Original Article
Dysregulation of IL-10 by macrophages and T cells in recurrent and malignant schwannomas

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Abstract: Schwannoma is a common, almost always benign tumor developed from Schwann cells, and can usually be removed by surgery. However, in a minority of patients, schwannoma can reappear at the same location for reasons not completely understood. Furthermore, schwannoma can undergo malignant transformation in a rare subset of patients, which is aggressive, rapidly growing and life threatening. To examine the underlying mechanism in schwannoma regrowth and malignant transformation, we examined the intratumor environment in primary and recurrent schwannomas of both benign and malignant types. We found that compared to the noncancerous, primary tumor, the recurrent schwannoma tumor after the removal of the first expressed significantly higher levels of IL-10. Moreover, the IL-10 level was further upregulated in malignant schwannomas. Malignant tumors also presented an infiltration of macrophages and T cells, which were not observed in benign tumors. Together, we discovered a previously unknown feature of recurrent and malignant schwannomas.

Keywords: IL-10, macrophage, T cell, schwannoma

Introduction

Schwannoma is a common, usually benign tumor developed from Schwann cells, and makes up about eight percent of all central nervous system tumors [1, 2]. Schwannomas are almost always noncancerous, and can usually be removed by surgery. However, in a minority of patients, schwannoma can reappear at the same location after surgery, for reasons not completely understood [3]. It is generally thought that the recurrent tumor might have resulted from incomplete surgical removal, but inexplicably, the recurring schwannoma is more difficult to treat by chemotherapy and radiotherapy than the original one [4-6]. In addition, in a rare subset of patients, schwannoma is presented as an aggressive, rapidly growing malignant form of tumor, also called malignant peripheral nerve sheath tumors, with a 5-year survival rate of between 39-85% [7-10]. Several risk factors, including the connection with the familial condition neurofibromatosis type 1 (NF1) and the use of gamma knife radiosurgeries, were associated with the malignant transformation of schwannomas [11, 12], but the lack of understanding of the precise underlying mechanisms prevents the development of better prognosis and treatment options.

The molecular and cellular participants in the intratumoral environment exert important tumorigenesis, inflammatory and/or immunoregulatory functions, which determines the disease outcome by promoting or limiting tumor growth [13]. The tumor microenvironment is generally considered inhibitory to immune activation due to multiple inhibitory mechanisms, such as antigen presentation by immunoregulatory IL-10^{high}IL-12^{low}-tumor-associated macrophages, defective dendritic cell function, inhibition of CD8$^+$ T cells by myeloid-derived suppressor cells, inhibition of immune cell proliferation by nutrient depletion and hypoxia [13, 14]. The tumor microenvironment in schwannoma has not been examined, but given its crucial role in other tumors, we hypothesized that potential differences in the intratumor environment may contribute to the increased difficulty in treating recurrent schwannomas and the transformation toward malignancy.

Among all factors that could potentially affect the intratumoral environment, we focused on interleukin-10 (IL-10) because of it mediates a wide range of immunoregulatory functions, including mediation of microbial tolerance in the digestive tract [15], counteraction of allergy [16], inhibition of proinflammatory cytokine (such as IFN-gamma, IL-2, TNF-alpha, and GM-CSF) production [17, 18], downregulation of adaptive T cell activation by downregulation of antigen presentation in dendritic cells and macrophages [19, 20], as well as mediating potentially proinflammatory B cell maturation and antibody production [21]. Furthermore, IL-10 overexpression is one of the hallmarks of tumor-associated macrophages [22, 23], which stimulate angiogenesis, enhance tumor cell invasion, motility and intravasasion, prevent tumor cell elimination by natural killer cells and T cells [24], and are correlated with poor prognosis [25]. The role of IL-10 in schwannoma is yet unclear.

In this study, we examined the intratumor environment in original and recurrent schwannomas of both benign and malignant types. We found that compared to the noncancerous, original tumor, the recurrent schwannoma tumor after the removal of the first expressed significantly higher levels of IL-10. Moreover, the IL-10 level was further upregulated in malignant schwannomas. Malignant tumors also presented an infiltration of macrophages and T cells, which were not observed in benign tumors. Together, we discovered a previously unknown feature of recurrent and malignant schwannomas.

