The Regulation of FOXP3 Expression and Its Therapeutic Potential in Non-Small Lung Cancer

Muhan Jiang *

Department Biosciences, Biomedical sciences, University College London, United Kingdom

* Corresponding Author Email: zcbtiaa@ucl.ac.uk

Abstract. Non-small cell cancer (NSCLC) is today one of the most threatening malignant tumors, and it is still rarely cured by current standard therapies with a high mortality rate. The transcription factor Forkhead box p3 (FOXP3) is a potential molecular target for NSCLC since it has been identified with an oncogenic effect in the tumor microenvironment (TME). It is well known that FOXP3 expression could characterize the regulatory T cells (Treg), which plays a critical role in regulating anti-tumor immune responses in TME. Through analyzing the basic mechanisms of FOXP3 and Treg in distinct tumor tissues, they have been found with contradictory expression and the frequency in different TME. In NSCLC, the suppressive function of Treg to immune systems and frequency of FOXP3 is accumulated to promote the development of tumors. Most recently, accumulating evidence reveals the therapeutic potential of targeting FOXP3 and the related signaling pathways in the treatment of NSCLC patients. Although unsolved side effects and limitations that still must be considered when targeting FOXP3 and FOXP3-related proteins, there is no doubt that they are potential therapeutic applications for NSCLC treatment. The main objective of this review is to mention the structure, function and recent progress in treatment targeting FOXP3 and FOXP3-related signaling pathways, especially to therapies with immune checkpoint inhibitors.

Keywords: NSCLC, Lung cancer, FOXP3, T regulatory cells, checkpoint inhibition.

1. Introduction

Nowadays, Lung cancer has been the dominant cause of death according to cancer in both sexes (30% in men, 26% in female) worldwide [1]. There are clinically two categories of Lung cancer: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Besides, NSCLC act as the major cause of lung malignant tumors for about 85%. NSCLC is generally classified into three types: squamous carcinoma, large cell carcinoma, and adenocarcinoma [1]. For patients with the primary tumor, lung carcinoma act as the dominant cause of death for cancer proliferation, linked with a low overall survival rate of 15% by current therapeutic treatments [2]. Although a wide range of proteins in immune systems has been investigated in cancer pathogenesis, lung cancer is still rarely cured with the nearly unexplored role of Forkhead box P3 (FOXP3) transcription factor in tumor microenvironment (TME). Accumulating evidence shows the importance of investigating the FOXP3 expression in malignant lung tumors as it is linked with T-cell lineage [1,2]. Notably, FOXP3 has been recognized as the critical regulator of the regulatory T cells, which could cause abnormal immunosuppression in TME. Thus, the significance of FOXP3 in cancer treatment has been revealed by its oncogenic effect, which alters both the progression and metastasis stage of lung cancer apparently. In addition, understanding the basic mechanism of FOXP3 expression in carcinogenesis is of great significance in shaping clinical therapy towards NSCLC.

2. Structure and function of FOXP3 gene

FOXP3 belongs to the family of forkhead box (FOX) transcription factors identified by a winged helix domain. Initially, the FOXP3 was identified in the study of scurfy mice, which is suffering from a lethal X-linked recessive immune dysregulation, highlighting that FOXP3 gene is involved in the p arm of the X chromosome. Twelve exons existed in the transcript of FOXP3, and eleven of them code a protein of 413 amino acids, has been proved with oncogenic effects in lung cancer [2]. Furthermore, FOXP3 is classified as a part of the B600 kDa-large molecule complex, which includes other enzymes,

such as histone acetyltransferases and some transcription factors as well [2]. Acting as one transcription factor, FOXP3 is composed of four center-located domains: the repressor (N-terminal) domain, the zinc finger and leucine zipper (ZL) domain, and the forkhead domain (FKH), which enhances the process of forming a unique head-to-head dimer (Fig. 1) [3]. Besides, the FKH domain also contains two sites to target FOXP3 location to the nucleus of its both C and N terminus.

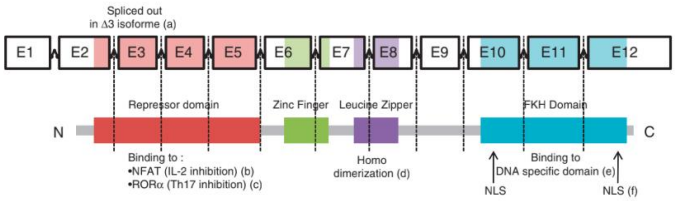


Fig. 1 Structure of FOXP3 gene [3]

3. Immunological regulation of FOXP3

As mentioned above, combining these transcription factors with FOXP3, involving the nuclear factor of activated T cells could consequently result in the inhibition of IL-2 expression, which damages effector capabilities of T-helper cells.[3] However, the expression of FOXP3 is distinct in human regulatory T cells (Treg) when compared with the mouse Treg in the immune system. The mouse Treg typically expresses as one full-length protein, highlighting the importance of identifying the differences between human Treg and mouse Treg in a therapeutic approach [2,3]. In contrast, two isoforms existed for the human Treg with almost equal amounts: one alternative splicing FOXP3, which lacks exon 3 (FOXPD3, molecular weight: 54kDa) and another one acting as the ortholog mouse Foxp3, with the full-length scale (FOXP3Fl, molecular weight:58kDa) [4]. From recent studies, FOXP exon 3 (aa72-106) has been discovered with the encoding function of a domain which could inhibit the retinoic acid receptor-related orphan receptors (ROR) and result in the formation of Th17 cells. Therefore, the full-length FOXP3FL regulates the differentiation of Th17 negatively and conversely when compared with the function of FOXP3D3, which indicates the importance of distinguishing the distinct situation of FOXP3 in Mouse models and Human trials. [2,3].

