Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer

Mabel Ryder[1,2], Ronald A Ghossein[3], Julio C M Ricarte-Filho[2], Jeffrey A Knauf[1,2] and James A Fagin[1,2]

[1]Endocrinology Service, Department of Medicine, [2]Human Oncology and Pathogenesis Program and [3]Department of Pathology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 296, New York, New York 10021, USA

(Correspondence should be addressed to M Ryder; Email: ryderm@mskcc.org)

Abstract

Thyroid cancers are infiltrated with tumor-associated macrophages (TAMs), yet their role in cancer progression is not known. The objectives of this study were to characterize the density of TAMs in well-differentiated (WDTC), poorly differentiated (PDTC), and anaplastic thyroid cancers (ATC) and to correlate TAM density with clinicopathologic parameters. Immunohistochemistry was performed on tissue microarray sections from WDTC ($n=33$), PDTC ($n=37$), and ATC ($n=20$) using macrophage-specific markers. Electronic medical records were used to gather clinical and pathologic data. Follow-up information of PDTC patients was available for 0–12 years. In total, 9 out of 33 WDTC (27%), 20 out of 37 PDTC (54%), and 19 out of 20 ATC (95%) had an increased density of $CD68^+$ TAMs (≥ 10 per 0.28 mm^2; WDTC versus PDTC, $P=0.03$; WDTC versus ATC, $P<0.0001$; PDTC versus ATC, $P<0.002$). Increased TAMs in PDTC was associated with capsular invasion ($P=0.034$), extrathyroidal extension ($P=0.009$), and decreased cancer-related survival ($P=0.009$) compared with PDTC with a low density of TAMs. In conclusion, the density of TAMs is increased in advanced thyroid cancers. The presence of a high density of TAMs in PDTC correlates with invasion and decreased cancer-related survival. These results suggest that TAMs may facilitate tumor progression. As novel therapies directed against thyroid tumor cell-specific targets are being tested, the potential role of TAMs as potential modulators of the thyroid cancer behavior will need to be considered.

Endocrine-Related Cancer (2008) **15** 1069–1074

Introduction

Infiltrating inflammatory cells are major constituents of tumor microenvironments, of which tumor-associated macrophages (TAMs) may comprise as much as 50% of the tumor mass. Understanding the functions of TAMs on tumor progression is complex due to the pleiotropic actions of macrophages on tumor progression (Lewis & Pollard 2006). Clinical studies of human breast (Leek *et al*. 1996, Yu 2003, Tsutsui *et al*. 2005, Bolat 2006), prostate (Lissbrant *et al*. 2000), and cervical (Schoppmann *et al*. 2002) cancers support a role of TAMs as tumor promoters based on the association of increased density of TAMs with tumor vascularization, metastases, and poor prognosis. Evidence for a key role of TAMs in cancer progression is further buttressed by the fact that selective depletion of TAMs in breast cancer mouse models disrupts transformation, progression, and invasion (Lin *et al*. 2006). The abundance of TAMs in the various thyroid cancer histotypes is relatively understudied (Herrmann *et al*. 1994), and there is little information on their possible role on thyroid cancer progression. The goal of this study was to examine the presence of TAMs in several histological thyroid cancer grades and, in an unbiased manner, correlate the presence of increased TAMs with clinicopathologic outcomes. We found a strong association between TAM abundance and advanced histological grade. We further show that TAM density was strongly associated with tumor invasiveness and cancer-related mortality in poorly differentiated thyroid cancers (PDTC).

DOI: 10.1677/ERC-08-0036

1351–0088/08/015–001069 *Online version via http://www.endocrinology-journals.org*

