1α, AMD3100, WZ811 or vehicle control at equimolar concentrations. To assess cell proliferation the Cell Proliferation ELISA, BrdU assay (Roche Applied Science, Mannheim, Germany) was performed at an absorbance at 370 nm according to the manufacturer’s protocol. All assays were analysed using an Infinite 200 microplate reader (Tecan Group Ltd., Crailsheim, Germany).

**Western blot analysis.** For protein isolation, cells were lysed in ice-cold RIPA buffer. Total protein concentrations were measured using a BioPhotometer (Eppendorf, Hamburg, Germany). Proteins were separated using SDS-polyacrylamid gel electrophoresis and transferred onto a nitrocellulose membrane for antibody-detection. Membranes were incubated overnight using the following primary antibodies: mouse monoclonal anti-CXCR4 (1:100 dilution; Abcam), rabbit polyclonal anti-CXCL12 (1:200 dilution; GeneTex). Membranes were incubated overnight using the following primary antibodies: mouse monoclonal anti-CXCR4 (1:100 dilution; Abcam), rabbit polyclonal anti-CXCR7 (1:200 dilution; GeneTex), rabbit polyclonal anti-CXCL12 (1:200 dilution; GeneTex), rabbit polyclonal anti-MMP-2 (1:500 dilution; GeneTex), rabbit polyclonal anti-MMP-9 (1:500 dilution; GeneTex). After 45 min of incubation, cells were washed and treated with either mouse anti- or rabbit-Alexa Fluor 488 (10 μg ml^-1; Thermo Fisher). Nuclear staining was achieved using DAPI (200 ng ml^-1). After fixation with 1% PFA, the cells were visualised using a fluorescence microscope at x400 magnification (Zeiss Axiosplan 2, Carl Zeiss).

**RT-PCR.** Total RNA was extracted from cells using the RNeasy Mini Kit according to the manufacturer’s protocol (Qiagen, Hilden, Germany). Complementary DNA (cDNA) was synthesised from 2 μg of total RNA using the qScript cDNA Synthesis Kit (QuantaBio, Beverly, MA, USA) following the manufacturer’s instructions. The cDNA was then adjusted to a final concentration of 1 ng ml^-1. For the subsequent quantitative real-time-PCR (qRT-PCR), 2 μl of cDNA template were mixed with 10 μl PerfeCTa Fast Mix II (QuantaBio), 0.2 μl probe (100 nM; Roche) and 0.2 μl of forward and reverse primer (200 nM each). All templates were run as triplicates. Glyceraldehyde-3-phosphate dehydrogenase served as internal reference gene. The primers used are listed in Supplementary Table 1. All experiments were performed on the DydadDisciple Chromo 4 (Bio-Rad) using the following set-up: 95°C for 2 min, followed by 40 cycles of denaturation at 95°C for 10 s and annealing and extension at 60°C for 30 s. Gene expression was quantified according to the 2^(-ΔΔCt) method (Livak and Schmittgen, 2001).

**Statistical analysis.** Correlations between the IRS of CXCR4 or CXCR7 and the respective clinicopathological parameters were evaluated by logistic regression analyses and the non-parametric Spearman correlation. Changes in chemokine receptor expression between groups were assessed using the non-parametric Mann–Whitney U test or the Wilcoxon matched-pairs signed rank test as indicated. In addition, the data were categorised according to the respective mean IRS and analysed by the Fisher’s exact test. Univariate survival analyses were performed by the log-rank (Mantel–Cox) test. Cox regression analyses were used to estimate hazard ratios (HR) with 95% confidence intervals (CI) for multivariate analyses including all variables. Moreover, a search for the best model was conducted using a stepwise variable selection procedure based on the Akaike Information Criterion (AIC). Cell culture experiments were repeated at least three times and evaluated for statistical significance using the non-parametric Mann–Whitney U test.

Statistical analyses were computed using GraphPad Prism (Version 6, GraphPad Software, San Diego, CA, USA) and the Statistical Software R version 3.1.0. A P-value <0.05 was considered statistical significant.