Materials and methods

Patient information

Thirty one schwannoma patients were recruited The Third Affiliated Hospital of Hebei Medical University. Twenty two patients were initially diagnosed with benign schwannoma, which were removed by surgery. Eight patients had later developed recurrent benign schwannoma at the same site and 2 patients had recurrent malignant schwannoma. One patient presented malignant first-time schwannoma. Benign and malignant schwannomas were determined by microscopic examination. Benign features included mild nuclear pleomorphism and absence of mitoses or necrosis, while malignant features included highly pleomorphic spindle cells with bizarre nuclei, scattered giant cell forms, and areas of necrosis and mitosis,

according to previously established standards [7, 26]. The study procedures were approved by the ethics board of The Third Affiliated Hospital of Hebei Medical University, and written informed consent was received from every patient.

Sample collection

Schwannoma tumors were surgically removed by experienced surgeons. All tumors were homogenized into single cell suspensions by adopting previously published protocols [27, 28]. Briefly, freshly resected tumor tissues were pre-washed with PBS, cut into 2 mm cubes and digested with 0.3 mg/mL collagenase (Worthington Biochemical) for 1 h at 37°C with shaking. The suspension was filtered through a 70 μm cell strainer (Corning) and was centrifuged. The top supernatant was collected for extracellular IL-10 measurement, while the cells were resuspended in PBS plus 1% BSA for intracellular IL-10 mRNA analysis.

IL-10 ELISA

Extracellular IL-10 was measured by Human IL-10 Quantikine ELISA kit (R&D Systems) following manufacturer's instructions. Sensitivity was 3.9 pg/mL while the assay range was 7.8-500 pg/mL. Measurements below sensitivity was assigned zero.

