Differential expression of Helios, Neuropilin-1 and FoxP3 in head and neck squamous cell carcinoma (HNSCC) patients

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Received: 22 August 2018 / Accepted: 6 April 2019 / Published online: 16 April 2019
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Abstract

In recent years, studies have begun to explore the immune involvement in head and neck tumors. Advanced stage head and neck squamous cell carcinoma (HNSCC) has a poor prognosis with low survival rates with high level of immune infiltrates. Tregs (regulatory T cells) play a crucial role in constructing an immunosuppressive tumor microenvironment. In the present study, we highlighted specific Treg markers and its factors in HNSCC solid tumors and peripheral blood of cancer patients. By histopathology and immunofluorescence staining, we observed differential expression of CD4, CD25, Foxp3, Helios and Neuropilin-1. Further, we analyzed the expression of Foxp3, Helios, Neuropilin-1 and GARP by qPCR and flow cytometry in whole blood and found to be elevated in HNSCC patients in comparison with healthy donors. Additionally, IFN- γ , TGF- β , IL-6, IL-2, IL-10 and TNF- α expressions were also found to be relatively increased in the head and neck cancer patients when compared with healthy donors. Our findings emphasize that Tregs may be involved in promoting tumor progression. Helios and Neuropilin-1 could be potent markers in identifying subsets of Tregs. Association of soluble factors could sculpture the activity of Tregs. With further research, Treg markers and its associated soluble factors could be employed to block Tregs trafficking to the tumor, thus enlightening a potential strategy for targeting human cancers.

Keywords Squamous cell carcinoma · HNSCCs · Tregs · Foxp3 · CD4 · Soluble factors

Introduction

Head and neck squamous cell carcinomas (HNSCCs) resemble most squamous cell carcinomas and can be found in the larynx, pharynx, oral cavity and nasal cavity. Head and neck cancer (HNC) is a major public health problem affected by various factors such as demographical, environmental, behavioral, gender, age and geographical factors. (Kulkarni 2018;

Poddar et al. 2018). When compared globally HNC constitutes more mortality in some developing countries. South central Asia has highest registered cases for lip and oral cavity cancers (Bray et al. 2018). HNC (both sexes) in India is found with more than 60% of patients with advanced disease compared to 40% in developed countries especially with people from lower and middle income groups (Francis 2018). HNSCCs have a poor prognosis with low survival rates for advanced stage tumors and negligible increases in survival rates over the past few decades (Torre et al. 2015). Other than cetuximab and nivolumab, no new targeted therapies or biomarkers have been approved for HNSCC (Jie et al. 2015; Ward-Hartstonge and Kemp 2017). This complex disease may be effectively targeted by immunotherapy once better understanding of the disease microenvironment is obtained (Melero et al. 2018). Understanding host cell signaling and identifying immune infiltrates can reveal novel approaches for cancer therapeutics (Li and Rudensky 2016). Further examination of the role of the immune system in the tumor microenvironment may lead to the identification of novel targets for the immunosuppression of tumor growth (Mohamed Adil et al. 2014; Whiteside

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2018b). Immunosuppression in cancer microenvironment plays a very important role in the progression of cancer and metastasis (Mohamed Adil et al. 2018). Recently, several clinical studies have targeted Tregs with immune check point inhibitor such as CTLA-4 and PD-1 for cancer immunotherapy (Shitara and Nishikawa 2018).

Limited studies have been found to investigate immune involvement and host response in HNC, though innumerable studies suggest that CD4+ T lymphocytes may play a role in the control and development of HNC. Tumor antigens associated with HNSCC have been recognized by specific CD4+ and CD8+ T lymphocytes (Hadrup et al. 2013). Tregs along with other immune cells may be involved in the mechanism by which HNSCC evades the immune system. According to the immunohistochemical studies, HNSCC tumor biopsies have a large T cell population, which potentially impacts the disease prognosis. Further preliminary data have demonstrated a rapid infiltration of T cells into these tumor sites (Barnes and Amir 2017). Foxp3 is specifically expressed in CD4+ CD25+ Tregs and is a critical regulator of CD4+ CD25+ Treg function and development (Lee and Lee 2018). Moreover, Helios has also been found to regulate Treg fitness and upregulate Foxp3 expression, though its precise role still remains debatable. Helios has been demonstrated to suppress Treg function and differentiation (Elkord et al. 2015; Grzanka et al. 2013; Sebastian et al. 2016; Takatori et al. 2015). Another cell surface molecule, Neuropilin-1 (NRP-1) is also expressed at very high levels in Tregs. Studies have shown that NRP-1 expressing Tregs migrate in response to VEGF-derived tumor cells (Sidaway 2017). Glycoprotein A repetitions predominant (GARP), a surface protein, has been expressed in activated Tregs. GARP has been shown to promote the secretion of TGF- β (Sun et al. 2016). Inhibitory cytokines, such as interleukin 10 (IL-10) and transforming growth factor beta (TGF- β) constitute one of the foremost mechanisms of immunosuppression used by Treg cells. Earlier, studies have demonstrated significant cross-talk between cancer-associated fibroblasts and tumor-infiltrating lymphocytes (TILs) in the tumor microenvironment and these TILs have been found to contain a higher percentage of Tregs and are found to suppress T cell proliferation in HNSCC patients (Ward-Hartstonge and Kemp 2017). In HNC Tregs are found at the tumor site and may contribute to poor prognosis. Although Tregs play an important role in immunosuppression, their clinical impact is unclear.