Recent studies indicate that Tregs expressing FOXP3 act as the dominant mediators in maintaining self-tolerance and preventing excessive immune responses in the immune system. For the normal FOXP3+ Tregs, most of them are derived from the differentiation of CD4+ and CD8- thymocytes, which highly express CD25 [4]. The CD25 expression from the MHC-self-reactive peptide complex could make responses from signaling of STAT 5 or IL2, which further stimulates the Tregs and enhance FOXP3 expression (Fig. 2) [4]. The function of the FOXP3 gene can be further included as the key to control cytokine production and proliferation, such as natural killer cells and antigen-presenting cells (APCs), which is recognized as the master control gene.

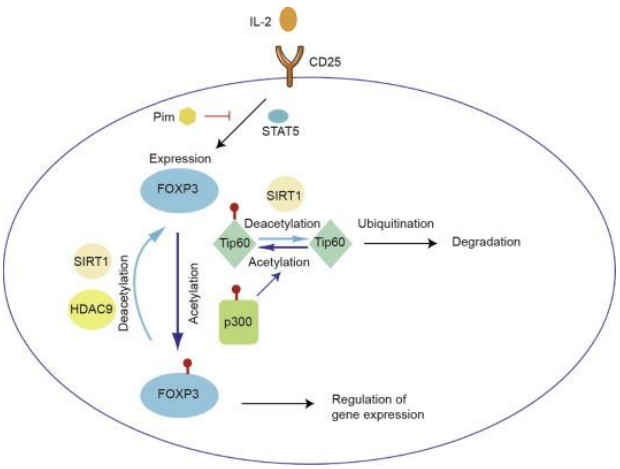


Fig. 2 The regulation of FOXP3 expression and Tregs [4].

4. Mechanisms of FOXP3 regulation in lung cancer

FOXP3 is both expressed abnormally in lung carcinoma tissues and Lung adenocarcinoma cell line A549, representing the LUAD and LSCC, respectively [2]. To shed light on the mechanisms of FOXP3 leading Tregs in the cancer microenvironment, they vary according to several factors. For example, the lung cancer cells, and tumor-filtrating macrophages generate overexpressed CC-chemokine ligand 22 (CCL22, a CCR4 ligand) in the lymph nodes or bone marrow, which further attract the Tregs with the expression of CC-chemokine receptor 4 (CCR4) [2].

In contrast to the normal immune system where Tregs do not readily proliferate upon activation by antibodies to CD3 and CD28, Tregs are demonstrated in related experiments to apparently proliferate very actively in a tumor environment where TLR4 signaling is activated significantly [5]. Meanwhile, Tregs are also activated when the dendritic cells in the tumor environment recognized tumor specific antigens, thus providing further evidence of a FOXP3 upregulation response activated by Tregs. Furthermore, the researchers demonstrated an increase in TGF-β expression that simultaneously led to Tregs activation by studying immature myeloid dendritic cells in the tumor [5].

To validate the ectopic expression of FOXP3 and the related effects of tumor growth in NSCLC, the FOXP3 lentivirus and colony assay were applied in both NSCLC cell lines (A549 and H460) in vitro [5]. The experiment result of the increased colony formation in A459-FOXP3 and H460-FOXP3 could be clearly identified through the comparation with the control group (Fig. 3) [5].

Moreover, the mediation of conversion of naïve T cells to FOXP3+Tregs through TGF-β expression was proved in an experimental model, which indicated the positive relationship between TGF-β and FOXP3 expression. In this experiment, the inclusion of 1 ng/ml TGF-β could lead to the stimulated expression of FOXP3 by 50% of T cells (Fig. 4) [6].

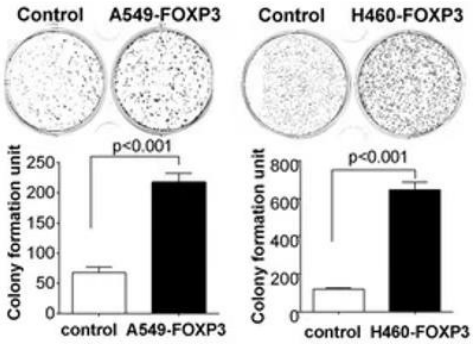


Fig. 3 The stimulation of tumor growth by FOXP3 expression in NSCLC [5].

By comparison of A549-FOXP3 and H460-FOXP3 with the control group, respectively, both reveal colony formation significantly in this colony formation assay.

