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CTL- *vs* T$_{reg}$ lymphocyte-attracting chemokines, CCL4 and CCL20, are strong reciprocal predictive markers for survival of patients with oesophageal squamous cell carcinoma

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Background: Tumoural infiltration of T lymphocytes is determined by local patterns of specific chemokine expression. In this report, we examined the roles of CCL4 and CCL20 in the accumulation of CD8$^+$ cytotoxic T lymphocytes (CTLs) and regulatory T (T$_{reg}$) lymphocytes in oesophageal squamous cell carcinoma (ESCC), and determined the correlations between chemokine expressions and ESCC patients' survival.

Methods: Reverse transcriptase–PCR and immunohistochemistry (IHC) staining were performed to examine the expressions of interested genes. Flow cytometry was adopted to check the expressions of CCL4- and CCL6-specific receptors, CCR5 and CCR6, on CTLs and T$_{reg}$ cells. In addition, transwell assay was carried on.

Results: The *CCL4* expression was significantly correlated with the expression of CTL markers (*CD8* and *Granzyme B*), whereas *CCL20* was positively correlated with T$_{reg}$ markers (*FoxP3* and *IL-10*). Consistently, CCR5 was found to be mainly expressed on CD8$^+$ T lymphocytes, while CCR6 showed prevalence on T$_{reg}$ lymphocytes and the frequencies of CCR5$^+$CD8$^+$ CTLs and CCR6$^+$ T$_{reg}$ cells were higher in TIL compared with PBMC. Respectively, CCL4 and CCL20 recruited CD8$^+$ and regulatory T cells *in vitro*. Importantly, high levels of CCL4 in the lesions of ESCC patients predicted prolonged survival. Furthermore, CCL4^{high}/CCL20^{low} group demonstrated better overall survival, whereas CCL4^{low}/CCL20^{low} and CCL4^{low}/CCL20^{high} groups showed the worst overall survival.

Conclusions: Our data showed that CCL4 and CCL20 recruit functionally different T lymphocyte subsets into oesophageal carcinoma, indicating CCL4 and CCL20 are potential predictors of ESCC patients' survival.

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With ∼482 300 new cases and 406 800 deaths each year, oesophageal carcinoma is the ninth most frequent cancer in the world and the fifth most frequent cancer in developed countries (Mariette et al, 2007; Sudo et al, 2014). Although a lot of new therapeutic modalities have been developed to treat oesophageal carcinoma, surgery is still used most frequently (Mariette et al, 2007). As surgical resection is only possible in <20% of patients and the relapse occurs in >60% of patients receiving chemoradiotherapy, the overall survival of patients with oesophageal carcinoma is <20% (Mariette et al, 2007; Sudo et al, 2014). Among the patients with oesophageal carcinoma, squamous cell carcinoma accounts for 90% of cases (Rustgi and El-Serag, 2014). Therefore, it's necessary to develop more promising therapeutic strategies for oesophageal carcinoma, especially oesophageal squamous cell carcinoma (ESCC). Although several key molecular targets have been demonstrated to be useful for prognosis prediction and novel therapeutic interventions, patients benefiting from such approaches remains challenging (Izzo et al, 2007; Yue et al, 2014).

It is well established that the communications between cancer cells and tumour microenvironment modulate cancer progression. The properties of tumour microenvironments have a strong impact on the disease progression, response to therapy and prognosis (Junttila and de Sauvage, 2013). Within tumour microenvironment, chemokines play central roles (Roussos et al, 2011). Apart from their impacts on tumour cells, chemokines decide the constitutions of tumour immune microenvironment (Roussos et al, 2011; Bindea et al, 2013), especially the types and intensities of infiltrated T lymphocytes (Harlin et al, 2009; Chew et al, 2012). Different T lymphocytes lead to opposite outcomes. Infiltration of CD8^{+} T lymphocytes usually favours better prognosis and prolonged survival, whereas tumoural presence of regulatory T (T$_{reg}$) lymphocytes means poor prognosis (Zhang et al, 2003; Curiel et al, 2004; Pages et al, 2005; Sato et al, 2005; Galon et al, 2006). Various chemokines are expressed in tumours. However, T lymphocyte subsets are selectively recruited into malignant tissues by specific chemokines (Franciszkiewicz et al, 2012; Gajewski et al, 2013). Therefore, exploring the chemokines deciding the tumoural accumulations of T lymphocyte subsets may provide new target for therapy and help to predict patients' prognosis.

In the past decades, a lot of efforts have been made to study the molecular signals and genetic mutations related with carcinogenesis and progression of oesophageal carcinoma (Hamano et al, 2011; Wu et al, 2014). However, the tumour microenvironmental factors and their relations with oesophageal carcinoma have been very limited debated. Particularly, the effects of chemokines on T lymphocyte recruitment and patients' prognosis are rarely reported. In this report, we explored the impact of CCL4 and CCL20, the respective ligands for CCR5 and CCR6, on migrations of CD8^{+} and T$_{reg}$ lymphocytes towards ESCC tissues, and on survival of ESCC patients.

MATERIALS AND METHODS

Clinical subjects. A total of 225 patients with ESCC were recruited, following the approval by the Ethics Committee Board of the First Affiliated Hospital of Zhengzhou University. Signed informed consent forms were obtained from all subjects. The detailed information of subjects is listed in Supplementary Table 1. In all, 225 tumour samples and paired 68 peripheral blood samples were collected during surgery in the First Affiliated Hospital of Zhengzhou University between January 2008 and July 2014. In addition, 75 adjacent tissues were sampled.