Materials and methods

Study design

Immunohistochemical (IHC) analyses were performed on paraffin-embedded human tissue microarray sections from blocks of well-differentiated thyroid cancers (WDTC; $n=33$), PDTC ($n=37$), and anaplastic thyroid cancers (ATC; $n=20$), and corresponding non-neoplastic and non-thyroiditis thyroid tissues ($n=46$) from WDTC ($n=30$) and PDTC ($n=15$) using two monoclonal antibodies that primarily label tissue macrophages: anti-CD68 KP-1 (pre-diluted according to the manufacturer; Ventana, Tucson, AZ, USA), and anti-CD163 (1:100, Vector, Burlingame, CA, USA). Each tumor was represented by three tissue cores taken from randomly chosen fragments of the tumor (0.6 mm diameter per core). Of the 33 WDTC, 19 were classical papillary thyroid cancer (PTC), 13 were follicular variant PTC (FVPTC), and 1 was a Hürthle cell carcinoma. Of that, 9 out of 19 classical PTC and 0 out of 13 FVPTC had an increased density of TAMs ($P=0.004$). PDTC were defined as carcinomas showing follicular cell differentiation (at the histological and/or IHC level, i.e., positive for thyroglobulin) with tumor necrosis and/or ≥ 5 mitoses per 10 high-power fields (400×). Positive controls included tonsillar tissue and giant cell tumors rich in histiocytes. The negative control consisted of the isotype mouse monoclonal control antibody (clone MOPC-1, Ventana), which showed no specific immunostaining. A single pathologist (R A G), who was blinded to the clinical assessments of each case, scored the immunostains by counting the number of $CD68^+$ TAMs in each of the three tissue cores from each patient's tumor sample (total core surface: 0.28 mm^2), and took the mean of three counts. Sections scored with ≥ 10 $CD68^+$ TAMs/0.28 mm^2 were designated positive for a high density of TAMs and those with <10 $CD68^+$ TAMs/0.28 mm^2 were designated as negative.

Genotype

Genotyping for oncogenic $BRAF^{V600E}$ was performed on WDTC and PDTC using 30 micron paraffin-embedded tissue sections. DNA was extracted using the Puregene DNA purification kit for 5–10 mg tissue (Gentra, Qiagen, Valencia, CA, USA). The presence of $BRAF^{V600E}$ was determined by Sequenom analysis (primer and primer extension sequences are available upon request).

Statistical analysis

Clinicopathologic data were recorded for each case in a blinded manner to the IHC staining results and approved by internal review board (IRB) protocol. Statistical analyses were performed using SPSS 14.0 for windows and GraphPad Prism 5.0. Noncontinuous variables were analyzed using Fischer's exact two-sided test and continuous variables were analyzed using unpaired two-sided *t*-tests. Survival analyses were performed using Kaplan–Meier log-rank tests. Significance was defined as $P<0.05$.

Results

TAM density in thyroid cancers of different histological grades

Of the 46 non-neoplastic thyroid tissue specimens, only 2 (4%) were positive for CD68 (mean number of $CD68^+$ macrophages was <5). In tumors, the anti-CD68 antibody showed dense cytoplasmic staining of mononuclear and multinucleated giant-type cells that morphologically resembled macrophages (small 'bean' shaped nuclei with a low nuclear:cytoplasmic ratio). In WDTC and PDTC, positively labeled cells were found within the lumen of the follicles (especially the multinucleated giant cells) and interspersed between the tumor cells. By contrast, in ATC, they were diffusely infiltrated throughout the core sections. The specificity of the stain was verified with anti-CD163 (Fig. 1). Nine out of 33 WDTC (27%), 20 out of 37 PDTC (54%), and 19 out of 20 ATC (95%) were positive for an increased density of $CD68^+$ TAMs (Table 1; WDTC versus PDTC, $P=0.030$; WDTC versus ATC, $P<0.0001$; PDTC versus ATC, $P=0.002$). The mean number of $CD68^+$ TAMs was <5 in WDTC, 9 in PDTC, and 39 in ATC. In the majority of tumor samples from individual patients, there was a consistent number of TAMs between the three cores. Figure 1 shows representative sections of each histological grade at low and high power magnifications.

Clinicopathologic correlations

As histological grade is an important determinant of tumor behavior and clinical prognosis, it was important to explore the effect of TAMs on tumor progression within a particular histological subtype, rather than comparing the impact of TAMs on clinicopathologic outcomes between grades. Although the number of WDTC with >10 TAMs/0.28 mm^2 was comparatively low (9/33; 27%), there was no statistical correlation between the presence of TAMs and extrathyroidal extension (ETE), capsular invasion and vascular invasion. However, the overall abundance of TAMs in the positive cases tended to be lower than that observed in the positive cases in PDTC, but the difference was not statistically significant. None of the patients with WDTC died of their disease (0 out of 33) and few tumors had

