Tumor-associated macrophages promote the metastatic potential of thyroid papillary cancer by releasing CXCL8

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Tumor-associated macrophages (TAMs) can promote cancer initiation and progression by releasing cytokines. Previously, we have found the density of TAMs correlated with lymph node metastasis in papillary thyroid carcinoma (PTC). However, the mechanisms of how TAMs promote PTC progression remain unclear. In this study, we first showed that the TAMs density in the tumor core was associated with progressive PTC features and TAMs conditioned medium enhanced PTC cells invasion. Cytokine profiling identified a mixed M1/M2 phenotype and CXCL8 was the most consistently abundant cytokine in PTC-derived TAMs. CXCL8 receptors, CXCR1 and CXCR2, were positively stained in PTC cell lines and tissues, though no association with lymph node metastasis or extrathyroid extension. PTC cell invasion was abrogated by anti-CXCL8-neutralizing antibody, whereas addition of exogenous recombinant human CXCL8 enhanced the invasiveness. More importantly, CXCL8 promoted PTC metastasis in vivo. No difference was found for TAMs-derived CXCL8 expression in patients with and without lymph node metastasis or extrathyroid extension. These findings indicated that TAMs may facilitate PTC cell metastasis through CXCL8 and its paracrine interaction with CXCR1/2.

Introduction

Tumor microenvironment is known to play a critical role in cancer initiation, progression and metastasis (1–3). A variety of non-malignant stromal cells comprise the tumor microenvironment; tumor-associated macrophages (TAMs) are one of the most important components (4,5). Evidence from clinical and epidemiological studies has shown a strong association between TAMs density and poor prognosis in various cancers, including thyroid cancer (4–6). Increased density of TAMs was shown in advanced thyroid cancers and was correlated with decreased cancer-related survival (7,8). In our previous study, we found the density of TAMs was correlated with lymph node metastasis in patients with papillary thyroid carcinoma (PTC) (6).

Macrophages are differentiated cells of the mononuclear phagocytic lineage (9). They are heterogeneous cells with distinct functions that respond differently to various microenvironmental signals and thus display distinct functions (10,11). TAMs function predominantly as tumor enhancers rather than inhibitors. Mechanistic studies have revealed that TAMs promote cancer progress and metastasis through the release of a variety of chemokines, inflammatory factors and growth factors (5,12,13). Several TAMs-derived cytokines have been associated with tumor progression (14,15). In colon adenocarcinoma, tumor necrosis factor-α (TNF-α) and interleukin-6 are secreted by infiltrating macrophages and correlate with increased tumor invasion (16). In skin cancer, TAMs have been shown to be a major source of matrix metalloproteinase-9, which contributes to tumor cell invasion (17). Macrophage-derived epidermal growth factor has been demonstrated to promote tumor cell invasion in breast cancer (18), while more recent studies indicate breast cancer metastasis can be mediated by secretion of CCL18 in TAMs and via PITPNM3 (19). A recent study showed TAMs were protumorigenic in advanced PTCs and targeting of CSF-1/CSF-1R inhibited TAMs recruitment and impaired BRAF-induced thyroid cancer progression (8). However, the molecular mechanisms of how TAMs facilitate thyroid cancer progression remain largely unknown.

Current study was designed to define the impact of TAMs-derived cytokines on PTC cell tumorigenesis. By cytokine profiling on primary isolated TAMs and functional study, we found TAMs promoted PTC invasion in a paracrine manner by releasing a panel of cytokines and CXCL8 was the most abundant.

Materials and methods

PTC tissue collection and immunohistochemistry

The board of medical ethics of Ruijin Hospital approved the study and all patients gave their written informed consent. Histologically confirmed PTC were collected and the sectioned paraffin-embedded tissues were used for immunohistochemistry analysis (6), using rabbit polyclonal antibodies against CXCR1 (1:200; Abcam, Cambridge, MA) and CXCR2 (1:150; Abcam) and mouse monoclonal antibody against CD68 (1:200; Dako, Glostrup, Denmark), respectively. Negative controls were incubated without primary antibody. A single pathologist, who was blinded to the clinical assessments of each case, scored immunostains and counted CD68+ TAMs under ×400 magnification. The number of TAMs, at least six independent fields at the peripheral and core areas were evaluated. The peripheral area was defined as the interface between the tumor and the adjacent non-neoplastic tissue. CD68+ cell counts were expressed as the mean ± SD per field. The CXCR1 and CXCR2 staining was classified as follows: 0, negative; +, <50% positive rate; ++, 50–60% positive rate and ++++, > 60% positive rate.