Isolation of tumour-infiltrating lymphocytes (TILs) and peripheral blood lymphocytes (PBLs). Freshly sampled tumour tissues were washed with RPMI-1640 (Invitrogen Life Technologies, Carlsbad, CA, USA) 3 times to remove traces of blood. Then, the tissues were cut into small pieces and incubated with 300 μg ml^{-1} collagenase (Roche, Indianapolis, IN, USA) and 50 μg ml^{-1} deoxyribonuclease I (Sigma-Aldrich, St Louis, MO, USA) for 2 h at 37 °C. Following this, the samples were mechanically disaggregated, supernatants were collected and TILs were separated by centrifugation at 2000 r.p.m. for 20 min on Ficoll-Paque Plus (Sigma-Aldrich). Peripheral blood lymphocytes were isolated from heparinised blood samples by Ficoll-Paque Plus density centrifugation.

Flow cytometry. Tumour-infiltrating lymphocytes and PBLs were suspended in flow buffer (PBS containing 2% fetal bovine serum), and incubated with anti-CD3, anti-CD8, anti-CD4, anti-CCR5 and anti-CCR6 antibodies (Biolegend, San Diego, CA, USA) against surface antigens for 30 min at 4 °C in the dark. Following that, samples were rinsed in flow buffer 2 times. Then, cells were fixed, permeabilised and stained with anti-FoxP3 antibody (Biolegend). After staining, cells were washed twice and analysed using a BD CantoII flow cytometer (Becton Dickinson, San Jose, CA, USA). To detect nonspecific signals, concentration- and isotype-matched nonspecific antibodies were used.

Reverse transcriptase–PCR. Tumour or marginal tissues were cut into 20 mm of pieces and mechanically grinded. Then, total RNA was extracted using Trizol solution (Invitrogen Life Technologies). Independently, RNA from each sample was reverse-transcribed using PrimeScript RT reagent Kit (Takara Bio, Otsu, Shiga, Japan). Subsequently, the expressions of interested genes were tested. Listed primers were used: 5′-GGAGCCAAAAGGGTCATCA TCTC-3′ sense primer and 5′-GAGGGGCCATCCACAGTCT TCT-3′ antisense primer for GAPDH, 5′-CGCTGTCAGATCCCC TTTGT-3′ sense primer and 5′-GAGGAAGGACCCTCTCCCTT-3′ antisense primer for CD8, 5′-GCAGGAAGATCGAAAGTGCG-3′ sense primer and 5′-TACAGCGGGGGCTTAGTTTG-3′ antisense primer for Granzyme B, 5′-GCTTCCTCGCAACT TTGTGG-3′ sense primer and 5′-GCGGAGAGGAGTCCTGAGTA-3′ antisense primer for CCL4, 5′-ATTGTGCGTCTCCTCAGTAAAAA-3′ sense primer and 5′-TGTGATGCTTAAACAAAGCAAAC-3′ antisense primer for CCL20, 5′-TCCAGGACAGGCCACAT TTC-3′ sense primer and 5′-GGGATTTGGGAAGGTGCAGA-3′ antisense primer for Foxp3, 5′-ACATCAAGGCGCATGTGAAC-3′ sense primer and 5′-GCCACCCTGATGTCTCAGTT-3′ antisense primer for interleukin-10 (IL-10). The initial step was performed at 94 °C for 5 min, the amplification was performed for 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s and elongation at 72 °C for 30 s. Following the last cycle, a terminal elongation step (5 min at 72 °C) was added and then the samples were kept at 4 °C. The PCR products were separated on 1.5% agarose gel and recorded. Targeted bands were analysed in ImageJ software (National Institute of Health, Bethesda, MD, USA) and optical densities were calculated. Then, relative expressions of genes were determined. The house-keeping gene GAPDH was used as reference.

Immunohistochemistry. Formalin-fixed, paraffin-embedded sections (3 μm) were deparaffinised in xylene, then rehydrated through graded alcohol and washed briefly in tap water. Endogenous peroxidase was blocked with methanol containing 0.3% hydrogen peroxide for 30 min. To retrieve antigenicity, sections were boiled in 10 mmol l^{-1} citrate buffer (pH 5.8) for 30 min in microwave (800 W). Following that, sections were incubated with goat serum diluted in PBS (pH 7.4) for 30 min at room temperature. Subsequently, sections were incubated at 4 °C overnight with the primary antibodies specific for CCL4 (dilution 1 : 200) or CCL20 (dilution 1 : 200) (Abcam, Cambridge, UK). Then, sections were rinsed with fresh PBS and incubated with horseradish peroxidase-linked secondary antibodies at room

temperature for 30 min. Finally, sections were stained with 3, 3′-diaminobenzidine (DAB) substrate (Dako, Carpinteria, CA, USA) and counterstained with Mayer's haematoxylin. Photos were recorded under microscopy (Leica, Wetzlar, Germany).

Evaluation of immunohistochemical (IHC) staining. All sections were assessed at $\times 20$ magnification by two experienced observers. The staining was evaluated based on the intensity (0 = none, weak = 1, moderate = 2 and high = 3) of chemokines staining and the density (0% = 0, 1–40% = 1, 41–75% = 2 and $>76\% = 3$) of positive tumour cells. The scores of sections were multiplied intensity and density. If the evaluations did not agree the sections were reevaluated and then classified according to the assessment given most frequently by the observers. The variances within sample were taken into consideration and the average scores were adopted. To determine the cutoff value, we checked the distribution of the frequencies of IHC scores (Supplementary Figure 4). Then, the cutoff value was assigned according to the value at peak. For CCL4, score $\leqslant 1$ was considered low expression. For CCL20, score $\leqslant 1.5$ was considered low expression.