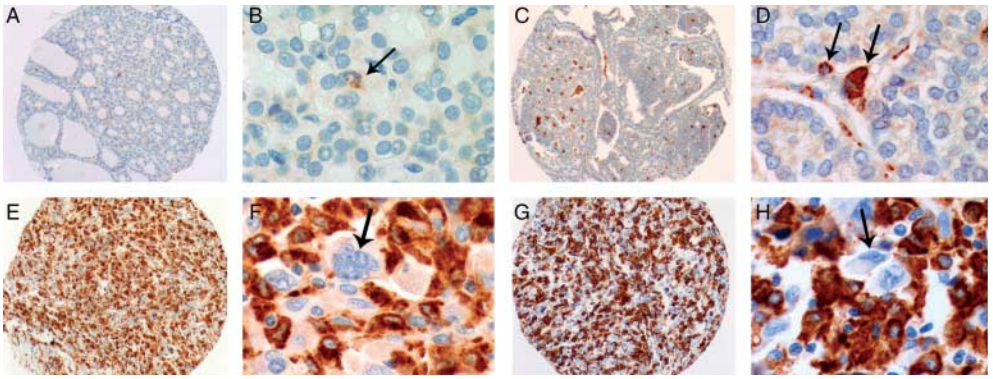

Figure 1 Immunostain for tumor-associated macrophages (TAM) markers in tissue microarrays. The signal is golden brown. (A) Very low density of $CD68^+$ TAM per tissue core (0.28 mm^2) in well-differentiated papillary carcinoma, follicular variant. (B) High power of (A) (arrow indicates isolated TAM). (C) Moderate density of $CD68^+$ TAM in poorly differentiated thyroid carcinomas. (D) High power of (C) (arrow indicates TAM). (E) Very high density of $CD68^+$ TAM in anaplastic carcinoma. (F) High power of E (arrow points to CD68 negative anaplastic bizarre carcinoma cell surrounded by TAM). (G) Same case as (E), (F) showing very high density of $CD163^+$ TAM. (H) High power of G (arrow points to CD163 negative anaplastic bizarre carcinoma cell surrounded by TAM).

increased TAMs, whereas 18 out of 20 patients with ATC died of thyroid cancer with a median survival of 3 months (data not shown), and all but one tumor had increased TAMs. Hence, we could not explore the possible role of increased TAMs on the biological behavior and clinical outcomes in these tumor types. By contrast, patients with PDTC had variable clinical outcomes and 54% of these tumors had increased TAMs, making this histotype the most suitable to examine the effects of increased TAMs on clinicopathologic parameters. Overall, patients with PDTC had a median cancer-related survival that ranged from 2.8 to 7.1 years. When stratified according to the presence of increased TAMs, PDTC with a higher density of TAMs were associated with increased capsular invasion ($P=0.034$), ETE ($P=0.009$), and decreased cancer-related survival ($P=0.009$), compared with patients with a low number of TAMs (Table 2 and Fig. 2). The mean cancer-related survival of patients with PDTC and increased $CD68^+$ TAMs was 5.4 ± 0.8 years, compared with 9.7 ± 1.2 years for patients with a low abundance of TAMs ($P=0.009$). The mean cancer-related survival for patients with ETE was 5.7 ± 0.8 years compared with 11.0 ± 1.2 years for patients without ETE ($P=0.005$; Fig. 2), consistent with the published data that ETE is a negative prognostic marker in thyroid cancers (Andersen *et al.* 1995). When the TAM-related survival data was adjusted for ETE, the correlation between increased TAMs and decreased cancer-related survival was lost (data not shown). There were no differences between patients with or without increased $CD68^+$ TAMs in gender, age, stage, or mitotic rate. Patients with increased $CD68^+$ TAMs had a trend toward larger tumors, more extensive tumor necrosis, a higher frequency of distant metastases, and increased flurodeoxyglucose (FDG)-avid positron emission tomography (PET) (+) disease, but these comparisons were not statistically significant (Table 2).

In the 33 WDTC, 16 (48%) were positive for $BRAF^{V600E}$, 11 from tumors without increased TAMs, and 5 from tumors with increased TAMs ($P=0.71$). Out of the 37 PDTC, 6 were positive for $BRAF^{V600E}$. This mutation was equally distributed between tumors with a low density of TAMs ($n=3$) and tumors with a high density of TAMs ($n=3$).