mRNA isolation and quantitative real-time RT-PCR

Intracellular IL-10 mRNA quantification was done by adapting a protocol for human lung cancers [28]. Total RNA from resuspended schwannoma cells were isolated by using RNeasy mini Kit (Qiagen) following manufacturer's instructions. mRNA reverse transcription was done using M-MLV reverse transcriptase (Promega). QRT-PCR was done using SYBR Green PCR Master Mix (Applied Biosystems). IL-10 copy numbers were normalized against beta-actin. The following primer sets were used: forward 5' AGAACCTGAAGACCCTCAGGC 3' and reverse 5' CCACGGCCTTGCTCTTGTT 3' for IL-10, and forward 5' ACCACACCTTCTACAATGA 3' and reverse 5' GTCATCTTCTCGCGGTTG 3' for beta-actin. The amplification included a 10-min 95°C initial denaturation, 40 cycles of 15-second 95°C denaturation and 1-min 60°C annealing and extension, a 10-min 60°C final extension, and a 4°C cooling step. Triplicates for each sample were performed. IL-10 mRNA was normalized relative to beta-actin mRNA.
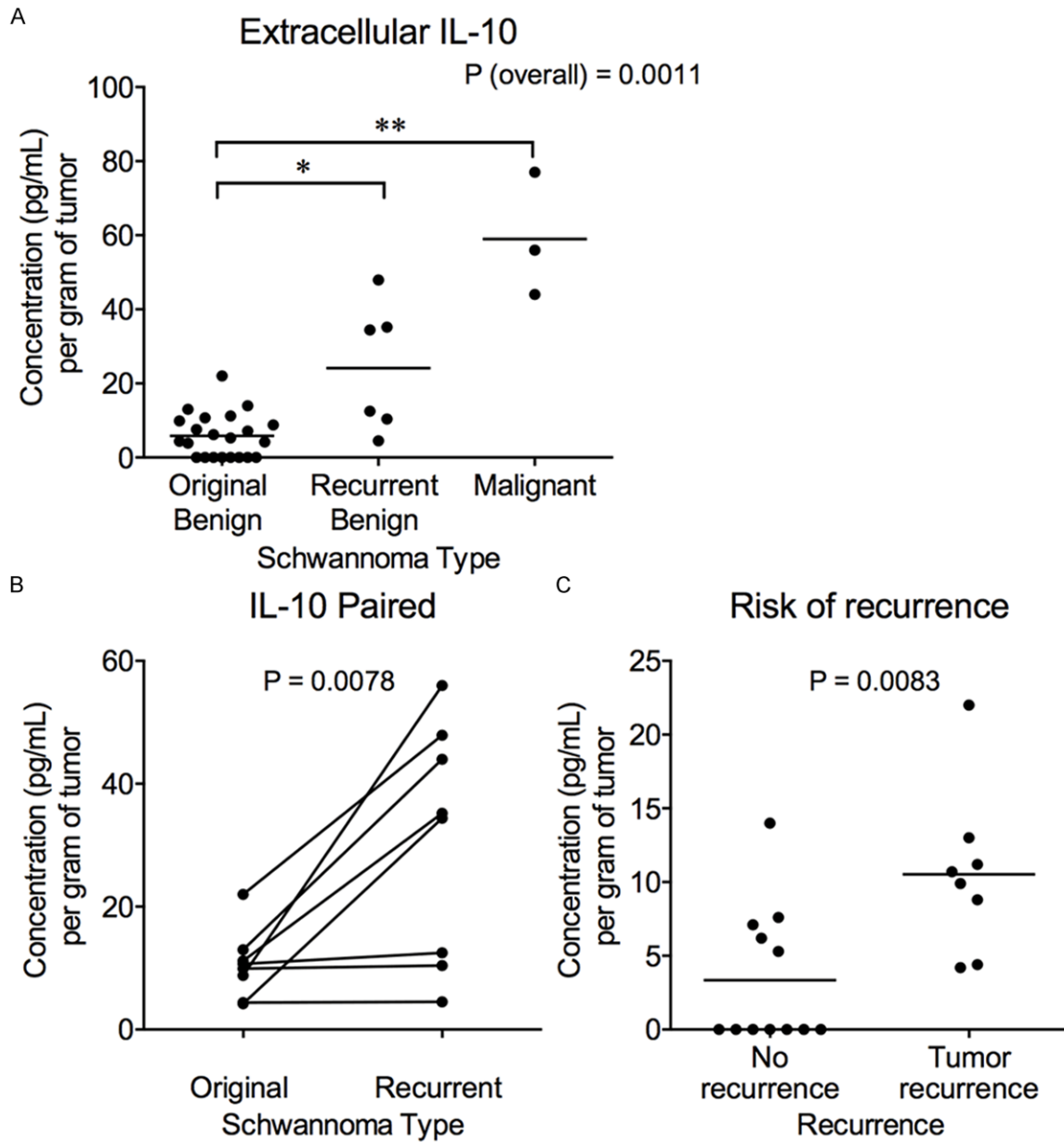
Figure 1. Extracellular concentration of IL-10 in various schwannoma subtypes. Freshly resected tumor were weighed, homogenized and centrifuged to separate cells from buffer. The extracellular IL-10 concentration in the supernatant was measured by ELISA. A. The extracellular IL-10 concentration in the original benign, recurrent benign and malignant tumors, normalized by the weight of the tumor resection. Kruskal Wallis one-way ANOVA followed by Dunn's multiple comparison test. **: $P < 0.01$. *: $P < 0.05$. B. The extracellular IL-10 concentration in patients with both original and recurrent tumors. Wilcoxon matched-pairs test. C. The extracellular IL-10 concentration in the original tumor in patients without recurrence, compared to that in patients with recurrence. Mann-Whitney test.

Flow cytometry

Anti-human CD68 and CD3 monoclonal antibodies were used to identify macrophages and T cells, respectively. Schwannoma cells from homogenized suspensions were incubated with 3 µg/mL antibodies for 20 min in 4°C. Cells were then washed twice and fixed in 2% formaldehyde for flow cytometry. Samples were acquired in a FACSCalibur cell sorter (BD) and analyzed in FlowJo (TreeStar).