Isolation of TAMs

TAMs were isolated as described previously (20,21). Briefly, surgically removed PTC tissue was cut into 1 mm3 fragments, followed by type II collagenase digestion (3 mg/ml; Sigma–Aldrich, Buchs, Switzerland) for 2 h at 37°C. The suspension was filtered through a 70 μm cell strainer (BD Falcon; BD Bioscience, Bedford, MA) to generate a single cell suspension. The suspension was centrifuged and washed twice with phosphate-buffered saline (PBS). Cells were cultured in serum-free RPMI 1640 for 40 min and non-adherent cells were removed with a medium change. Conditioned medium (CM) was collected as cell culture supernatants in serum-free 1640 medium 24 h after TAMs isolation.

Cell lines

K1 cells were maintained in Dulbecco’s modified Eagle’s medium: Ham’s F12: MCDB105 (2:1:1) (Gibco, Grand Island, NY) medium supplemented with 10% fetal bovine serum. TPC-1 and BCPAP cells were maintained in 1640 medium (Gibco) supplemented with 10% fetal bovine serum. Peripheral blood monocytes from healthy donors were isolated by density gradient

Abbreviations: CM, conditioned medium; mRNA, messenger RNA; PBS, phosphate-buffered saline; PTC, papillary thyroid carcinoma; qRT–PCR, quantitative real-time reverse transcription–polymerase chain reaction; TAM, tumor-associated macrophage; TGF-β2, transforming growth factor-β2; TNF-α, tumor necrosis factor-α.

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centrifugation using Ficoll-Hypaque (Pharmacia, Peapack, NJ) according to the manufacturer’s instruction.

**Cytokine array analysis of TAMs-CM**

A total of 80 cytokines were assessed by using human cytokine array (RayBiotech, Norcross, GA) according to the manufacturer’s instructions. Briefly, the slides were blocked for 30 min, incubated with TAMs-CM (100 μl per well) overnight at 4°C, then followed by biotin-conjugated cocktail of antibodies and horseradish peroxidase-conjugated streptavidin for 2 h, respectively. The signals were detected with GMS 418 array scanner (Genetic Microsystems, Woburn, MA) after incubation with Alexa flour 555-conjugated streptavidin in darkness for 2 h.

**Cell invasion assay**

Cell invasion was examined in Boyden chambers (Corning, Corning, NY) with 8 μm pore inserts coated with matrigel (BD Biosciences, Franklin Lakes, NJ) as previously reported (22). After trypsination, PTC cells were plated on the inserts and cultured at 37°C in the upper chambers with 200 μl of serum-free medium. The growth medium containing 10% fetal bovine serum was used as a chemoattractant in the bottom well. Following 24 h of incubation, cells on the upper surface were removed and the invaded cells on the lower surface were stained with 0.01% crystal violet (Sigma Chemical Co., St Louis, MO). The invaded cells were counted as cells per field under phase contrast microscopy. Assays were carried out in triplicate.

**Immunofluorescence**

TAMs and PTC cell lines were adhered to 8-well culture slides (BD Biosciences, Heidelberg, Germany), fixed in 4% paraformaldehyde at room temperature for 20 min and incubated with primary antibodies against CD68 (1:100; Dako), CXCR1 (1:200; Abcam) and CXCR2 (1:150; Abcam), respectively, at 4°C overnight. The secondary antibody was fluoresce-labeled donkey anti-rabbit antibody (1:400; Invitrogen, Carlsbad, CA). Nuclei were stained with 4′,6-diamidino-2-phenylindole. Image was taken at ×400 magnification on an Olympus-BX51 microscope. TAMs immunofluorescence was repeated in three randomly selected patients, and PTC cell lines were repeated three times.