Table 1 Correlation between the density of $CD68^+$ tumor-associated macrophages (TAMs) and tumor grade in human well-differentiated (WDTC), poorly differentiated (PDTC), and anaplastic thyroid cancers (ATC)

Tissue type	No. of cases	No. of positive cases (%)	Mean # $CD68^+$ TAMs/0.28 mm^2	*P* value
WDTC	33	9 (27%)	<5	0.030[a]
				<0.0001[b]
PDTC	37	20 (54%)	9	0.002[c]
ATC	20	19 (95%)	39	

IHC was performed on tissue microarray sections composed of 0.6 mm core biopsies. Positive cases were defined as 10 or more $CD68^+$ TAMs per core (0.28 mm^2).
[a]WDTC compared with PDTC.
[b]WDTC compared with ATC.
[c]PDTC compared with ATC.

Table 2 Comparison of clinical and histopathologic parameters in patients with a low versus a high density of $CD68^+$ tumor-associated macrophages (TAMs) in poorly differentiated thyroid cancer (PDTC) patients

	Density of $CD68^+$ TAMs		
Variables	**Low ($n=17$)**	**High ($n=20$)**	***P* value**
Age	63±4 (range 16–85)	61±4 (range 25–93)	0.760
Gender			
Male	4	9	0.300
Female	13	11	
Stage[a]			
I	3	0	0.419
II	1	1	
III	4	2	
IV	9	12	
Tumor size (cm)	4.3±0.4 ($n=17$)	5.4±0.6 ($n=14$)	0.135
Necrosis[b]			
Focal	8	4	0.149
Extensive	7	12	
Mitosis, # per high-power field	4.0±2.3 ($n=12$)	8.2±7.0 ($n=16$)	0.168
Capsular invasion			
Absent	5	0	**0.034**
Present	4	7	
No capsule	5	6	
Extrathyroidal extension			
Absent	10	2	**0.009**
Present	6	14	
Extrathyroidal vascular invasion			
Absent	8	4	0.128
Present	5	10	
FDG-PET			
No uptake	9	5	0.063
Positive uptake	4	12	
Distant metastases			
None	5	2	0.197
Present	9	15	

[a]Staging based on American Joint Committee on Cancer Staging manual, 6th edition.
[b]Two tumors with a low TAM density and three tumors with a high TAM density had no evidence of tumor necrosis. For each variable, where *n* does not equal the total number of cases in the TAM group, there was insufficient or missing data from some cases in that particular category and therefore some cases were not included in the analysis.
Bold figures represent statistically significant correlations.

Discussion

Our study demonstrates, for the first time, that an increased density of TAMs is associated with tumor progression in advanced thyroid cancers. There is a remarkably strong correlation between increased TAMs and histological grade, and between TAMs, tumor invasiveness, and decreased cancer-related survival in PDTC. These results suggest that TAMs promote thyroid cancer progression. If correct, this represents a conceptually novel paradigm that has the potential to influence the development of experimental therapies for advanced disease.

The mechanisms by which TAMs are recruited to the tumor microenvironment in thyroid cancer are not known. Expression of oncoproteins involved in thyroid cell transformation may directly stimulate the recruitment of TAMs through increased expression of macrophage chemoattractants. Expression of oncogenic BRAF was found to be particularly potent at inducing their expression as compared with RET/PTC (Mesa *et al*. 2006), suggesting that cancers with this mutation may be particularly prone to macrophage infiltration. In the present series, neither WDTC nor PDTC with *BRAF* mutations were preferentially associated with a high density of TAMs, indicating that other TAM recruiting factors are likely at play. However, because there was a low percentage of PDTC with *BRAF* mutations in this series, the relationship between TAMs and tumor genotype should be viewed as inconclusive in this tumor grade. Regardless of how they are recruited, TAMs secrete a rich repertoire of chemokines and growth factors that may exert paracrine effects on tumor cells to facilitate