Materials and methods

Patients and controls

Tissue samples were obtained from 30 HNC patients and peripheral blood samples were obtained from 21 HNC

patients. The patient age ranged between 32 and 80 years across both sex. Average mean age is 57 years for male and 55 years for female patients. Control samples were collected from healthy donors in ratio accordance of HNC samples (23–73 years; both sex). Average mean age of the total donor was 40 years. All the donor tissue samples collected from the oral buccal cavity and blood collected, were examined. All patients and healthy controls provided written informed consent prior to participating in the study. This study was approved by the institutional ethical committee of B.S.A.C.I.S.T. (BSAU: REG-OFF: 2015/01SLS).

Histology of HNSCC

Tumor tissues obtained from patients diagnosed with HNSCC and control donor tissues were included in the study. Tissues were fixed overnight in 10% formalin, dehydrated in ethanol, and embedded in paraffin wax. Paraffin-embedded tissues were cut using a microtome (Leica Biosystems, Germany), and the tissue sections were mounted on slides coated with 1% aminopropylethoxysilane (ACP) (Sigma Aldrich). These sections were used for hematoxylin and eosin (H&E) staining and immunofluorescence.

Hematoxylin and eosin staining

Tissue sections were de-waxed with xylene and then re-hydrated and stained with hematoxylin (Harris-modified, Sigma) and eosin Y (Sigma). After staining, the tissue sections were dehydrated and mounted in mounting media (Sigma). Images were acquired using bright-field microscopy (AXIO, Zeiss, Germany).

Immunofluorescence

Tissue sections were de-waxed using xylene and then re-hydrated and stained using an indirect immunofluorescence method. The primary antibodies used in this study included Foxp3 (Sc-28705), Neuropilin-1 (Sc-5307), Helios (Sc-390357), CD 4 (Sc-7219), and CD 25/IL-2R α (sc-365511) (Santa Cruz Biotechnology). Following incubation with primary antibodies, the slides were incubated with fluorescent-conjugated secondary antibodies and then washed. The secondary antibodies used in this study are as follows: mouse anti-rabbit IgG-PE (Santa Cruz, Sc3753) and rabbit anti-mouse IgG3-FITC (Santa Cruz, Sc358949) (Table 1) The IgG-PE secondary antibody was used on Foxp3- and CD4-stained samples, and the IgG3-FITC secondary was used on Helios- and Neuropilin-1-stained samples. Images were acquired using fluorescence microscopy (AXIO, Zeiss). Immunofluorescence staining was performed to assess and quantify CD4+, CD25+, Foxp3+, Helios+ and Neuropilin-1+ (NRP-1) cell populations.

Table 1 List of antibodies used for immunofluorescence studies

Primary antibodies		
CD 4 + (H-370) Sc-7219	Rabbit polyclonal IgG	Santa Cruz Biotechnology, USA
FoxP3 (H-370) Sc-28705c	Rabbit polyclonal IgG	Santa Cruz Biotechnology, USA
Helios (E-7) Sc- 390,357	Mouse polyclonal IgG	Santa Cruz Biotechnology, USA
Neuropilin-1 (A-12) Sc-5307	Mouse monoclonal IgG1	Santa Cruz Biotechnology, USA
CD25 + /IL-2R α (C-11) Sc-365511	Mouse monoclonal IgG1	Santa Cruz Biotechnology, USA
Secondary antibodies		
Mouse anti-rabbit IgG-PE		Santa Cruz Biotechnology, USA
Rabbit anti-mouse IgG-FITC		Santa Cruz Biotechnology, USA