**Quantitative real-time reverse transcription–polymerase chain reaction**

Quantitative real-time reverse transcription–polymerase chain reaction (qRT–PCR) was performed in a LightCycler480 instrument (Roche Diagnostics, Basel, Switzerland), using the SYBR Premix Ex TaqTM (TaKaRa, Shiga, Japan). All reactions were performed in triplicate. Following an initial denaturation at 95°C for 30 s, 40 cycles of PCR amplification were performed at 95°C for 5 s and 60°C for 20 s. The specificity of the reaction was verified by melt curve analysis and agarose gel electrophoresis. The comparative CT method (AACT method) was used to determine the quantity of the target sequences. Oligonucleotide sequences of qRT–PCR primers were as follows: CXCL8, forward-5′-TGTCGCTCTCTGTCTCCTCA-3′ actin, forward-5′-ACTGCGAGGAGGA-3′ reverse-GTGGAGAGAGAGAGAGAGA-3′ reverse-GTACCCTCTTCTGCGTGC.

**Animal study**

Male NOD/SCID mice of ages 5–12 weeks were bred and maintained under defined conditions at the Animal Experiment Center of Shanghai Jiaotong University, School of Medicine, and all procedures were approved by the Animal Care and Use Committee of Shanghai Jiaotong University, School of Medicine, conforming to the legal mandates and national guidelines for the care and maintenance of laboratory animals. To evaluate the effects of CXCL8 on metastatic potential, BCPAP cells were injected (3 × 10⁵ cells) intravenously into the tail vein of mice on day 0 and exogenous recombinant human 72-amino acid CXCL8 (rCXCL8) (1 μg per mouse, 2 μg per mouse and PBS as control) was injected intraperitoneally from day 1 to day 3. On the day of injection, rCXCL8 was given 2 h before tumor cell injection and the subsequent doses were given in intervals of 24 h. On day 40, the mice were killed and dissected. Metastatic lesions were counted and measured. Hematoxylin and eosin-stained sections from the metastatic lesions were analyzed.

**Statistical analysis**

Prism 5.0 (GraphPad Software, La Jolla, CA) and SPSS (Version 13.0 software; SPSS, Chicago, IL) were used to perform the analysis. Data was expressed as mean ± SD. The Mann–Whitney test was used for non-numerical variables, whereas Student’s t-test was used for numerical variables. Kaplan–Meier survival curves were plotted, and log rank test was performed. A P value <0.05 was accepted as a significant difference.

**Results**

**TAMs density in the tumor core area was associated with lymph node metastasis and extrathyroid extension in PTC patients**

As shown in Table I and Supplementary Figure S1, available at Carcinogenesis online tumor core area showed a higher density
of TAMs staining than peripheral area of PTC tumor (25.32 ± 12.62 versus 22.83 ± 6.61, \( P = 0.0428 \)). TAMs density in the core area was significantly associated with lymph node metastasis (17.58 ± 6.29 versus 34.18 ± 12.22, lymph node negative versus lymph node positive, \( P = 0.000 \)), extrathyroid extension (23.53 ± 11.96 versus 29.68 ± 13.30, no extrathyroid extension versus extrathyroid extension, \( P = 0.024 \)) and TNM (i.e. T, primary tumor, N, regional lymph nodes, M, distant metastasis) stage (22.71 ± 11.08 versus 32.64 ± 13.94, stage I–II versus stage III–IV, \( P = 0.000 \)). We did not observe any significant association between TAM density in peripheral area and PTC tumor features.

**TAMs promoted PTC cell invasion**

The isolation of TAMs from PTC tumor generated populations with over 95% CD68-positive cells (Figure 1A). CM derived from culturing isolated PTC-TAMs for 24h was tested for its effect on PTC cell invasiveness. Boyden chamber assays were performed using three well-characterized PTC cell lines. As shown in Figure 1B, the number of invaded cancer cells increased significantly when exposed to TAMs-CM. Compared with the control, TAMs-CM enhanced the invasiveness of K1, TPC-1 and BCPAP cells by 50, 53.76 and 73%, respectively. We did not observe a significant change of cell proliferation or apoptosis in PTC cell lines treated with TAMs-CM (Supplementary Figures S2 and S3, available at Carcinogenesis Online).