**RESULTS**

**Patients and outcome.** Overall 86 patients were included in our study. The patients’ characteristics are illustrated in Table 1. The median overall survival in our cohort of patients was 170 months (range 5–287) with a median follow-up of 191 months (range 5–287) based on the reverse Kaplan–Meier procedure. The last follow-up was performed in January 2013. During this period of time 19 patients died. Owing to loss of follow-up, 11 patients were unavailable for subsequent survival analysis. The calcitonin basal blood levels prior to surgery were available for 67 patients, with a median of 304 pg ml^-1. The genesis of the malignancy, whether sporadic or inherited, was known for the majority of patients. Only for eight patients the genetic profile was unavailable. While there were differences in surgical treatment between the patients, owed to the given recommendations at the respective time, the great majority of patients underwent radical, total thyroidectomy.

**Expression of CXCR4 correlates with advanced tumour stage and metastatic phenotype.** First, we compared the IRS of the two chemokine receptors in MTC samples and corresponding non-neoplastic thyroid tissues, lymph node as well as distant metastases. Whereas primary tumour samples and corresponding metastases...
CXCR4 was compared across groups of different clinicopathological parameters and CXCR4 expression, the IRS for CXCR7, whereas three lymph node metastases demonstrated a weak expression (Figure 1A and B). In contrast, almost all tissue specimens stained strongly positive for CXCR4, normal thyroid tissue samples exhibited predominantly no expression of CXCR4 ($P < 0.001$; Figure 1A and B). In contrast, almost all tissue specimens stained negatively for CXCR7 in both tumour and corresponding unaffected tissue cores. Only one primary tumour exhibited a strong expression of CXCR7, whereas three lymph node metastases demonstrated a weak expression for CXCR7 (data not shown). To further investigate a possible association between different clinicopathological parameters and CXCR4 expression, the IRS for CXCR4 was compared across groups of different clinicopathological variables. Interestingly, we found a strong association between high CXCR4 expression and large tumour size (T1/2 vs T3/4: $P < 0.01$; Figure 2A) as well as advanced tumour stages (UICC I/II vs UICC III/IV: $P < 0.001$; Figure 2B). In addition, patients with lymph node or distant metastases exhibited a significantly higher expression of CXCR4 in their primary tumour cores when compared to patients’ samples without metastases (N neg. vs N pos.: $P < 0.001$; M0 vs M1: $P < 0.01$; Figure 2C and D). Of note, CXCR4 was also significantly higher expressed in the lymph node metastases than in the corresponding primary tumour ($P < 0.001$; Figure 1B). Moreover, tumours from patients with a sporadic genesis of their disease and advanced age at the time of first diagnosis exhibited markedly higher expression levels of CXCR4 stained strongly positive for CXCR4, normal thyroid tissue samples exhibited predominantly no expression of CXCR4 ($P < 0.001$; Figure 1A and B). In contrast, almost all tissue specimens stained negatively for CXCR7 in both tumour and corresponding unaffected tissue cores. Only one primary tumour exhibited a strong expression of CXCR7, whereas three lymph node metastases demonstrated a weak expression for CXCR7 (data not shown).

**Table 1. Patient characteristics (n = 86)**

| Variables                      | No. of patients (%) |
|--------------------------------|---------------------|
| Total                          | 86                  |
| Age (Median (range); years)    | 48 (6–83)           |
| Gender                         |                     |
| Male                           | 42 (49)             |
| Female                         | 44 (51)             |
| Genetic                        |                     |
| Sporadic                       | 40 (47)             |
| MEN2A                          | 38 (44)             |
| Unknown                        | 8 (9)               |
| Basal calcitonin levels prior to surgery |     |
| <12 pg ml$^{-1}$               | 9 (10)              |
| >12 pg ml$^{-1}$               | 58 (68)             |
| Unknown                        | 19 (22)             |
| Type of surgery                |                     |
| Hemithyroidectomy              | 11 (13)             |
| with unilateral ND             | 3 (3)               |
| Subtotal thyroidectomy         | 7 (8)               |
| with bilateral ND              | 2 (2)               |
| Total thyroidectomy            | 16 (19)             |
| with unilateral ND             | 16 (19)             |
| with bilateral ND              | 31 (36)             |
| Side affected                  |                     |
| Unilateral                     | 70 (81)             |
| Bilateral                      | 16 (19)             |
| Tumour stage                   |                     |
| T1/2                           | 67 (78)             |
| T3/4                           | 19 (22)             |
| Lymph node metastasis          |                     |
| N negative                     | 43 (50)             |
| N positive                     | 43 (50)             |
| Distant metastasis             |                     |
| M0                             | 68 (79)             |
| M1                             | 18 (21)             |
| UICC stage                     |                     |
| UICC I/II                      | 41 (48)             |
| UICC III/IV                    | 45 (52)             |

Abbreviations: MEN2A = multiple endocrine neoplasia type 2A; ND = neck dissection; UICC = Union Internationale Contre le Cancer.