Statistical analysis

All analyses were performed using Prism 6.0 (GraphPad Software). Two-tailed *P* value less than 0.05 was considered statistically significant.
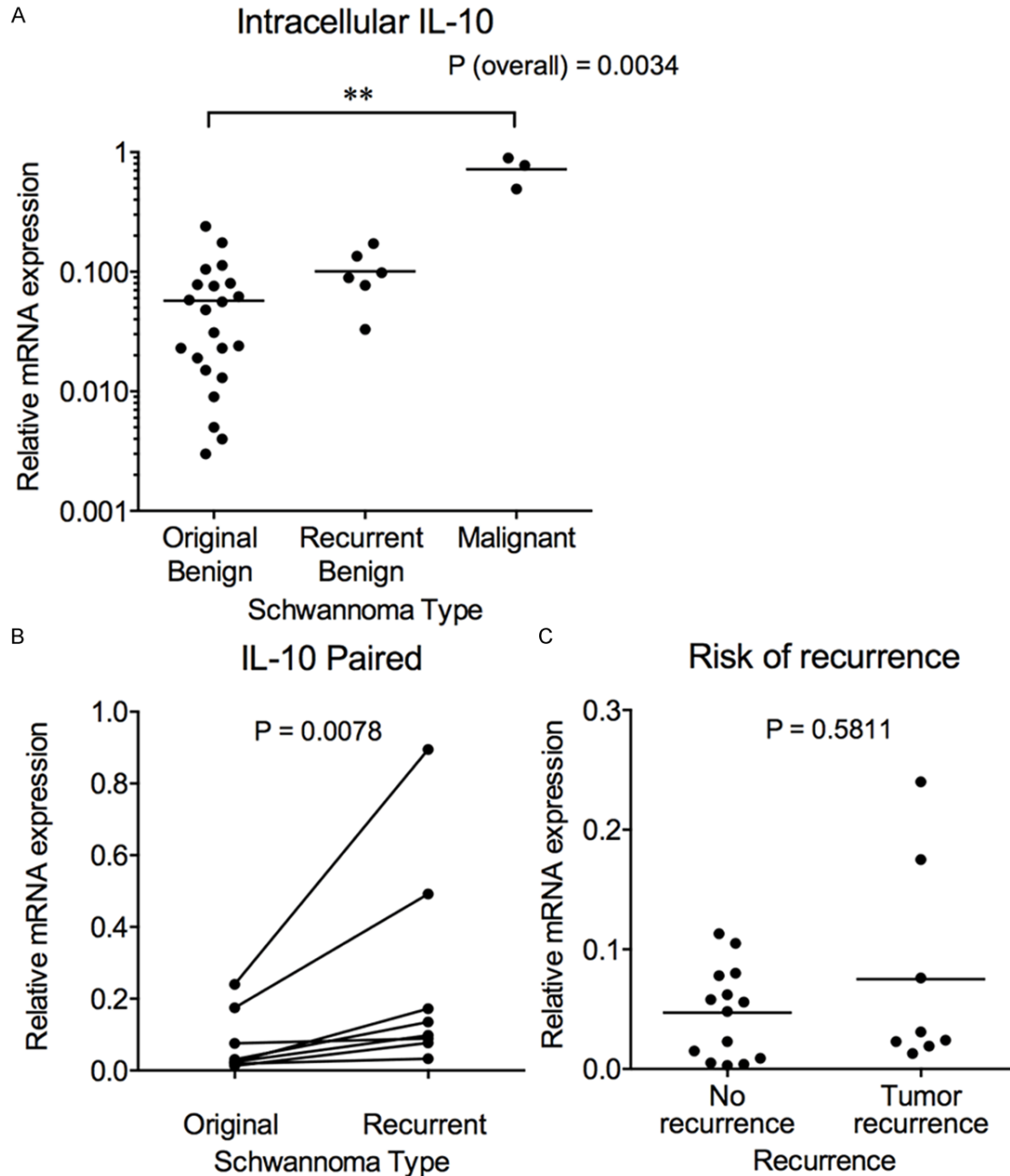
Figure 2. Intracellular IL-10 mRNA level in various schwannoma subtypes. IL-10 mRNA from lysed cells was measured by quantitative real-time PCR and was normalized against beta-actin mRNA. A. The intracellular IL-10 mRNA level in the original benign, recurrent benign and malignant tumors, normalized by the weight of the tumor resection. Kruskal Wallis one-way ANOVA followed by Dunn's multiple comparison test. **: P < 0.01. B. The intracellular IL-10 mRNA level in patients with both original and recurrent tumors. Wilcoxon matched-pairs test. C. The intracellular IL-10 mRNA level in the original tumor in patients without recurrence, compared to that in patients with recurrence. Mann-Whitney test.