**Cytokine array analysis revealed high level of CXCL8 in TAMs-CM**

To identify the cytokines produced by PTC-derived TAMs, we performed cytokine array assays using TAMs-CM generated from eight patients. Elevated levels of TNF-\( \alpha \), TNF-\( \beta \), macrophage inflammatory protein, transforming growth factor-\( \beta \)2 (TGF-\( \beta \)2), CXCL8 (also known as interleukin-8), and cokain were observed. Of note, CXCL8 level was consistently high in all the TAMs-CM (Figure 2A and B). Examination of CXCL8, TNF-\( \alpha \) and TGF-\( \beta \)2 messenger RNA (mRNA) levels by qRT–PCR in 15 TAMs and PTC cell lines confirmed their overexpression was predominantly confined to TAMs (Figure 2C), with CXCL8 ranking number one. We therefore focused on CXCL8 in the following study.

**Expression of CXCL8 receptors in PTC tissues and PTC cell lines**

Both CXCR1 and CXCR2 have been reported as the receptors of CXCL8. We analyzed immunostaining of CXCR1 and CXCR2 in a total of 26 PTC tissues and 6 normal thyroid tissues. A diffuse intracytoplasmic granular staining demonstrated positive CXCR1 and CXCR2 immunoreactivity. None of the normal thyroid tissue showed CXCR1/CXCR2 immunostaining in thyroid follicular epithelial cells. We found 83% of PTCs were ++ or +++ for CXCR1 staining and 71.5% for CXCR2 staining (Figure 3A). Mann–Whitney test showed no difference between PTC patients with and without lymph node metastasis (CXCR1, \( P = 0.801 \); CXCR2, \( P = 0.917 \)), as well as PTC patients with and without extrathyroid extension (CXCR1, \( P = 0.823 \); CXCR2, \( P = 0.815 \)). For PTC cell lines, we performed fluorescence staining and found positive cell membrane localization of CXCR1 and CXCR2 expression (Figure 3B).

**CXCL8 promoted PTC cell invasion**

To confirm that CXCL8 directly contributes to PTC cell invasion, TAMs-CM was pretreated with anti-CXCL8-neutralizing antibody.

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**Fig. 1.** Conditioned medium from TAMs promoted the invasion of papillary thyroid cancer cells. (A) Immunofluorescent staining of CD68 in TAMs. Original magnification, \( \times 400 \). (B) TAMs-CM promoted PTC cell invasion. PTC cell lines (K1, TPC-1, BCPAP) were plated on the upper cell culture inserts with serum-free medium (Control) or with serum-free medium following 24h of TAMs culture (TAMs-CM). The lower chamber contained 10% fetal bovine serum as a chemoattractant. Bars corresponded to mean ± SD. \(* P < 0.05\) and \( ** P < 0.01\), compared with the cells treated with serum-free medium. Each experiment was repeated three times.
TAM-derived CXCL8 promotes PTC invasion

Antibody-mediated neutralization of CXCL8 partially abrogated the invasion-promoting capacity of TAMs-CM. PTC cell invasion was reduced by 43.6, 29.3 and 36.6% in K1, TPC-1 and BCPAP cells, respectively, at 1 μg/ml anti-CXCL8 antibody and by 72.0, 39.6 and 52.8%, respectively, at higher concentration (10 μg/ml) (Figure 4A). Consistent with this observation, treatment of PTC cells with exogenous recombinant CXCL8 (rCXCL8) enhanced invasion in a dose-dependent manner (Figure 4B). At the highest treatment dosage, K1, TPC-1 and BCPAP cell invasion was enhanced by 61.4, 97.8 and 110%, respectively. We did not observe a significant change of cell proliferation or apoptosis in PTC cell lines treated with exogenous CXCL8 (Supplementary Figures S4 and S5, available at Carcinogenesis Online). Collectively, these data suggested that TAMs-derived CXCL8 played an important role in promoting PTC cell invasion.

CXCL8 enhanced PTC metastasis in vivo

Kaplan–Meier analysis demonstrated a significant decline of survival curve for mice treated with rCXCL8 than PBS (P = 0.0249) (Figure 5). The percentage of mice with metastasis and/or death was greater for rCXCL8-treated mice than control, while no significant difference was observed for rCXCL8-1 μg or rCXCL8-2 μg. Moreover, rCXCL8 induced larger metastatic lesions than control (1.82 ± 0.87, 8.46 ± 6.01, 10.03 ± 7.99 mm for PBS, 1 μg, 2 μg, respectively) (Supplementary Table S1, available at Carcinogenesis Online).