Purification of T lymphocyte. Tumour-infiltrating lymphocytes were isolated from fresh tumour tissues of ESCC patients as the protocols described above. T lymphocyte were suspended in flow buffer, stained with ant-CD4, anti-CD8, anti-CCR5 and anti-CCR6 antibodies in dark and rinsed. Then, T lymphocyte subsets were independently isolated using MoFlo XDP cytometer (Beckman Coulter Inc., Indianapolis, IN, USA).

Transwell assay. Directional migration of T lymphocytes was evaluated in 24-well plates with 5-μm pore size polycarbonate filters (Corning Inc., Corning, NY, USA). Purified T lymphocytes from TILs of ESCC patients were counted. Then, 4×10^5 of T lymphocytes were added to the upper chambers with $100 \, \mu l$ RPMI-1640 media with 1% serum. The lower chambers were then filled with $600 \, \mu l$ RPMI-1640 media containing 1% serum or supplemented with recombinant human chemokine (CCL4 or CCL20) (Pepro-Tech, Rocky Hill, NJ, USA). The plates were incubated at $37 \, ^\circ C$ in a 5% CO_2 atmosphere for 2.5 h. After

that, cells in the bottom chambers were collected. The $CD8^+$ T lymphocytes were counted by a limited 60-s run on cytometer. For analysis of T_{reg} cells, migrated cells were stained with anti-CD4 and anti-FoxP3 antibodies, and then the numbers of interested cells were calculated by flow cytometry.

Statistical analysis. Analyses were performed using the SPSS for Windows version 19.0 (IBM, Armonk, NY, USA). Data were expressed as mean $\pm$ s.d. Student's t-test and one-way ANOVA were conducted to compare the differences between variables. Spearman's test was adopted to determine the correlation between chemokine genes and T cell-associated markers. Kaplan–Meier survival curves and log-rank statistics were used to evaluate overall survival. Multivariate regression analysis was performed using the Cox proportional hazards model. Values with $P < 0.05$ (two tailed) were considered significant.

RESULTS

The expression levels of CCL4 and CCL20 in ESCC tissues correlate with local expression of, respectively, CTL and T_{reg} lymphocytes markers. As reported, CCL4 is involved in the migration of not only $CD8^+$ T lymphocytes but also T_{reg} lymphocytes (Harlin *et al*, 2009; Schlecker *et al*, 2012). CCL20 mainly dictates the movement of T_{reg} lymphocytes (Chen *et al*, 2011; Cook *et al*, 2014). However, the impacts of CCL4 and CCL20 on T lymphocytes in ESCC have not been reported. To elucidate how the tumoural accumulation of T lymphocytes was affected by CCL4 and CCL20 in ESCC, we performed RT–PCR assay to detect the associations between chemokines and T lymphocyte markers in malignant sites. As shown in Figure 1A, the expressions of CCL4 were positively related with cytotoxic T lymphocyte (CTL) markers, CD8 and Granzyme B ($r_{CD8} = 0.257$, $P < 0.005$; $r_{GranB} = 0.273$, $P < 0.01$). Meanwhile, the intensity of CCL20 was clearly correlated with T_{reg} lymphocyte markers: FoxP3 and IL-10 ($r_{FoxP3} = 0.240$, $P < 0.01$; $r_{IL-10} = 0.387$, $P < 0.005$; Figure 1B). Furthermore, the mRNA levels of CTL markers were not
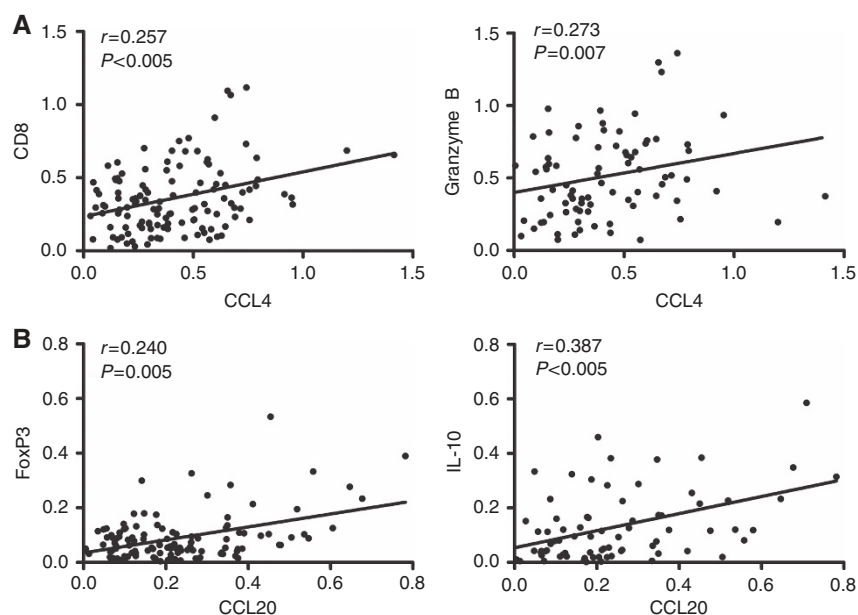

Figure 1. Expression levels of CCL4 and CCL20 correlate with, respectively, local expressions of (A) CTL markers (CD8 and Granzyme B) and (B) regulatory T lymphocytes markers (FoxP3 and IL-10). A total of 123 fresh tumour tissues from ESCC patients were collected and processed. RNA was extracted. Reverse transcriptase–PCR assay of interested genes was performed. Spearman's test was performed to determine the correlations among gene expressions.

statistically associated with that of CCL20 ($r_{CD8} = -0.024$, $P > 0.05$; $r_{GranB} = 0.107$, $P > 0.05$; Supplementary Figure 1B), whereas the expressions of FoxP3 and IL-10 were independent on CCL4 levels ($r_{FoxP3} = 0.103$, $P > 0.05$; $r_{IL-10} = 0.159$, $P > 0.05$; Figure 1B). These data suggest that CCL4 and CCL20 are probably implicated in the migration and retention of CD8$^+$ CTLs and T$_{reg}$ lymphocytes, respectively, into ESCC lesions.