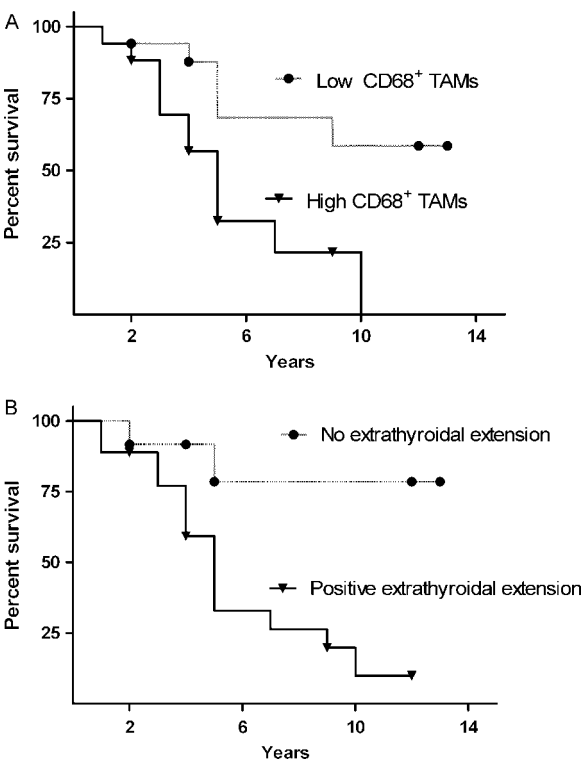

Figure 2 Top: Kaplan–Meier log-rank cancer-related survival analyses of PDTC patients with or without increased TAMs ($P=0.009$). Bottom: Kaplan–Meier curve for patients with and without ETE at initial surgical presentation ($P=0.005$).

progression. For example, TAM-derived chemokines may directly enhance tumor cell growth (Mantovani 1994) and may indirectly influence thyroid cancer cell expression of chemokine receptors, such as CXCR4 (Hwang *et al*. 2003, Castellone *et al*. 2004), that are important for tumor spread. Secretion of matrix metalloproteases by TAMs can remodel the extracellular matrix, which in turn enables tumor cell mobility, migration, and invasion at both local and distant sites (van Kempen & Coussens 2002). Decreased oxygen tension at necrotic sites may stimulate the recruitment of both phagocytic and angiogenic TAMs to scavenge debris and to stimulate tumor angiogenesis respectively (Lewis *et al*. 2007). Activation of an angiogenic switch is an absolute requirement for tumor progression, and TAMs have been shown to trigger this process in breast cancer animal models (Folkman *et al*. 1989, Lin *et al*. 2006).

The remarkable functional plasticity of TAMs in tumor microenvironments may explain several observations in our study. The correlation between increased TAMs, capsular invasion, ETE and the trend toward distant metastases in PDTC suggests that TAMs are not merely prognostic markers of tumor progression, but may actively participate in the biology of the process. The fact that the density of TAMs and ETE are not independent variables for cause-specific survival suggests that these two events may indeed be functionally related. A study by Fiumara *et al*. (1997) found no association between the presence of TAMs and ETE, lymph node disease, and distant metastases in WDTC. However, this study was limited to patients with low-grade tumors that are generally associated with favorable outcomes. The presence of both increased TAMs and extensive tumor necrosis in all ATC (data not shown) and the trend toward this association in PDTC suggests that TAMs may also be involved in thyroid cancer angiogenesis. Indeed, Dhar *et al*. (1998) showed an association between increased TAMs and tumor vascularity in WDTC.

There are presently vigorous efforts by many research groups to develop novel therapies for thyroid cancer, which specifically interfere with signaling pathways activated by mutated oncoproteins. The findings in this paper may impact these efforts in at least two ways. First, if TAMs are important drivers of the biological behavior of advanced tumors, therapies that do not target them may not be effective. Secondly, there is evidence that certain cytokines, such as tumor necrosis factor-α which may be expressed by macrophages, can induce resistance to drugs that target the BRAF–MEK–ERK pathway by inhibiting apoptosis following BRAF inhibition (Gray-Schopfer *et al*. 2007).

In summary, our results demonstrate that increased TAMs in high-grade thyroid cancers are associated with invasive cancers and decreased cancer-related survival. This study underscores the importance of expanding our understanding of thyroid cancer progression from the one that is narrowly focused on intrinsic oncogenic pathways to investigations that more accurately encompass the whole tumor microenvironment, including tumor-promoting TAMs.

Declaration of interest

The authors declare that there are no conflicts of interest which would prejudice the impartiality of this study.

Funding

This paper was supported in part by grants from the American Thyroid Association (M R), NIH CA50706 (J A F), and a Byrne award (J A F).

References

Andersen PE, Kinsella J, Loree TR, Shaha AR & Shah JP 1995 Differentiated carcinoma of the thyroid with extrathyroidal extension. *American Journal of Surgery* **170** 467–470.