Results

Extracellular IL-10 concentration is elevated in recurrent and malignant schwannomas

We first examined the extracellular IL-10 concentration directly ex vivo. The supernatant from homogenized single cell suspensions from freshly resected schwannoma tumor was collected and IL-10 enzyme-linked immunosorbent assay (ELISA) was performed. For normalization across tumors with different sizes, data were expressed as the concentration of IL-10
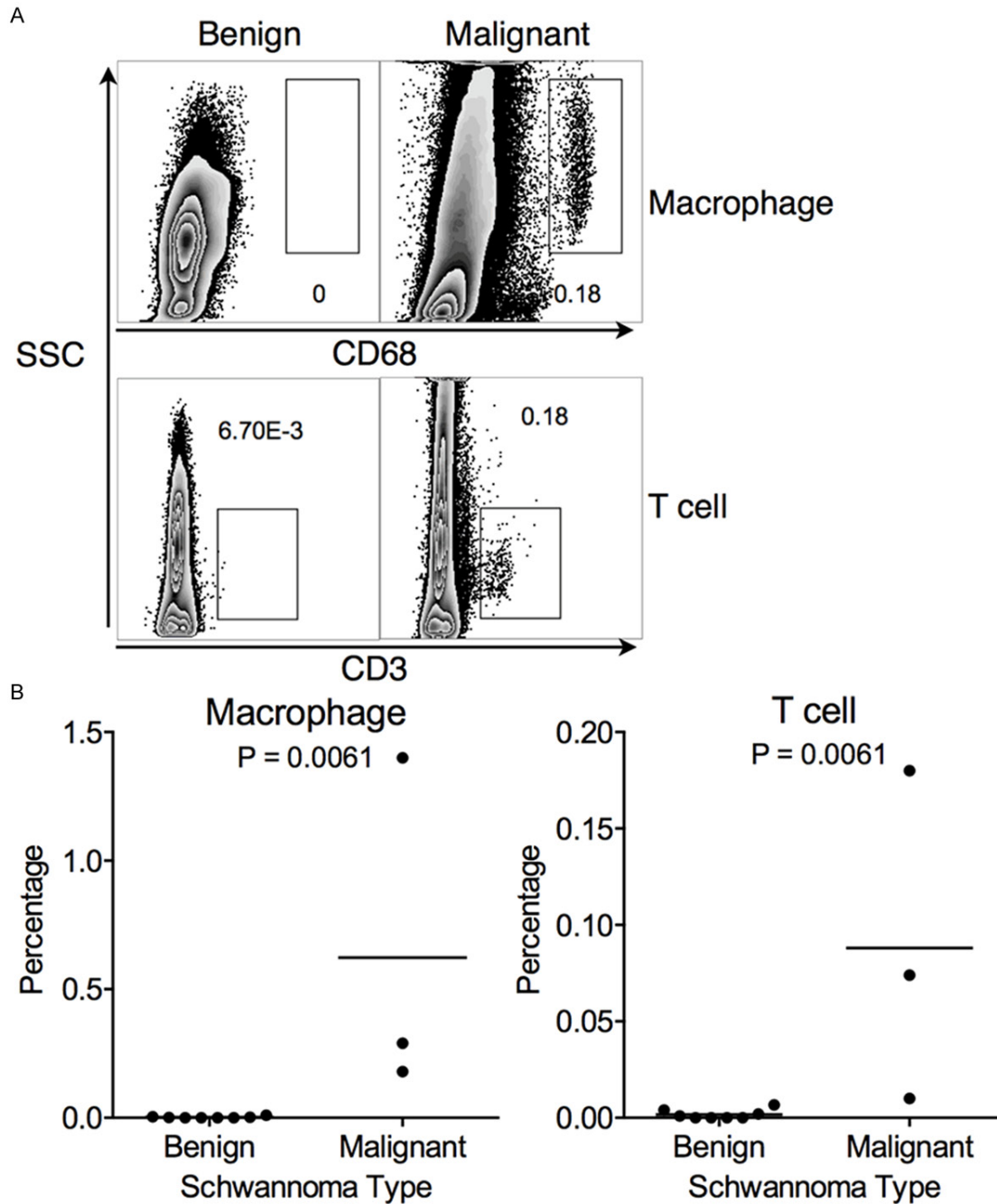
Figure 3. Presence of macrophages and T cells in benign and malignant schwannomas. Cells were stained with anti-human CD68 and CD3 and analyzed by flow cytometry. A. Staining pattern of CD68 and CD3 in one representative original benign and one representative malignant tumors. B. The frequencies of CD68^{+} macrophages in original benign and malignant tumors. C. The frequencies of CD3^{+} T cells in original benign and malignant tumors. Mann-Whitney test.