CCL4- and CCL20-specific receptors, CCR5 and CCR6, are differently expressed on T lymphocyte subsets in ESCC. As specific receptors are critical for T lymphocytes moving towards tumour tissues in response to the recruitment of chemokines, monitoring the expression differences of chemokine receptors is helpful for elucidating the roles of specific chemokines. First, we detected the frequencies of CCR5 (CCL4-specific receptor) and CCR6 (CCL20-specific receptor) on peripheral CD8$^+$ T and T$_{reg}$ lymphocytes. As shown in Figure 2A, the proportions of CCR5-positive cells in CD8$^+$ T lymphocytes were significantly greater than in CD4$^+$FoxP3$^+$ T$_{reg}$ lymphocytes ($21.60\% \pm 15.83\%$ vs $11.61\% \pm 14.34\%$; $P < 0.01$). The percentages of CCR6-positive cells in regulatory T lymphocyte groups were $38.83\% \pm 22.76\%$, and the counterparts in CD8$^+$ T lymphocytes were $3.95\% \pm 5.03\%$ ($P < 0.001$; Figure 2B). These observations indicate that CCR5 preferentially expresses on CD8$^+$ T lymphocytes, whereas CCR6 shows biased expression on T$_{reg}$ lymphocytes. Following that, we examined tumour-infiltrating T lymphocytes. Compared with $34.38\% \pm 13.35\%$ in peripheral blood, the percentages of CD8$^+$ T lymphocytes were obviously increased to $42.84\% \pm 15.24\%$ ($t = 3.156$, $P < 0.005$; Supplementary Figure 2). Similarly, the portions of T$_{reg}$ lymphocytes were sharply elevated to $16.65\% \pm 9.77\%$ in TILs from $6.67\% \pm 6.71\%$ in PBLs (Supplementary Figure 2). The difference is statistically significant ($t = 6.42$, $P < 0.0001$). These observations indicated that CD8$^+$ T and T$_{reg}$ lymphocytes are efficiently recruited into ESCC sites. In tumour-infiltrating CD8$^+$ T lymphocytes, CCR5 expressed on greater portions of cells (Figure 2A). The difference between CCR5-expressing CD8$^+$ T lymphocytes in TILs and PBLs is

statistically significant ($45.90\% \pm 25.28\%$ vs $21.60\% \pm 15.82\%$, $P < 0.001$; Figure 2A). In T$_{reg}$ lymphocytes in tumour sites, CCR5 expression was also observed. However, the intensities of CCR5$^+$ T$_{reg}$ lymphocytes in TILs were not obviously enhanced, compared with the counterparts in PBLs ($19.32\% \pm 17.41\%$ vs $11.61\% \pm 14.34\%$, $P > 0.05$; Figure 2A). Moreover, CCR5$^+$ T$_{reg}$ lymphocytes were less frequent than CCR5$^+$CD8$^+$ T lymphocytes in tumours ($P < 0.0001$). Conversely, CCR6 expression was robustly increased in tumour-infiltrating T$_{reg}$ lymphocytes (Figure 2B). The portions of CCR6$^+$ T$_{reg}$ lymphocytes in TILs were much greater than that in PBLs ($57.49\% \pm 29.58\%$ vs $38.83\% \pm 22.76\%$, $P < 0.05$), or CCR6$^+$CD8$^+$ T lymphocytes in TILs ($57.49\% \pm 29.58\%$ vs $10.77\% \pm 10.04\%$, $P < 0.0001$; Figure 2B). Together with the RT–PCR data, these results indicate that CCL4 plays major roles in recruiting CD8$^+$ T lymphocytes into malignancies, whereas CCL20 mainly directs the tumoural movement of T$_{reg}$ lymphocytes.