Elevated TAMs-derived CXCL8 mRNA expression was not associated with lymph node metastasis or extrathyroid extension in PTC patients

Because CXCL8 was associated with enhanced in vitro invasion, we first analyzed mRNA expression in TAMs from primary tumors derived from PTC patients with (n = 27) and without lymph node metastasis (n = 16). As shown in Supplementary Figure S12, available at Carcinogenesis Online, there was no significant difference between PTC patients with and without lymph node metastasis (P = 0.8911). And no significant difference between PTC patients with (n = 15) and without (n = 28) extrathyroid extension (P = 0.2409). Examination of other clinicopathological features also failed to find significant correlations with CXCL8 mRNA level.

Discussion

In this study, we found the density of TAMs in the core area of PTC tumor was associated with tumor metastasis and invasion; CM from PTC-derived TAMs promoted PTC cell line invasion by releasing a panel of cytokines; CXCL8 was the most abundant cytokine, which mediated TAMs-dependent PTC cell invasion and enhanced PTC metastasis in vivo. These results suggest that TAMs might regulate PTC tumor behavior through CXCL8-CXCR1/2 pathway.

TAMs have been proposed to facilitate cancer progression through several mechanisms such as suppressing immune activation, facilitating extracellular matrix remodeling, promoting angiogenesis and tumor growth as well as enhancing tumor cell migration and invasion (4, 5, 13). Little is known regarding possible roles of TAMs in thyroid cancer while only correlation studies have been reported (6,7,23). The first published examination of TAMs reported a negative correlation of macrophage phagocytosis with vascular invasion and distant metastasis in well-differentiated thyroid cancer (23). More recently, increased TAMs number in high-grade thyroid cancers was found to be associated with invasive cancers and decreased cancer-related survival (7). The same group showed TAMs were protumorigenic in advanced PTCs and targeting of CSF-1/CSF-1R inhibited TAMs recruitment and impaired BRAF-induced thyroid cancer progression most recently (8). Our group demonstrated that increased infiltration...
of TAMs in PTC primary tumors was correlated with advanced TNM stage (T, extent of the primary tumor; N, absence or presence of the disease into the lymph nodes metastasis; M, absence or presence of distant metastasis) and lymph node metastasis (6). However, none of these studies examined the mechanism(s) through which TAMs function to enhance tumor progression in thyroid cancer. In this study, we demonstrated that TAMs-CM promoted invasion of three established PTC cell lines, suggesting cytokines produced by TAMs may facilitate thyroid cancer progression. Of note, we found no effect of TAMs-CM on the proliferation or apoptosis of PTC cells (Supplementary Figures S2 and S3, available at Carcinogenesis Online).

In an effort to identify the primary mediator driving in vitro invasion of PTC cells, we performed antibody array analysis on CM generated from eight independent PTC TAMs isolation. We found high levels of TNF-α, TNF-β, macrophage inflammatory protein, TGF-β2, CXCL8 and eotaxin. This cytokine pattern identified a mixed M1- and M2-related phenotype. Although M1 macrophage is linked with tumoricidal activity, M2 macrophage is associated with cancer progression and metastasis (24). However, TAMs were reported to be composed of several distinct populations that share features of both M1 and M2 phenotype (25,26). This is consistent with our findings. The heterozygous phenotype might represent a transition status after macrophage infiltration. Of 80 profiled cytokines on the array, CXCL8 was consistently the most abundant cytokine detected. Positive CXCR1/2 staining was also detected on PTC tumor section. CXCL8 is a proinflammatory CXC chemokine that was first purified and cloned from lipopolysaccharide-stimulated human mononuclear cell supernatants in 1987. CXCL8, alternatively known as interleukin-8, was originally identified as a neutrophil chemoattractant and played as inflammatory mediator (27) and angiogenesis promoter (28). Subsequently, CXCL8 has been shown to contribute to human cancer progression through mitogenesis, angiogenesis (29) and metastasis (30) in a variety of cancers, such as melanoma (31), carcinoma of breast (32), stomach (33), prostate (34), nasopharynx (35) and liver (36). Elevated serum CXCL8 level has also been found to be a prognostic marker in cancer patients (37,38). In our experiments we found blocking CXCL8 signaling abrogated PTC cell invasion, whereas exogenous CXCL8 increased PTC cell invasion and promoted PTC metastasis in NOD/SCID mice. Therefore, CXCL8 appears to mediating thyroid cancer progression in a paracrine manner.