Analysis of relative mRNA expression by qPCR

Total RNA was extracted from all blood samples using TRIzol (Ambion, Thermo Fisher Scientific). mRNA was prepared using the conventional method, and cDNA was prepared using the high capacity cDNA reverse transcription kit (Applied Biosystems, Thermo Fisher Scientific) in a Mastercycle rNexus cycler (Eppendorf). RT-qPCR was performed on a CFX 96 (BIO-RAD) to detect relative mRNA expression levels. Primers (Table 2) were obtained from G Biosciences and IDT.

Flow cytometry

RBCs were lysed using RBC Lysis Buffer (BioLegend) according to the manufacturer's instructions. Anti-coagulated whole blood was obtained, stained for CD4-PerCP, CD25-PE (Biolegend) incubated for 45 min. Then, intracellular staining was performed according to the manufacturer's instructions with the following Foxp3-Alexa Fluor 647 Helios-FITC, NRP-1-PE/CY7 (BioLegend) antibodies with permeabilization buffer (Beckman Coulter) and then fixed with BD cytofix (BD Biosciences). Data were acquired on all fluorescence channels on a MOFLO XDP instrument

(Beckman Coulter) and analyzed. Positive staining and gating strategies were determined by comparison to isotype control.

Statistical analysis

Graph Pad Prism 7.0 and Microsoft Excel 2013 were used to conduct statistical analyses. All experiments were evaluated using paired *t* test assuming Gaussian distribution for the determination of statistical significance. For all the analyses $*P < 0.05$ was considered as statistically significant.

Results

Patient characteristics

Table 3 lists the clinicopathological characteristics of the 30 studied patients with HNSCC. The patients were classified based on sex and age. Tissue sections were classified based on the TNM classification of malignant tumors. The TNM staging system, established by the American Joint Committee on Cancer (AJCC) is used to describe the stages of cancer. In this study, T1 tumors made up 6.7% of all samples, T2 tumors made up 66.7%, and T3 tumors made up 26.7%.

Table 2 List of primers

GENE	Forward primer	Reverse primer
β -actin	CTACGTCGCCCTGGACTTCGAGC	GATGGAGCCGCCGATCCACACGG
Foxp3	GCACCTTCCCAAATCCCAGT	GGCCACTTGCAGACACCAT
Helios	CACCTCAGGACCCATTCTGTGGG	CCATGGTGACTCATGACCTGGGG
Neuropilin-1	ACAGGTAGACTTGGGCCTTCTGCG	ACCACAACATCTGTGGGGTTGGTG
GARP	GGTATAGTGGGAACATTTGCTTTG	ACAGGGCACTTTGTCTTGGTGTGTGC
CD127	AGTGATCAGGGATGGATCGAAC	TCCAACCGGCAGCAATGTAT
IL--6	ACTCCACTGGAATTTGCTTGCCAGG	GCCAGTGCCTCTTTGCTGCTTTCA
IL- 2	GTCACAAACAGTGCACCTAC	CCCTGGGTCTTAAGTGAAAG
IL-10	GCCTAACATGCTTCGAGATC	CTCATGGCTTTGTAGATGCC
IFN- γ	CTGGCAGCATCGCTTTAAACTCGTA	GGAGGTGGGGGCTTTTATTACGG
TGF- β	CACCCGCGTGCTAATGG	ATGCTGTGTGACTCTGCTTGAAC
TNF α	ACCACGCTCTTCTGCCTGCTG	TCTGGTAGGAGACGGCGATGC

N0-stage lymph node involvement was observed in 20% of the samples, while N1, N2, and N3 involvement was identified in 53.3%, 26.7%, and 0% of the samples, respectively. Cell infiltration was observed in the tumor region (excluding hemorrhagic, necrotic, and fibrotic regions) by H & E staining (Fig. 1). The Metastasis M0 was 33.33%, M1 was 40% and Mx was 26.7%. (Table 3).