CCL4 and CCL20 promote migrations of ESCC patients' CD8$^+$ T and T$_{reg}$ lymphocytes, respectively, in vitro. To further validate the roles of CCL4 and CCL20 in T lymphocyte chemotaxis, we performed transwell experiments. First, CD8$^+$CCR5$^-$, CD8$^+$CCR5$^+$, CD4$^+$CCR6$^-$ and CD4$^+$CCR6$^+$ T lymphocytes (the purities were over 90%) were purified from TILs. Then, the purified T lymphocytes were separately added into transwell chambers, whose bottom wells were filled of media containing 1% FBS alone or with corresponding chemokines (recombinant human CCL4 or CCL20). The migrated cells in bottom wells were collected, stained and calculated. As shown in Figure 3, CCL4 obviously enhanced the migrating ability of the CCR5-expressing subset in CD8$^+$ T lymphocytes, whereas media alone had little impact on the movement of CD8$^+$ T lymphocytes. Similarly, the directional movement of T$_{reg}$ cells was relying on CCL20 and CCR6 (Figure 3). These observations verify that CCL4–CCR5 and CCL20–CCR6 axes are critical for the migrations of CD8$^+$ T and T$_{reg}$ lymphocytes, respectively.
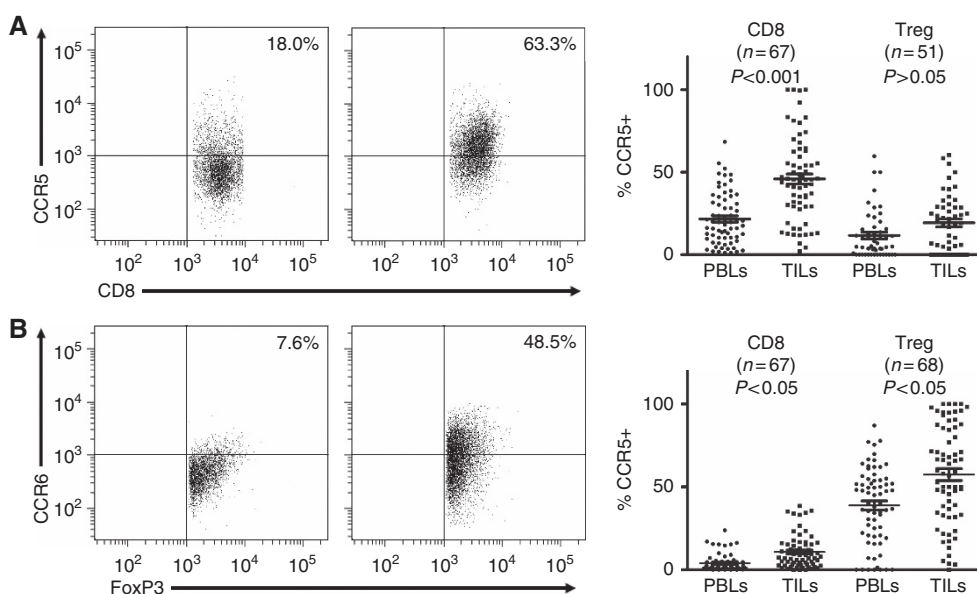

Figure 2. CCR5 and CCR6 show differential expressions on CD8$^+$ and regulatory T lymphocytes in PBLs and matched TILS. Paired PBL and TIL samples were stained with antibodies specific for CD3, CD4, CD8, FoxP3, CCR4 and CCR6. The CD8$^+$ T lymphocytes (CD3$^+$CD8$^+$) and T$_{reg}$ lymphocytes (CD3$^+$CD4$^+$FoxP3$^+$) were separately gated, and then the expressions of CCR5 and CCR6 were determined with multicolour flow cytometry. (**A**) Representative plots of CCR5 staining on CD8$^+$ T lymphocytes in matched PBLs and TILs (left panel). Paired analysis of CCR5 expressions on CD8$^+$ T and T$_{reg}$ lymphocytes in paired PBLs and TILs (right panel). (**B**) Representative plots of CCR6 staining on T$_{reg}$ lymphocytes in matched PBLs and TILs (left panel). Paired analysis of CCR6 expressions on CD8$^+$ T and T$_{reg}$ lymphocytes in paired PBLs and TILs (right panel).
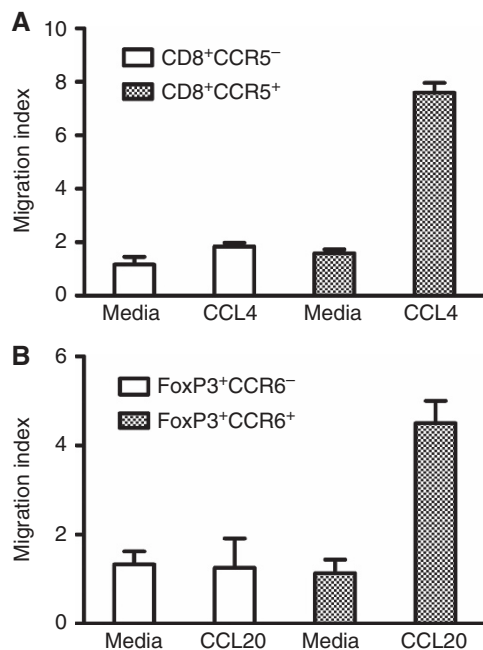
Figure 3. CCL6 and CCL20 individually enhance the migrations of CD8$^+$ and regulatory T lymphocytes *in vitro*. (**A**) CD8$^+$CCR5$^-$ and CD8$^+$CCR5$^+$ T lymphocytes were isolated from TILs of ESCC patients using MoFlo XDP cell sorter (Purity >90%), counted and suspended in media containing 1% FBS. Then, cells were placed in transwell inserts (5 μm pore size) with media containing 1% FBS alone or supplemented with recombinant CCL4 (20 ng ml^{-1}) in the bottom chambers. Migration index was calculated by dividing the number of cells that migrated in each group by the number migrating in CD8$^+$CCR5$^-$/media alone group. (**B**) Purified CD4$^+$CCR6$^-$ and CD4$^+$CCR6$^+$ T lymphocytes from TILs of ESCC patients were counted, suspended and placed in transwell inserts (5 μm pore size) with media containing 1% FBS alone or added with CCL20 (50 ng ml^{-1}) in the bottom chambers as indicated. After incubation, FoxP3$^+$ T$_{reg}$ cells that migrated through the transwell were counted as described in the Materials and Methods. Migration index was calculated by dividing the number of cells that migrated in each group by the number migrating in FoxP3$^+$CCR6$^-$/media alone group. Data shown represent mean ± s.d.