CXCR4 expression is a negative prognostic marker in MTC. To further explore a prognostic relevance of CXCR4 in MTC univariate survival analyses were performed. Accordingly, a high CXCR4 expression was strongly associated with a poor overall survival in MTC patients ($HR = 4.474$; 95% CI: 1.671–11.98; $P < 0.001$; Table 2). In addition, other clinicopathological markers such as old age at first diagnosis, sporadic MTC, present lymph node or distant metastases and advanced T3/T4 tumour stages predicted poor prognosis (Table 2).

In the subsequent multivariate analysis including all variables, no clinicopathological parameter came up as an independent negative prognostic marker (Table 2). However, the implementation of a variable selection procedure based on the AIC identified the presence of distant metastases at the time of first diagnosis to be an independent prognostic factor (Table 2). Importantly, the AIC decreased during the stepwise variable selection by 10 units, proving a better goodness of fit of the selection model and indicating its higher relevance in our set of data (Burnham and Anderson, 2003).

**Rh-SDF1z induces CXCR4-dependent tumour cell invasion.** To date, only one study using a papillary thyroid carcinoma cell line demonstrated a potential role of the SDF1z/CXCR4 axis in initiating tumour cell invasion and migration in thyroid carcinoma (Zhu et al., 2016). Since this functional relevance has not yet been reported for MTC, we took advantage of the MTC cell line TT, which expresses both chemokine receptors CXCR4 and CXCR7 as revealed by immunocytochemistry and western blot analyses (Figure 3A and B). To investigate the impact of SDF1-driven invasiveness, we treated TT cells with increasing concentrations of rh-SDF1z and measured the changes in invasive growth. Rh-SDF1z induced a significant increase in the number of invading cells by a fold change of 1.5 as compared to cells treated with vehicle control ($P < 0.01$; Figure 4A and B).

Next, we treated the cells with the well-described CXCR4 antagonists AMD3100 and WZ811. The incubation with the respective compounds markedly reduced the invasive capacity of the MTC cells. For both compounds the effect was more pronounced at higher concentrations suggesting a dose-dependent mechanism. The incubation with WZ811 at 100 nM reduced the amount of invasive cells to 0.59 fold of the amount of control-treated cells ($P < 0.05$) while the incubation with 1 μM caused a decrease in invasive cells by a fold change of 0.45 ($P < 0.01$; Figure 4A and B). Similarly, the effect of AMD3100 became more obvious at 1 μM than at 100 nM inducing a fold change of 0.53 of invading cells as opposed to 0.68 when compared to vehicle control-treated cells ($P < 0.01$; Figure 4A and B). Interestingly, the inhibitory effect of the respective chemokine receptor antagonists persisted when the cells were co-incubated with rh-SDF1z (data not shown).

**Rh-SDF1z induces invasiveness through cell cycle activation and EMT.** To further elucidate the possible mechanisms behind the increase in invasiveness after incubation with rh-SDF1z we performed cell cycle analyses using FACS technology. The
Figure 1. Expression of CXCR4 in MTC. (A) Representative tissue samples with immunohistochemical staining for CXCR4 and calcitonin in MTC (left), respective lymph node metastasis (middle) and non-neoplastic thyroid gland (right). All shown samples were classified as a strong expression for the given marker in accordance with the IRS. The bar at the top left corner indicates 50 μm. (B) Expression levels of CXCR4 in non-neoplastic thyroid tissue specimens and respective corresponding MTC, lymph node and distant metastases. Boxplots display the median IRS with the upper and lower quartile, as well as maximum and minimum for CXCR4. CXCR4 expression in the different tissue samples was compared between groups using the Wilcoxon matched-pairs signed rank test. Bars indicate the respective pairs. CXCR4 = C-X-C chemokine receptor type 4; DM = distant metastases; IRS = immunoreactivity score; LN = lymph node metastases; MTC = medullary thyroid carcinoma; NT = non-neoplastic thyroid gland; ***P < 0.001.