Analysis of Tregs markers

The purpose of this study was to establish and investigate the predictive value of Tregs in tumor-infiltrating lymphocytes (TILs) and to analyze the presence of Foxp3+, Helios+, and NRP-1+ in tissue samples from HNSCC patients. Tissue sections were stained for immunofluorescence and assessed the presence of Tregs infiltration and evaluated the expression of CD4, CD25, Foxp3, Helios, and Neuropilin-1 (Fig. 2). Quantification was performed by a pathologist and an oral pathologist via scoring of both H & E-stained and

immunofluorescence-stained tissues. Pathological examination revealed that tumor tissues expressed increased infiltration levels of CD4+ cells than control tissues. Stage III tumor-infiltrating lymphocytes (TILs) were found to have low Foxp3 expression compared to Stage I/II. Further Foxp3 was also expressed in normal donor tissues. The expression of Helios was increased in Stage II/III carcinoma tissues. NRP-1 expression was also found increased in stage II/III patients compared to the healthy donors.

Tregs and immune factors in the peripheral immune environment

To understand the prognostic nature of Tregs in the tumor microenvironment, we assessed whole blood samples from HNC patients and healthy donors. Numerous soluble factors within the tumor environment, including cytokines, chemokines and other associated factors support tumor growth and formation. We analyzed the expression of Foxp3, revealing that Foxp3 expression was higher in HNC

Table 3 Clinical pathological features (tumor site and TNM stage)

Characteristics (cancer patients)	No. patients	Percentage
Sex	30	100
Male (32–70 yrs)	18	60
Female (45–80 yrs)	12	40
Primary tumor site		
Oral cavity	23	76.7
Larynx	2	6.7
Pharynx	2	6.7
Oropharynx	2	6.7
Nasopharynx	1	3.3
Tumor staging (TNM)		
T1	2	6.7
T2	20	66.7
T3	8	26.7
T4	0	0
Lymph node involvement		
N0	6	20
N1	16	53.3
N2	8	26.7
N3	0	0
Metastases		
M0	8	26.6
M1	0	0
Mx	22	73.4
Characteristics (donor)	No. donor	Percentage
Sex	30	100
Male (27–54)	15	50
Female (23–73)	15	50

Fig. 1 H&E staining of squamous carcinoma tissue from cancer patients showing increased infiltration of the immune cells as compared to normal epithelial tissues from human donor. The image is the representative of image of $N=30$ samples analyzed

Fig. 2 Immunofluorescence studies on squamous epithelial carcinoma tissue and normal donor epithelial tissue stained to analyze the expression of CD4, NRP-1, Foxp3, Helios and CD25. The image is one the representative of image of $N=30$ samples analyzed

patients than in healthy donors. Further, Helios expression was correlated with Foxp3 expression, which was elevated in HNC patients. Additionally, NRP-1 expression was also found to be high in cancer patients, coinciding with the expression of both Foxp3 and Helios in HNC patients indicating the infiltration of Tregs population in cancer patients. Expression of *GARP* was found to be increased in HNC patients compared with healthy donors, indicating the significance of tumor-infiltrating Tregs cells, suggesting that Helios and NRP-1 markers may be prognostic markers for Tregs in HNSCCs (Fig. 3a). Treg-mediated modulation of the immune system and interactions with

different immune-activating molecules are yet to be defined. We also analyzed the mRNA expression levels of pro-inflammatory and anti-inflammatory cytokines (IL-2, IL-6, IL-10, TGF- β , TNF- α , IFN- γ) in whole blood samples (Fig. 3b). IL-2 and IL-6 showed increased expression in cancer samples. Meanwhile, TGF- β , TNF- α and IL-10 expression levels were significantly high in the cancer patients compared with control samples. IL-10 did not possess any significant change as it is been implicated in the regulation of inflammation and immunity in the tumor microenvironment. IFN- γ was also highly expressed in HNC patients as compared to control.

Fig. 3 a The relative mRNA expression levels of Fcpx3, Helios, Neuropilin-1 and GARP in whole blood from head and neck cancer patients and healthy donors. $*P < 0.05$ ($N = 21$). **b** The relative mRNA

expression levels of IL-2, IL-6, IL-10, TGF- β , TNF- α and IFN- γ in whole blood from head and neck cancer patients and healthy donors. The results of independent experiments are shown. $*P < 0.05$ ($N = 21$)

Increased expression of Treg markers in human cancer

To specifically elucidate the expression levels of Tregs markers in cancer patients, whole blood samples were analyzed by multicolor flow cytometry. Five markers CD4, CD25, Fcpx3, Helios and NRP-1 were analyzed (Fig. 4a). The CD4 + CD25 + cell population was high in cancer patients. The CD4 + CD25 + Fcpx3 + cell population were

high in HNSCC patient samples when compared to healthy donor samples. Also, Helios + population was high in cancer patient samples compared to that of the healthy donor samples. The CD4 + CD25 + Fcpx3 + Helios + cell population was also high in cancer patients compared to that in the healthy donors, suggesting increased population of activated Tregs. Also, when compared, the CD4 + CD25 + Fcpx3 + NRP-1 + cells population in cancer patients were higher than healthy donors (Fig. 4b). The Helios and NRP-1 provide an

excellent marker to help clinicians during immunotherapies for patients with HNSCC.