Elevated production of both CCL4 and CCL20 in ESCC tissues compared with marginal sites. Next, we checked the expressions of CCL4 and CCL20 in tumour and marginal tissues. The IHC analysis showed that only 68.0% of marginal tissues (51 out of 75) were stained positively for CCL4, compared with 93.75% of tumour tissues (120 out of 128) (Figure 4A). The average scores of CCL4 were different between marginal and tumour tissues (1.26 ± 1.50 vs 3.22 ± 2.61; $t = 5.914$, $P < 0.0001$; Figure 4B). For CCL20 staining, 89.84% of tumour samples (115 out of 128) were CCL20 positive in contrast with 54.67% of marginal tissues (41 out of 75) (Figure 4A). The average IHC scores of tumour tissues were obviously higher than marginal tissues (2.28 ± 1.89 vs 0.89 ± 1.25; $t = 5.648$, $P < 0.0001$; Figure 4B). This result was also reflected in the RT–PCR analysis. In paired samples, mRNA intensities of CCL4 and CCL20 were significantly increased in tumour tissues ($t_{CCL4} = 2.268$, $P < 0.05$; $t_{CCL20} = 2765$, $P < 0.01$; Supplementary Figure 3).

Intratumoural expression of CCL4 and CCL20 predicts overall survival of ESCC patients. First, we checked the associations of the patterns of chemokine expression with clinical and pathologic parameters (Supplementary Table 2). The expressions of CCL4 were not associated with patients' gender, age, clinical stage,

T stage, lymph node metastasis and tumour length. Similarly, the CCL20 levels were not associated with these factors, except for lymph node invasion (Supplementary Table 2). Increased expressions of CCL20 were observed in patients with lymph node metastases ($P < 0.05$). The lymph node invasion was significantly associated with patients' survival ($P < 0.05$; Figure 5A and Table 1). Prompted by these data, we investigated the effects of CCL4 and CCL20 on clinical outcomes. For CCL4, increased expression showed a significant association with prolonged overall survival (Figure 5B and Table 1). The medium survival time of CCL4^{high} group was 53 months, whereas it was only 26.5 months in CCL4^{low} group ($P < 0.001$). Between low and high CCL20-expressing patients, the difference in survival was not significant ($P > 0.05$; Figure 5C and Table 1). Nevertheless, further classification of patients into four groups, according to both expressions of CCL4 and CCL20, revealed a prognostic role of CCL20. As shown in Figure 5D, the CCL4^{high}/CCL20^{low} group demonstrated best overall survival compared with other three groups ($P < 0.005$), whereas the CCL4^{low}/CCL20^{high} and CCL4^{low}/CCL20^{low} groups showed poor survival. To determine whether chemokines were predicting markers for patients' survival, multivariate analysis was performed. The results revealed that CCL4 expression is a strong predictive factor for increased survival ($P < 0.005$; Table 1). Lymph node invasion was an independent predictive factor for survival as well ($P < 0.05$; Table 1).

DISCUSSION

The infiltration of T lymphocytes into tumour tissues is a common phenomenon, but its magnification and character are widely different (Wehler *et al*, 2011; Salerno *et al*, 2013; Sherwood *et al*, 2013; Sugiyama *et al*, 2013). T lymphocyte accumulation in tumour tissues has significant impacts on tumour differentiation, metastasis, disease progress and patients' survival (Zhang *et al*, 2006; Chen *et al*, 2011; Liu *et al*, 2011; Winkler *et al*, 2011; Gu-Trantien *et al*, 2013). The subtypes and intensities of T lymphocytes infiltrated into special sites are tightly related with specific chemokines (Oldham *et al*, 2012; Oo *et al*, 2012; Parsonage *et al*, 2012). Although several studies have examined the expressions of chemokines and chemokine receptors by oesophageal carcinoma cells (Verbeke *et al*, 2012; Tachezy *et al*, 2013), the relationship between oesophageal carcinoma-associated chemokine and T lymphocyte accumulation is rarely reported. More importantly, the clinical consequence of chemokine-directed tumoural recruitment of T lymphocytes has not been noticed in oesophageal carcinoma. In this report, we conducted the first study on the effects of CCL4 and CCL20 on T lymphocyte subgroup migration and patients' survival in ESCC. In ESCC, CCL4 expression was correlated with the infiltrations of CD8$^+$ T lymphocytes. The tumoural expression of CCL20 was associated with T$_{reg}$ lymphocyte accumulation. Meanwhile, CCR5 (CCL4-specific receptor) was mainly expressed in oesophageal cancer-associated CD8$^+$ T lymphocytes, and CCR6 (CCL20-specific receptor) was preferentially expressed by oesophageal cancer-associated regulatory T lymphocytes. Most importantly, the patterns of intratumoural expressions of CCL4 and CCL20 were strongly predictive of patients' survival.