Bolat F 2006 Microvessel density, VEGF expression, and tumor-associated macrophages in breast tumors: correlations with prognostic parameters. *Journal of Experimental & Clinical Cancer Research* **25** 365–372.

Castellone MD, Guarino V, De F V, Carlomagno F, Basolo F, Faviana P, Kruhoffer M, Orntoft T, Russell JP, Rothstein JL *et al.* 2004 Functional expression of the CXCR4 chemokine receptor is induced by RET/PTC oncogenes and is a common event in human papillary thyroid carcinomas. *Oncogene* **23** 5958–5967.

Dhar DK, Kubota H, Kotoh T, Tabara H, Watanabe R, Tachibana M, Kohno H & Nagasue N 1998 Tumor vascularity predicts recurrence in differentiated thyroid carcinoma. *American Journal of Surgery* **176** 442–447.

Fiumara A, Belfiore A, Russo G, Salomone E, Santonocito GM, Ippolito O, Vigneri R & Gangemi P 1997 *In situ* evidence of neoplastic cell phagocytosis by macrophages in papillary thyroid cancer. *Journal of Clinical Endocrinology and Metabolism* **82** 1615–1620.

Folkman J, Watson K, Ingber D & Hanahan D 1989 Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* **339** 58–61.

Gray-Schopfer VC, Karasarides M, Hayward R & Marais R 2007 Tumor necrosis factor-α blocks apoptosis in melanoma cells when BRAF signaling is inhibited. *Cancer Research* **67** 122–129.

Herrmann G, Schumm-Graeger P-M, Muller C, Atai E, Wenzel B, Fabian T, Henning K, Usadel KH & Huber K

1994 T lymphocytes, CD68-positive cells and vascularization in thyroid carcinomas. *Journal of Cancer Research and Clinical Oncology* **120** 651–656.

Hwang JH, Hwang JH, Chung HK, Kim DW, Hwang ES, Suh JM, Kim H, You KH, Kwon OY, Ro HK *et al.* 2003 CXC chemokine receptor 4 expression and function in human anaplastic thyroid cancer cells. *Journal of Clinical Endocrinology and Metabolism* **88** 408–416.

van Kempen LC & Coussens LM 2002 MMP9 potentiates pulmonary metastasis formation. *Cancer Cell* **2** 251–252.

Leek RD, Lewis CE, Whitehouse R, Greenall M, Clarke J & Harris AL 1996 Association of macrophage infiltration with angiogenesis and prognosis in invasive breast carcinoma. *Cancer Research* **56** 4625–4629.

Lewis CE & Pollard JW 2006 Distinct role of macrophages in different tumor microenvironments. *Cancer Research* **66** 605–612.

Lewis CE, De Palma M & Naldini L 2007 Tie2-expressing monocytes and tumor angiogenesis: regulation by hypoxia and angiopoietin-2. *Cancer Research* **67** 8429–8432.

Lin EY, Li JF, Gnatovskiy L, Deng Y, Zhu L, Grzesik DA, Qian H, Xue XN & Pollard JW 2006 Macrophages regulate the angiogenic switch in a mouse model of breast cancer. *Cancer Research* **66** 11238–11246.

Lissbrant IF, Stattin P, Wikstrom P, Damber JE, Egevad L & Bergh A 2000 Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *International Journal of Oncology* **17** 445–451.

Mantovani A 1994 Tumor-associated macrophages in neoplastic progression: a paradigm for the *in vivo* function of chemokines. *Laboratory Investigation* **71** 5–16.

Mesa C Jr, Mirza M, Mitsutake N, Sartor M, Medvedovic M, Tomlinson C, Knauf JA, Weber GF & Fagin JA 2006 Conditional activation of RET/PTC3 and BRAFV600E in thyroid cells is associated with gene expression profiles that predict a preferential role of BRAF in extracellular matrix remodeling. *Cancer Research* **66** 6521–6529.

Schoppmann SF, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C, Kriehuber E, Nagy K, Alitalo K & Kerjaschki D 2002 Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *American Journal of Pathology* **161** 947–956.

Tsutsui S, Yasuda K, Suzuki K, Tahara K, Higashi H & Era S 2005 Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncology Reports* **14** 425–431.

Yu JL 2003 Host microenvironment in breast cancer development: inflammatory and immune cells in tumour angiogenesis and arteriogenesis. *Breast Cancer Research* **5** 83–88.