Figure 2. Association between CXCR4 expression and different clinicopathological parameters. (A–F) Boxplots display the median IRS with the upper and lower quartile, as well as maximum and minimum for CXCR4 in the primary tumour core grouped according to the given clinicopathological parameter. CXCR4 expression levels were compared using the non-parametric Mann–Whitney U test. (G) Cox regression analyses demonstrate the correlation between increasing expression levels of CXCR4 and the likelihood of an advanced tumour stage or metastatic phenotype. CXCR4 = C-X-C chemokine receptor type 4; IRS = immunoreactivity score; LN = lymph node; *P < 0.05; **P < 0.01; ***P < 0.001.
treatment with rh-SDF1α resulted in a significant decrease of cells remaining in the G1 phase, while significantly more cells entered the G2/M phase (Figure 4C). Interestingly, these changes in cell cycle activation did not translate into an increase in cell proliferation (data not shown). Moreover, incubation of MTC cells with CXCR4 antagonists AMD3100 and WZ911 initiated no changes in cell cycle profiles or proliferation (data not shown).

Next, we investigated possible changes in mRNA expression levels of genes associated with EMT and tumour cell invasion. Whereas rh-SDF1α induced no changes in the expression of SNAI1, the expression levels of BST2, FGF9 and Vimentin were significantly upregulated after rh-SDF1α treatment ($P<0.01$; Figure 4D, Supplementary Table 3). On the other hand, expression levels of E-cadherin were markedly reduced after incubation with rh-SDF1α ($P<0.05$; Figure 4D, Supplementary Table 3).

**DISCUSSION**

Carcinomas of the thyroid gland are the most common endocrine malignancies in the world. They are responsible for more patients’ deaths than all other endocrine-associated malignancies combined (Ernani et al, 2016). Although surgery may be curative in the early stages of the disease, the majority of patients harbour distant metastases at the time of diagnosis (Hu et al, 2014). Locoregional and distant spread that occur during the early stages of the disease is not rarely the first clinical sign. Once the disease has spread the treatment options with curative intent are limited. In this regard, the discovery and new appreciation of the activating mutation of the RET proto-oncogene in hereditary and sporadic MTC patients has caused paramount changes in adjuvant therapeutic protocols. It has led to the replacement of mainly dacarbazine- and doxorubicin-based chemotherapeutic regimes by small molecule TKI (Lalam and Awada, 2011). However, after the initial euphoria had faded and the limited effects on progression-free survival became apparent, a new appraisal of the commonly altered signalling pathways in carcinogenesis has started to evolve (Licitra et al, 2010; Haddad, 2013; Hu et al, 2014).

CXCR4 has been linked to different tumour entities as a negative prognostic marker and important factor in carcinoma cell homing and subsequent metastasis (Chen et al, 2015; Krieg et al, 2015; Mego et al, 2016). Thus far, little is known about the CXCR4/ CXCR7/CXCL12 axis in MTC. To our knowledge, only one study has investigated the expression of CXCR4 and CXCR7 in MTC. In a study performed by Zhu et al only 10 MTC tissue specimens were immunohistochemically stained for CXCR4 and CXCR7 but no subsequent correlation with clinicopathological variables or functional analyses were performed (Zhu et al, 2016). Therefore, our study is the first that investigated the stage-dependent expression of CXCR4 and CXCR7 in MTC patients and further stratified their functional implications in vitro.

In our set of patients, CXCR4 was significantly higher expressed in MTC than in non-neoplastic thyroid tissue specimens. In addition, a high CXCR4 expression in the primary tumour was associated with a high probability of an advanced tumour stage, high UICC stage and metastatic phenotype. In the univariate analysis, elevated CXCR4 expression levels were also associated with a significantly worse prognosis. In the multivariate analysis however, only the presence of distant metastases proved to be an independent negative prognostic parameter. Taken together, these findings underscore the paramount importance of metastatic spread for the patients’ outcome on the one hand and on the other draft a concept by which CXCR4 influences this powerful prognostic marker. So while metastatic disease determines the outcome, CXCR4 facilitates its formation.

CXCR7 on the other hand was almost not expressed in our MTC tissue probes, which is in line with the study by Zhu et al. There, only endothelial cells of adjacent blood vessels stained partially positive for CXCR7 (Zhu et al, 2016).