Although CCL4 has not been extensively studied, its importance in the recruitment of CD8$^+$ T lymphocytes has been highlighted (Harlin *et al*, 2009). Conversely, CCL20 plays critical roles in the migration of T$_{reg}$ lymphocytes (Yamazaki *et al*, 2008; Cook *et al*, 2014). Consistently, our data showed that CCL4 was dominantly involved in the trafficking of CD8$^+$ T lymphocytes and CCL20 mainly regulated the infiltration of T$_{reg}$ lymphocytes in ESCC. The difference in chemotaxis of CD8$^+$ T and T$_{reg}$ lymphocytes is
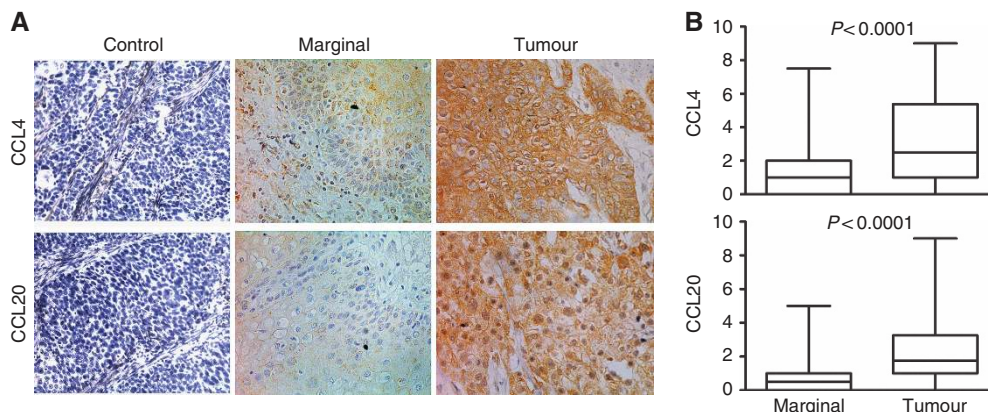
Figure 4. Expressions of CCL4 and CCL20 are elevated in tumour tissues. Both marginal and tumour tissues were checked by immunohistochemistry for the expressions of CCL4 and CCL20. Then, the immunohistochemical scores were calculated as described in the Materials and Methods. (**A**) Representative photographs of marginal and tumour tissue sections stained with specific primary antibodies or non-immune rabbit IgG, × 400. (**B**) Scores of CCL4 and CCL20 were significantly higher in malignant tissues ($n = 128$) than that in marginal tissues ($n = 75$).
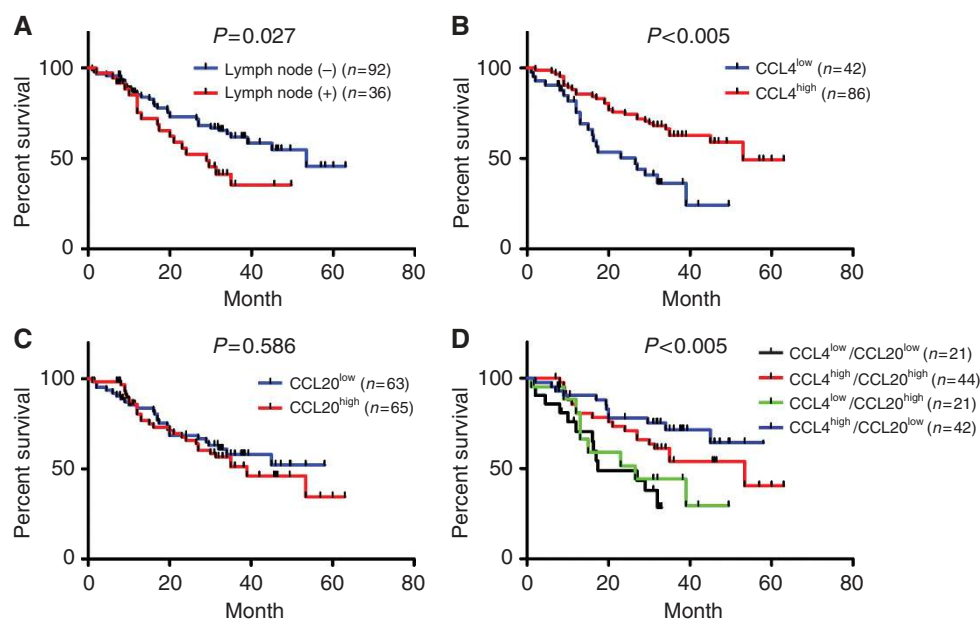

Figure 5. Intratumoural expression levels of CCL4 and CCL20 predict patients' survival. (**A**) Kaplan–Meier survival curve comparing the survival of patients with or without lymph node metastasis. (**B** and **C**) Kaplan–Meier survival curve comparing the high and the low CCL4 (**B**) or CCL20 (**C**) expression groups. (**D**) Kaplan–Meier survival curve according to the expressions of both CCL4 and CCL20.

related with the expressing patterns of specific chemokine receptors. As the only receptor for CCL4, CCR5 shows preferred expression on $CD8^+$ T lymphocytes in cancer patients (Musha *et al*, 2005; Parsonage *et al*, 2012). In contrast, CCR6, the specific receptor of CCL20, is highly expressed on T_{reg} lymphocytes (Chen *et al*, 2011). Consistent with these reports, we found that CCR5 and CCR6 were primarily expressed on $CD8^+$ T and T_{reg} lymphocytes, respectively, in ESCC patients (Figure 2). In ESCC tissues, the portions of $CCR5^+CD8^+$ T and $CCR6^+ T_{reg}$ lymphocytes were significantly greater than that of $CCR6^+CD8^+$ T and $CCR5^+ T_{reg}$ lymphocytes, separately (Figure 2). These observations suggested that the specific expressions of receptors are related with the selective recruitments of $CD8^+$ T and T_{reg} lymphocytes by CCL4 and CCL20 into ESCC lesions.