Discussion

Tregs play an important role in immune suppression; however, their clinical significance in HNSCCs is currently unknown. In this study, we assessed the expression levels of markers of Tregs along with Helios and NRP-1. The CD4⁺ cell population was highly expressed in patients with squamous cell carcinomas compared to normal donors. We also found that well-differentiated carcinomas displayed greater levels of lymphovascular invasion than moderately differentiated cancer tissues. Clinical prognosis of HNSCC patients is correlated with various changes in the peripheral blood. Intratumoral Treg infiltration may be influenced by the type of cancer as well as by immune-associated microenvironmental soluble factors such as TGF- β , IFN- γ and many others. Earlier reports on hepatocellular carcinoma (HCC) demonstrated that HCC patients had high levels of tumor-infiltrating Tregs with high Foxp3⁺ expression, leading to high intratumoral Tregs concentration, which plays a critical role in tumor progression (Tu et al. 2016; Whiteside 2018a). HNC tissues were shown to have high CD25 expression in comparison with donor tissues. Studies on patients with solid tumors have reported increased Treg levels in both the tumor tissue and peripheral blood (TOKUNO et al. 2009). Similarly, within the total lymphocyte population, the percentage of CD4⁺CD25⁺ T cells were higher in lung cancer patients than in control patients (Karagöz et al. 2010). We found that, increased Foxp3 expression was correlated with increased CD4 and CD25 expression in HNC tissues. Likewise, the Helios and NRP-1 marker were highly expressed in HNC tissues than in healthy donor tissues suggesting specific marker to identify the subsets of Tregs. Immunohistochemical studies demonstrated that tumor biopsies had large T cell population and these cells may significantly impact disease prognosis. T cell infiltration should be obtained as preliminary patient data (Mihm and Mulé 2015). Tregs play a role in helping carcinomas evade the immune system. HNSCC patients have been found to have low white blood cells and increased numbers of suppressive Tregs in peripheral circulation. Furthermore, HNSCC TILs consist of more Treg cell than that found in the peripheral bloodstream of HNSCC patients (Moskovitz et al. 2018). Drennan et al. (2013) assessed the frequency and suppressive activity of Tregs in the tumor microenvironment (Drennan et al. 2013). In the present study, the relative mRNA expression levels of Foxp3, Helios, Neuropilin-1, and GARP were found to be elevated in the peripheral whole blood samples from cancer patients than those in the healthy donor samples. Treg-based Foxp3 mutations are associated with IPEX and hence