**Table 2. Overall survival analysis**

| Variables                  | HR   | CI (lower–upper 95%) | $P$-value |
|----------------------------|------|----------------------|-----------|
| **Univariate survival analysis** |      |                      |           |
| Age at first diagnosis     | 3.740| 1.477–9.469          | **0.005** |
| Sex                       | 1.893| 0.749–4.785          | 0.176     |
| T1/2 vs T3/4              | 3.641| 1.115–11.89         | **0.032** |
| N negative vs N positive   | 4.773| 1.879–12.13          | **0.001** |
| M0 vs M1                  | 21.45| 6.304–72.98          | < 0.001   |
| Sporadic vs MEN2A          | 6.289| 2.193–18.03          | < 0.001   |
| Calcium basal blood level  | 3.688| 0.856–15.89          | 0.080     |
| CXCR4 expression           | 4.474| 1.671–11.98          | **0.003** |
| **Multivariate survival analysis** |      |                      |           |
| Age at first diagnosis     | 3.1662| 0.089–1.116        | 0.074     |
| Sex                       | 0.953| 0.328–2.766         | 0.930     |
| T1/2 vs T3/4              | 1.074| 0.360–3.202         | 0.898     |
| N negative vs N positive   | 2.193| 0.401–11.987        | 0.365     |
| M0 vs M1                  | 2.219| 0.558–8.823         | 0.258     |
| Sporadic vs MEN2A          | 4.523| 0.507–40.300        | 0.176     |
| Calcium basal blood level  | 0.946| 0.313–2.860         | 0.922     |
| CXCR4 expression           | 1.045| 0.867–1.261         | 0.643     |

**Multivariate survival analysis after stepwise variable selection**

| Variables | HR   | CI (lower–upper 95%) | $P$-value |
|-----------|------|----------------------|-----------|
| M0 vs M1  | 11.47| 2.941–44.71          | < 0.001   |

Abbreviations: CI = confidence interval; CXCR4 = C-X-C chemokine receptor type 4; HR = hazard ratio; MEN2A = multiple endocrine neoplasia type 2A. The bold values are statistically significant.

**Figure 3. Expression levels of CXCR4 and CXCR7 in MTC cell line TT.** (A) Immunocytochemical staining of CXCR4 and CXCR7 in MTC cell line TT using Alexa Fluor 488 as secondary antibody. DAPI was used for visualisation of the nucleus. Overlays demonstrate the composite images for both staining procedures. Antibody specificity was confirmed with isotype controls (control). Images were captured using a fluorescence microscope at $\times$ 400 magnification. Bar at the top left corner indicates 50 µm. (B) Western blot analysis for both CXCR4 and CXCR7 in MTC cell line TT. GAPDH served as loading control. CXCR4/7 = C-X-C chemokine receptor type 4/7.
Figure 4. rh-SDF1α induces tumour cell invasion, cell cycle activation and EMT. (A) Representative pictures of matrigel invasion membranes stained with DAPI for nuclear visualisation after treatment of human MTC cell line TT with CXCR4 antagonising compounds AMD3100 and WZ811 as well as chemokine receptor agonist rh-SDF1α. (B) The number of invading cells was estimated by counting cells in five visual fields of at least three separate membranes and illustrated as fold change to control. (C) After treatment with rh-SDF1α, cell cycle analyses were performed using PI staining and FACS. Cell populations are grouped according to the distinct cell cycle phase. (D) Changes in mRNA expression levels of EMT-associated genes were evaluated using qRT–PCR where GAPDH served as housekeeping gene. Fold changes were calculated using the $2^{-\Delta\Delta CT}$ method. All plots display the mean with s.e.m. Numerical data were analysed using the non-parametric Mann–Whitney U test. CT values are displayed in Supplementary Table 3. Ctrl = vehicle control for the highest concentration; *P<0.05; **P<0.01; ***P<0.001.

Considering the pivotal role of CXCR4 for tumour cell dissemination, the significantly higher expression of the chemokine receptor in lymph node metastases compared to the expression in the corresponding primary MTC specimens fits perfectly into our expanding knowledge of the CXCR4/CXCL12 axis in carcinoma. Mechanistically, the homeostatic microenvironment chemokine CXCL12 functions as a chemoattractant for CXCR4-expressing cells and modulates intracellular calcium flux, cell cycle activation and chemotaxis (Mego et al., 2016). This in turn leads to the initiation of different cell signalling pathways, which promote EMT, the forming of an invasive phenotype and tumour cell progression (Sobolik et al., 2014; Tu et al., 2016; Wu et al., 2016).