per gram of tumor. We found that the original benign tumor contained significantly less IL-10 than the recurrent benign tumor or the malignant tumor (**Figure 1A**). For the eight patients who had a recurrence after first surgical resection, we compared their extracellular IL-10 in the original tumor and the recurrent tumor. All eight subjects had upregulated extracellular IL-10 in the recurrent tumor, although the degree of upregulation was highly variable (**Figure 1B**). We also examined the association between extracellular IL-10 concentration and

the risk of recurrence, and found that patients with recurring schwannoma had significantly higher extracellular IL-10 in the original tumor, than patients who did not have recurrence. Overall, these data demonstrated that extracellular IL-10 concentration in schwannoma is correlated with disease severity and recurrence.

Cells in recurrent and malignant schwannoma expressed significantly higher IL-10

To examine the cellular expression in schwannoma tumor, we examined IL-10 mRNA levels by quantitative real-time PCR. We found that the IL-10 mRNA level in malignant schwannomas was significantly increased compared to that in the original benign tumor (**Figure 2A**). In the eight subjects with recurrence, the IL-10 mRNA level was significantly upregulated in the recurrent tumor (**Figure 2B**), but no difference was observed between patients with or without recurrence, in terms of the IL-10 mRNA level in the original tumor (**Figure 2C**). Together, these data demonstrated an upregulation of IL-10-production by cells in malignant schwannoma.

CD68+ macrophages and CD3+ T cells were observed in malignant schwannoma but not benign schwannoma

IL-10 is primarily expressed by innate and adaptive immune cells, such as macrophages, dendritic cells, mast cells, natural killer cells, eosinophils, neutrophils, T cells and B cells [29]. Presence of intracellular IL-10 suggests infiltration of immune cells. Therefore, we examined the presence of macrophages and T cells, two common tumor-infiltrating cell types with potential roles in angiogenesis and anti-tumor immunity (**Figure 3A**). We found that little to none CD68+ macrophages and CD3+ T cells were found in benign schwannoma, but in striking contrast, macrophages and T cells were both present in all three malignant schwannoma patients examined (**Figure 3B**). These data suggest that macrophages and T cells could infiltrate malignant schwannoma and potentially play a role in disease pathogenesis and progression.

Discussion

Malignant transformation of schwannoma is a rare but life-threatening condition, with only a few studies available and most of which are case reports. As a result, we have very limited understanding in its etiology and progression. Currently, it is generally thought that schwannoma recurrence after removal of the original tumor was due to incomplete first resection, which leads to regrowth at the original site. The use of gamma knife radiosurgery at the first resection and the presence of NF1 are associated malignant transformation [5, 8, 11]. Prognosis was also correlated with other general tumor characteristics, such as tumor grade, location, and degree of differentiation [8, 9, 30]. These prognostic associations and risk factors offer only limited power of prediction and incomplete insight into the underlying mechanism, and do not explain the differences in the responsiveness toward various treatments, in original and recurrent tumor, as well as in malignant tumors. To solve this problem, we obtained original and recurrent schwannoma resections of both benign and malignant types. The critical role of tumor microenvironment in modulating tumor growth and anti-tumor immunity is well established. Proinflammatory mechanisms, such as infiltration of cytotoxic T cells, improved tumor antigen presentation, and presence of proinflammatory cytokines, were associated with better prognosis and higher 5-year survival rate, while anti-inflammatory mechanisms, including the presence of inhibitory tumor-associated macrophages, regulatory T cells, and other suppressor cells, secretion of inhibitory cytokines, and downregulated MHC class I molecule and costimulatory molecules, were associated with poor prognosis [14]. We therefore examined the inflammatory status of schwannoma tumor microenvironment by focusing on IL-10, an inhibitory cytokine known to suppress anti-tumor immunity [31]. We showed that IL-10 concentration in schwannoma is correlated with disease severity and might act as a risk factor for recurrence and malignant transformation. We showed by extracellular supernatant ELISA and intracellular mRNA that IL-10 was more abundant in recurrent benign and malignant tumors compared to the original benign tumor, demonstrating an association with disease severity. Surprisingly, patients with recurrence showed higher levels of extracellular IL-10 concentration in the original tumor, which suggest that besides incomplete resection, tumor recurrence was also associated with the inflammatory status of the patient, a feature shared with