However, it is important to note that CXCL8 can be produced by a variety of cell types, including macrophages, endothelial cells and

![Fig. 3. Positive staining of CXCR1 and CXCR2 in PTC tissues and cell lines. (A) Immunohistochemical staining of CXCR1 and CXCR2 in PTC tissue sections. No significant difference between PTC patients with and without lymph node metastasis (LN+, LN−) or with and without extrathyroid extension (EX+, EX−). (B) Immunofluorescent staining of CXCR1 and CXCR2 in PTC cell lines.](Downloaded from https://academic.oup.com/carcin/article-abstract/35/8/1780/317968 by guest on 12 February 2019)
cancer cells (39, 40). Studies have also shown CXCL8 can be produced by thyroid follicular cell, both normal (41, 42) and malignant (43). Interestingly, the pro-protein of CXCL8 is processed differently in non-immune cells and monocyte/macrophages. Two isoforms of CXCL8 protein were reported and the length was 77 amino acids and 72 amino acids, respectively. The 72-amino acid form of CXCL8, which lacks the Leu-Pro-Arg sequence, has been shown by several groups to be more potent than human CXCL8 (77 aa) as chemotactic factor (44). Moreover, in this study, we found TAMs produced much more CXCL8 than PTC cancer cell not only at mRNA level but also at protein level (data not shown). Collectively, we proposed TAMs-derived CXCL8 should take an active role in thyroid cancer progression, possibly by paracrine binding on CXCR1/2 on the PTC cell. Using TAMs CXCL8 mRNA expression, we failed to find a significant correlation between expression levels and clinicopathological features of PTC patients. This finding seems to contradict with the in vitro findings reported here. Four possible explanations may account for this difference. First, the sample size (n = 46) was small, less than half that was used in our previous analysis (6). A more likely explanation, however, was that TAMs number/density rather than CXCL8 mRNA level impacts PTC progression. The data shown in Supplementary Figure S12, available at Carcinogenesis Online, other cytokines including TNF-α and TGF-β2 regulated PTC invasion and metastasis as well. PTC cells showed minimum invasion even after blocking CXCL8, suggesting PTC invasion was not solely dependent on CXCL8. Another limitation of this study was the lack of nodal metastasis in the mice model of PTC. Neither orthotopic nor subcutaneous mice model of PTC develops distant or nodal metastases. In the current study, we injected PTC cell through tail vein of NOD/SCID mice and analyzed metastatic lesions.

In summary, we found TAMs promoted PTC metastasis by releasing a panel of cytokines in a paracrine manner; CXCL8 was the most abundant; blocking CXCL8 significantly reduced PTC metastasis in vitro and exogenous CXCL8 enhanced PTC metastasis in vivo. Our data are the first to report that TAMs facilitate PTC cell invasion through the CXCL8 pathway. They suggest that targeting the CXCL8 signaling pathway within the tumor microenvironment may provide therapeutic benefit.

Fig. 4. TAMs-CM-mediated PTC cell invasion was dependent on CXCL8. (A) Anti-CXCL8-neutralizing antibody abrogated TAMs-CM function. PTC cell lines were plated on the upper chamber with TAMs-CM in the presence of anti-CXCL8-neutralizing antibody or an isotype-matched IgG. The lower chamber contained 10% fetal bovine serum as a chemoattractant. *P < 0.05 and **P < 0.01, compared with cells treated with TAMs-CM. (B) rCXCL8 enhanced PTC cell invasion in a dose-dependent manner. PTC cell lines treated without or with different concentrations of rCXCL8 were plated on the upper cell culture inserts. The lower chamber contained 10% fetal bovine serum as a chemoattractant. *P < 0.05 and **P < 0.01, compared with the cells treated with serum-free medium.
Fig. 5. Exogenous rCXCL8 promoted PTC metastasis in vivo. (A) Kaplan–Meier survival curve for the mice bearing BCPAP cancer with and without rCXCL8 treatment. (B) Gross pulmonary metastatic lesion. (C) Hematoxylin and eosin staining of pulmonary metastatic lesion.

Supplementary material

Supplementary Table S1 and Figures S1–S12 can be found at http://carcin.oxfordjournals.org/

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References

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