Our functional in vitro experiments demonstrated for both CXCR4 antagonising compounds AMD3100 and WZ811 a significant reduction in the number of invading cells. These observations are in line with the current literature where multiple studies have shown an anti-metastatic effect for both compounds. WZ811 exhibits a profound anti-tumourigenic potential in chronic lymphocytic leukaemia and negatively influences CD3-positive T-cell migration both in vitro and in vivo (Jung et al., 2014; Li et al., 2016). AMD3100 on the other hand is known to reduce the metastatic potential in small cell lung carcinoma (Taromi et al., 2016), breast cell carcinoma (Wendel et al., 2012) and oral squamous cell carcinoma (Uchida et al., 2007). Its predominant clinical application, however, is its capacity to mobilise haematopoietic stem cells from the bone marrow after transplantation for haematopoietic malignancies. Since cell migration in and out of the bone marrow follows opposite chemokine gradients, AMD3100 has proven itself a useful asset, especially in patients predicted to be poor stem cell mobilisers (Bilgin and de Greef, 2016; Danylko et al., 2016).

The fact that a co-incubation with rh-SDF1α could not overcome the inhibitory effect of the two CXCR4-specific antagonists in a cell model where both CXCR4 and CXCR7 were equally expressed, suggests that a functioning CXCR4 is mandatory for the CXCR4/CXCL12 axis to fully evolve in MTC and cannot be entirely compensated by CXCR7 alone. Notably, one study by Kalatskaya et al even suggests AMD3100 as a ligand to CXCR7 with allosteric agonist properties, questioning not only its CXCR4-specificity but also its functionality (Kalatskaya et al., 2009). These observations support the hypothesis that CXCR7 functions as regulator to CXCR4 G protein signalling (Sierro et al., 2007; Sánchez-Martín et al., 2013). However, further functional experiments are warranted to fully elucidate the complex interaction between CXCR4, CXCR7 and their mutual ligand CXCL12.

The incubation of MTC cell line TT with rh-SDF1α resulted in an activation of cell cycle and subsequent increase in the number of invading cells. Of note however, the increase in cell count in the G2/M phase did not translate into an increase in cell proliferation as investigated by BrdU colorimetric ELISA assay. The discrepancy may be attributed to the slow doubling time of the cell line and the fact that all in vitro studies were conducted in serum-starved culture medium. Owing to these circumstances, the respective lengths of the incubation periods of the MTC cell line were limited as the cultured cells succumbed to serum starvation and did not incorporate BrdU in a sufficient manner to corroborate the results from the FACS analyses. The CXCR4/CXCR7/CXCL12 axis has been implicated with EMT in different tumour types, which in turn was held responsible for
enhanced invasive growth and metastatic spread. As a marker for EMT, we investigated the changes in expression levels for Vimentin and E-cadherin following rh-SDF1x treatment. We could demonstrate that the incubation of MTC cell line TT with rh-SDF1x resulted in a significant upregulation of Vimentin while the expression of E-cadherin was markedly reduced, suggesting a cellular transition from epithelial to mesenchymal phenotype. Additionally, mRNA levels of EMT-associated transcriptional factors BST2 and FGFR9 were markedly upregulated after rh-SDF1x treatment.

BST2 is a known activator of NF-kB, whose activation promotes tumour cell invasion and metastasis through EMT (Matsuda et al., 2003; Wu and Bonavida, 2009). In breast carcinoma BST2 has been identified as an independent marker for metastasis, its overexpression causing anchorage independent growth and facilitating invasiveness (Woodman et al., 2016). GF9 on the other hand has been associated with EMT via the upregulation of vascular endothelial growth factor (Teishima et al., 2014) and its expression is linked to poor prognosis and a metastatic phenotype in different solid tumours (Ueng et al., 2010; Oghto et al., 2014; Ren et al., 2016). The increased mRNA expression of these markers may be seen as an indicator of the underlying cellular machinery of the CXCR4/CXCR7/CXCL12-mediated metastatic push, but still requires further investigation to fully appreciate the molecular mechanics.

Since invasive growth and the subsequent formation of distant metastases are the primary deciding factors for fatal outcome, a better understanding of the molecular pathways of tumour cell dissemination and invasion is mandatory in developing new therapeutic strategies. Here, we propose the CXCR4/CXCR7/CXCL12 axis as an important functional determinant in MTC tumour biology. With its distinct expression profile and strong association with disseminated disease, CXCR4 can be considered as a new and viable therapeutic target in advanced MTC. Especially its involvement in the induction of EMT offers new valuable insight into the oncogenesis of MTC and should provide encouragement for further preclinical studies.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies this paper on British Journal of Cancer website (http://www.nature.com/bjc)