Chemokines have been reported to impact the survival of cancer patients (Popple *et al*, 2012; Zeng *et al*, 2013). Apart from their effects on tumour cells themselves, the impacts of chemokines on

patient survival are, at least partially, associated with T lymphocyte recruitment (Wehler *et al*, 2011; Chew *et al*, 2012; Mulligan *et al*, 2013). T lymphocyte infiltration is intimately associated with prognosis (Correale *et al*, 2012; Katz *et al*, 2013). Intense infiltration of $CD8^+$ T lymphocytes usually indicates favourable prognosis and longer survival times (Seo *et al*, 2013; Hermans *et al*, 2014), whereas the accumulation of T_{reg} lymphocytes is related with poor survival (Chen *et al*, 2011; Suzuki *et al*, 2013). Our data showed CCL4 and CCL20 were related with tumoural infiltration of $CD8^+$ T and T_{reg} lymphocytes, respectively, in ESCC. Upregulation of $CD8^+$ T lymphocyte-associated CCL4 predicted favoured overall survival (Figure 5B). However, CCL20 was not significantly related with patients' survival (Figure 5C). CCL20 is also involved in the recruitment of T helper 17 (Th17) lymphocytes (Zhao *et al*, 2014). The Th17 lymphocytes could promote the activation of $CD8^+$ T lymphocytes by recruiting dendritic cells (Martin-Orozco *et al*, 2009). Chen *et al* (2012) reported that

Table 1. Univariate and multivariate analysis of overall survival

Clinical factors	Deaths/no. of patients	Univariate analysis		Multivariate analysis	
		Log-rank P		Risk ratio (95% confidence interval)	P
Gender					
Male	40/93	0.542		0.816 (0.430–1.548)	0.534
Female	13/35				
Age, years					
⩽60	21/53	0.810		1.169 (0.659–2.071)	0.594
>60	32/75				
Clinical stage					
I–IIa	33/87	0.082		0.550 (0.067–4.535)	0.579
IIb–IV	20/41				
Tumour invasion					
T1	4/16	0.226		1.150 (0.811–1.633)	0.433
T2	21/49				
T3	25/53				
T4	3/10				
Lymph node metastasis					
Negative	34/92	**0.027**		1.946 (1.109–3.479)	**0.021**
Positive	19/36				
Tumour length, cm					
⩽3	28/64	0.999		0.961 (0.639–1.447)	0.849
3–5	18/44				
>5	7/20				
CCL4 expression					
Low	22/42	**0.001**		0.386 (0.220–0.678)	**0.001**
High	31/86				
CCL20 expression					
Low	25/63	0.586		0.653 (0.098–4.337)	0.659
High	28/65				
CCL4/CCL20					
Low/low	13/22	**0.003**		1.139 (0.646–2.007)	0.653
Low/high	9/20				
High/low	11/41				
High/high	20/45				

The bold value indicates the difference reaches statistical significance.

CCL20 attracted Th17 lymphocytes in oesophageal carcinoma. In our study, CCL20 was correlated with Th17 lymphocyte-related molecules, IL-17 and IL-23 ($r_{IL-17} = 0.196$, $P < 0.05$; $r_{IL-23} = 0.519$, $P < 0.001$; Supplementary Figure 1C), although the coefficient between CCL20 and IL-17 was relative weak. This observation indicated that CCL20 in ESCC probably recruits T_{reg} and Th17 lymphocytes that have opposite functions. The dual effects of CCL20 on tumour immunity may help to explain why CCL20 lacks the prognostic value in ESCC patients. Interestingly, patients' survival was further affected when CCL4 and CCL20 were together analysed. For patients with high levels of CCL4, high expression of CCL20 decreased survival (Figure 5D). This phenomenon is probably related with the interaction between CD8$^+$ T and T_{reg} lymphocytes. Katz *et al* (2013) reported that the ratio between T_{reg} and CD8$^+$ T lymphocytes was the key to determine survival when both cell types were taken into consideration, and enhanced T_{reg} lymphocyte accumulation hampered the function of CD8$^+$ CTLs. Our data imply that chemokine-directed T lymphocyte infiltration exerts great impacts on ESCC patients' survival.

In addition, we noticed that lymph node metastasis and CCL4 expression were independent predictors for ESCC patients' survival (Figure 5). Relapse occurs in great numbers of patients with oesophageal carcinoma and leads to death (Sudo *et al*, 2013; Mariette *et al*, 2014; Sudo *et al*, 2014). However, the efficient prognosis predictors would help guide treatment and management

of oesophageal carcinoma patients according to individual risk, finally improving patients' survival. Lymph node metastasis is the only important prognostic factor in ESCC patients (Sehitogullari *et al*, 2011; Cho *et al*, 2014). Our data confirmed the predictive ability of lymph node invasion in ESCC patients (Figure 5A). CCL4 was an independent predictor for survival as well (Figure 5B). Although not significantly associated with survival, CCL20 helped further classify patients into high-, middle- and low-risk groups (Figure 5D). Therefore, CCL4 and CCL20 are valuable predictive factors and helpful for the improvement of personalised therapy.

In summary, our data demonstrate that the chemokine character of ESCC tumour microenvironment determines local recruitments of functionally distinct T lymphocyte subsets and predicts patients' survival. T lymphocyte subgroups, CD8$^+$ CTLs and T_{reg} lymphocytes, were separately recruited towards malignant lesions by CCL4 and CCL20. CCL4 proved to be a strong independent predictive marker of ESCC patients' survival, with particularly strong prognostic value when analysed jointly with CCL20. CCL4 and CCL20 might prove valuable not only as survival predictors, but also as targets for ESCC therapy. Our data highlight the importance of chemokines in immune surveillance and survival of ESCC patients.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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