Foxp3⁺ Treg cells are thus essential for immune suppression (Ramsdell and Ziegler 2014). Helios has also been found to regulate the fitness of Tregs (Sebastian et al. 2016). Helios reportedly controls the suppressive function and differentiation of Tregs. Helios has also been found to upregulate Foxp3 expression and linked to T cell activation and cellular division (Grzanka et al. 2013; Takatori et al. 2015). NRP-1 is another cell surface molecule that is expressed and can be used to identify Tregs (Singh et al. 2015). Nrp-1 is expressed at a later developmental stage in Tregs than Helios and Foxp3 (Singh et al. 2015; Szurek et al. 2015). Glycoprotein A repetitions predominant (GARP), a surface protein, is expressed in activated Tregs. GARP has been shown to promote the secretion of TGF- β (Sun et al. 2016). The expression of GARP, which is specifically increased in tumor-infiltrating Tregs, has been found to correlate with the clinicopathological characteristics of lung cancer patients (Jin et al. 2017). Similarly, NRP-1 is also expressed in a variety of cancers (prostate, melanoma, glioblastoma, neuroblastoma, lung, astrocytoma, pancreatic and colon carcinomas) (Graziani and Lacal 2015; Jubb et al. 2012). These observations suggest that NRP-1 is a potential anti-angiogenic and anti-tumor target. We also found that the mRNA expression levels of pro-inflammatory and anti-inflammatory cytokines, such as IL6, IL2, IL-10, TGF β , IFN- γ , and TNF- α , correlate with Treg infiltration in the tumor microenvironment. This suggests that the soluble factors sculpture the activity of Tregs. The differentiation of Tregs requires TGF- β (Pourgholamnejad et al. 2016). TGF- β induces Foxp3, which is essential for Treg development; however, TGF- β also stimulates many suppressive mechanisms depending upon the environment and the type of immune stimulation (Aggarwal et al. 2017). TGF- β converts naïve T cells into iTregs, plays a role in the inflammatory response, protects and maintains Tregs during the immune response (Tu et al. 2014). The expression of Foxp3 correlates with squamous cell differentiation and also a marker of chronic inflammation (Phé et al. 2015). Foxp3 expression is a main prognostic factor for survival and the expression of Treg-specific Foxp3. A study showed that Foxp3 expression is correlated with IFN- γ expression in ovarian cancer (Wolf et al. 2005). IFN- γ with its anti-tumor immunity also has a contrary dark side of promoting tumor (Mojicet et al. 2017). Although increased Treg invasion appears to be a characteristic feature of most tumors, little is known about the cellular and molecular mechanisms responsible for this invasion. Overproduction of IL-6 in vivo impedes the generation of iTregs from naïve T cells but does not restrict Treg function. Naïve CD4⁺T cells continuously exposed to IL-6 in vivo retain their ability to differentiate into Tregs (Fujimoto et al. 2011). Internal Treg stability and peripheral Treg maintenance are controlled by IL-2, closely tying Treg activity to the effector immune response and allowing Treg cells to respond to inflammation

Fig. 4 **a** CD4+CD25+ cells analyzed for the Foxp3+Helios+ cells, Foxp3+NRP-1+ cells and Foxp3+Helios+ cells in whole blood from head and neck cancer patients and healthy donors ($N=21$). **b** Percentage of Histogram of CD4+CD25+ cells, CD4+CD25+Foxp3+ cells, CD4+CD25+Helios+ cells, CD4+CD25+Foxp3+Helios+ cells, CD4+CD25+NRP-1 cells and CD4+CD25+Foxp3+NRP-1+ cells in whole blood from head and neck cancer patients and healthy donors were analyzed by flow cytometry ($N=21$)

(Moon et al. 2015). IL-2 is required for the maintenance and stabilization of Foxp3 expression (Chinen et al. 2016). TGF- β expression is reduced in cancer patients but found to play dual role. IL-10 has been implicated in the regulation of inflammation and immunity in the tumor microenvironment. Our results illustrate that analysis of these factors may be indicative of HNSCC prognosis and may lead to the development of novel treatment strategies for immunotherapy. Fluorescence staining for Treg markers in the whole blood of cancer patients and healthy donors revealed that Helios, and Nrp-1 were correlated with the increased expression of Foxp3+ Treg markers in cancer patients. Helios+Foxp3+CD25+CD4+ and Nrp-1+Foxp3+CD25+CD4+ Treg cells were significantly increased in whole blood samples from cancer patients. Based on these findings, Helios and NRP-1 markers of Tregs may be useful prognostic markers for determining lymphocytic invasion with potent immunosuppressive features in cancer. Further study is required to determine the roles of cytokine factors in the modulation of Tregs in the tumor microenvironment. Likewise the soluble factors could trigger the tregs based upon the immune response resulting in a systematic response. Overall we tried to elucidate role of Helios and NRP-1 marker of Tregs in Indian HNSCC patients and soluble factors governing it.

Conclusion

Tregs function to promote tumor development by cooperating with various soluble factors to aid in formation of the tumor microenvironment. In this study we try to uncover the clinical significance of Tregs in patients with HNSCC. Previous individual studies on Helios and NRP-1 have reported as potential markers for Tregs and its subsets. We explored its association and co-existence of Helios and NRP-1 relating Foxp3 along with soluble factors. Cognizance of tumor trafficking may enable the development of therapies that inhibit tumor formation. Tregs have emerged as a potential immune modulator of HNSCCs and specific markers are required to distinguish it (Thornton et al. 2018). This is the first preliminary quantitative study conducted among the south Indian population to analyze the markers of Tregs and its soluble factors associated in HNSCC patients.

Acknowledgements We would like to thank DST-SERB (No: YSS/2014/000424) Govt. of INDIA and the first author thanks the Maulana Azad national fellowship scheme (UGC) (F1-17.1/2014-15/MANF-2014-15-MUS-PON-44115).

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

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