other tumors and cancers [32, 33]. Together, our study revealed a previously unknown mechanism in schwannoma regulation.

We also observed that both extracellular and intracellular IL-10 could be found in schwannoma at varying amounts. The source of extracellular IL-10 could come from circulation or be produced endogenously, by infiltrating immune cells, and interestingly, we indeed found a presence of CD68^{+} tumor-associated macrophages and CD3^{+} infiltrating T cells, almost exclusively in malignant tumor. The precise roles of immune cell infiltration in schwannoma development are currently unknown, but their presence suggests an involvement that needs to be further studied. In the future, histological staining, as well as flow cytometry analysis, will be performed to examine the localization, surface marker expression and cytokine production of these macrophages and T cells.

Disclosure of conflict of interest

None.

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References

[1] Propp JM, McCarthy BJ, Davis FG, Preston-Martin S. Descriptive epidemiology of vestibular schwannomas. Neuro Oncol 2006; 8: 1-11.

[2] Jeon JH, Hwang HS, Jeong JH, Park SH, Moon JG, Kim CH. Spinal schwannoma; analysis of 40 cases. J Korean Neurosurg Soc 2008; 43: 135-8.

[3] Ahmad RA, Sivalingam S, Topsakal V, Russo A, Taibah A, Sanna M. Rate of recurrent vestibular schwannoma after total removal via different surgical approaches. Ann Otol Rhinol Laryngol 2012; 121: 156-61.

[4] Li Z, Wang H, Li J, Teng L. Recurrent retroperitoneal Schwannomas displaying different differentiation from primary tumor: case report and literature review. World J Surg Oncol 2010; 8: 66.

[5] Kimmel RA, Doherty J, Slattery WH, Linthicum FH. Recurrent schwannoma postirradiation: histological review reveals mixed schwannoma and meningioma. Otolaryngol. Head Neck Surg 2013; 148: 886-8.

[6] Asuquo ME, Nwagbara VI, Akpan SO, Otobo FO, Umeh J, Ebughe G, Chukwuegbo CC, Ugbem TI. Recurrent benign schwannoma of the scalp: Case report. Int J Surg Case Rep 2013; 4: 65-7.

[7] Wilkinson JS, Reid H, Armstrong GR. Malignant transformation of a recurrent vestibular schwannoma. J Clin Pathol 2004; 57: 109-10.

[8] Porter DE, Prasad V, Foster L, Dall GF, Birch R, Grimer RJ. Survival in malignant peripheral nerve sheath tumours: a comparison between sporadic and neurofibromatosis type 1-associated tumours. Sarcoma 2009; 2009: 756395.

[9] White HR. Survival in malignant schwannoma. An 18-year study. Cancer 1971; 27: 720-9.

[10] Cashen DV, Parisien RC, Raskin K, Hornicek FJ, Gebhardt MC, Mankin HJ. Survival data for patients with malignant schwannoma. Clin Orthop Relat Res 2004; 69-73.

[11] Shin M, Ueki K, Kurita H, Kirino T. Malignant transformation of a vestibular schwannoma after gamma knife radiosurgery. Lancet 2002; 360: 309-10.

[12] Evans DG, Baser ME, McGaughran J, Sharif S, Howard E, Moran A. Malignant peripheral nerve sheath tumours in neurofibromatosis 1. J Med Genet 2002; 39: 311-4.

[13] Quail DF, Joyce JA. Microenvironmental regulation of tumor progression and metastasis. Nat Med 2013; 19: 1423-37.

[14] Gajewski TF, Schreiber H, Fu YX. Innate and adaptive immune cells in the tumor microenvironment. Nat Immunol 2013; 14: 1014-22.

[15] Braat H, Rottiers P, Hommes DW, Huyghebaert N, Remaut E, Remon JP, van Deventer SJ, Neirynck S, Peppelenbosch MP, Steidler L. A phase I trial with transgenic bacteria expressing interleukin-10 in Crohn's disease. Clin Gastroenterol Hepatol 2006; 4: 754-9.

[16] Grimbaldeston MA, Nakae S, Kalesnikoff J, Tsai M, Galli SJ. Mast cell-derived interleukin 10 limits skin pathology in contact dermatitis and chronic irradiation with ultraviolet B. Nat Immunol 2007; 8: 1095-104.

[17] D'Andrea A. Interleukin 10 (IL-10) inhibits human lymphocyte interferon gamma- production by suppressing natural killer cell stimulatory factor/IL-12 synthesis in accessory cells. J Exp Med 1993; 178: 1041-8.

[18] Couper KN, Blount DG, Riley EM. IL-10: the master regulator of immunity to infection. J Immunol 2008; 180: 5771-7.

[19] Mittal SK, Roche PA. Suppression of antigen presentation by IL-10. Curr Opin Immunol 2015; 34: 22-7.

[20] Knolle PA, Uhrig A, Hegenbarth S, Löser E, Schmitt E, Gerken G, Lohse AW. IL-10 downregulates T cell activation by antigen-presenting liver sinusoidal endothelial cells through decreased antigen uptake via the mannose receptor and lowered surface expression of accessory molecules. Clin Exp Immunol 1998; 114: 427-33.

[21] Hardy RR, Hayakawa K. B cell development pathways. Annu Rev Immunol 2001; 19: 595-621.

[22] Mantovani A, Romero P, Palucka AK, Marincola FM. Tumour immunity: effector response to tumour and role of the microenvironment. Lancet 2008; 371: 771-83.

[23] Biswas SK, Mantovani A. Macrophage plasticity and interaction with lymphocyte subsets: cancer as a paradigm. Nat Immunol 2010; 11: 889-96.

[24] Noy R, Pollard JW. Tumor-associated macrophages: from mechanisms to therapy. Immunity Elsevier 2014; 41: 49-61.

[25] Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. J Pathol 2002; 196: 254-65.

[26] Yousem SA, Colby TV, Urich H. Malignant epithelioid schwannoma arising in a benign schwannoma. A case report. Cancer 1985; 55: 2799-803.

[27] Solinas G, Schiarea S, Liguori M, Fabbri M, Pesce S, Zammataro L, Pasqualini F, Nebuloni M, Chiabrando C, Mantovani A, Allavena P. Tumor-conditioned macrophages secrete migration-stimulating factor: a new marker for M2-polarization, influencing tumor cell motility. J Immunol 2010; 185: 642-52.

[28] Wang R, Lu M, Zhang J, Chen S, Luo X, Qin Y, Chen H. Increased IL-10 mRNA expression in tumor-associated macrophage correlated with late stage of lung cancer. J Exp Clin Cancer Res 2011; 30: 62.

[29] Ouyang W, Rutz S, Crellin NK, Valdez PA, Hymowitz SG. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. Annu Rev Immunol 2011; 29: 71-109.

[30] Strauss DC, Hayes AJ, Thway K, Moskovic EC, Fisher C, Thomas JM. Surgical management of primary retroperitoneal sarcoma. Br J Surg 2010; 97: 698-706.

[31] Sato T, Terai M, Tamura Y, Alexeev V, Mastrangelo MJ, Selvan SR. Interleukin 10 in the tumor microenvironment: a target for anticancer immunotherapy. Immunol Res 2011; 51: 170-82.

[32] Govindaraj C, Scalzo-Inguanti K, Madondo M, Hallo J, Flanagan K, Quinn M, Plebanski M. Impaired Th1 immunity in ovarian cancer patients is mediated by TNFR2+ Tregs within the tumor microenvironment. Clin Immunol 2013; 149: 97-110.

[33] Sakuishi K, Ngiow SF, Sullivan JM, Teng MW, Kuchroo VK, Smyth MJ, Anderson AC. TIM3(+) FOXP3(+) regulatory T cells are tissue-specific promoters of T-cell dysfunction in cancer. Oncoimmunology 2013; 2: e23849.