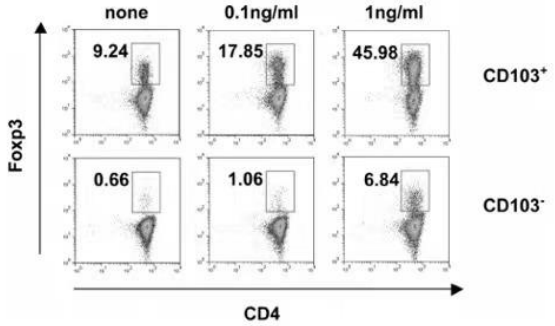


Fig. 4 The expression of FOXP3 is induced by TGF-β [6].

CD4+ T cells from DO11.10 SCID mice were added with increasing concentration of TGF-β at day6. The stained T cells for CD4 and FOXP3 are analyzed by FACS, and the numbers indicate the percentage of FOXP3+T cells in CD4+T cells.

4.1. Alternative splicing regulation

A recent study has indicated that alternative splicing is the primary cause of complex FOXP3 isoforms, as well as one of the major abnormal regulations occurred in NSCLC. As mentioned above, exon 8 is present in the repressor domain of FOXP3, where alternative splicing is most likely to occur. By analyzing RNA-seq data from the TCGA database containing 32 cancer types, the results pointed out that FOXP3D3 is the most predominantly expressed isoform in almost all malignancies, with the exception of acute myeloid leukaemia [2]. This phenomenon can prove that the expression of the FOXP3 isoform is closely linked with the overall poor overall survival rate and higher advanced stages of lung cancer. In this field, the importance of investigating the influence of FOXP3D3 in tumor malignancy for NSCLC has been revealed as it is linked with the advanced T stage.

In addition, the isoform of FOXP3 is also a major cause of chemoresistance in bladder cancer, and the association between lung cancer and bladder cancer has been demonstrated by the involvement of the same environmental hazards [3]. In this condition, the influence of this isoform of FOXP3 can be speculated to have similar effects on lung cancer. Through the experiments applied in animal models about the expression level of the two FOXP3 isoforms, the FOXP3D3 has been proven to be nine times higher than FOXP3FL in tumor cells. Besides, FOXP3D3 is the dominant isoform in NSCLC patient samples, which has a significant oncogenic effect on cancer metastasis and proliferation by modulating the hedgehog pathway.

4.2. FOXP3 promote metastasis of lung tumors by GINS1.

GINS complex subunit 1 (GINS1) is one of the GINS complexes, which plays a critical role in initiating DNA replication and maintaining of elongation [2]. In various types of tumors, including NSCLC, GINS1 is overexpressed and results in tumorigenesis in NSCLC with the co-stimulation of Metastasis-associated lung adenocarcinoma transcript 1 (MALAT1). MALAT1 belongs to the member of Long non-coding RNAs (lncRNA), and is related to lung cancer, which directly contributes to the cancer progression.

In recent studies, the overexpression of the GINS complex has been detected in several malignant tumors, which include NSCLC [3]. Considering the fact that apoptosis reaction is inhibited by GINS1 to promote metastasis of cancer cells, GINS1 could be recognized as one of the biomarkers for tumorigenesis. In NSCLC, FOXP3 plays a critical role by acting as the transcription factor of GINS1, which undergoing a positive relationship with tumor progression. Notably, the expression of FOPX3 affects the proliferation of lung adenocarcinoma and lung squamous cell carcinoma differently. The FOXP3 could enhance the level of Cyclin D1, a checkpoint gene for the cell cycle G1phase and S phase, which stimulates tumor proliferation and poor prognosis in LUAD. In contrast, the apoptosis of proliferated tumor is exhibited by the upregulation of FOXP3 in LSCC [2].

The FOXP3 was proved to be indispensable for the MALAT1-mediated GINS1 transcription in the experiment of Luciferase analysis. The overexpressed FOXP3 could reverse the GINS promoter activity downregulated by MALAT1 knockdown in NSCLC cells.

4.3. FOXP3-induced renewal of cancer stem cells

Moreover, the cancer stem cells (CSCs) in NSCLC grow significantly due to enhanced FOXP3 expression, which further drives uncontrolled tumor initiation and progression [2,3]. In multiplex cancer, the Glioma-associated oncogene homolog (GIL1), an effector of the Hedgehog signaling pathway, can be used as a biomarker showing the effect of CSCs on cancer proliferation. Meanwhile, the FOXP3 expression level was reported to cause the up regulation of GIL1, proving that FOXP3 could affect the increasing number of CSCs. The binding of GLI1 to FOXP3 promoter increases FOXP3 transcription as well as the expression of Notch1 mechanically, thus consequently stimulating the CSCs renewal [2]. Besides, the NSCLC cell stemness could also be enhanced by the binding between FOXP3 and the promoter of one long non-coding RNAs 1232 (lINC01232), which further controls the transforming growth factor receptor 1 (TGFBR1). After modulating the activity of

TGFBR1, the TGF-β signaling pathway is further activated, positively upregulating stemness and related to the progression of malignant lung tumors [6].

5. Therapeutical strategies for NSCLCS

5.1. Chemotherapy

In several earlier reports, the investigation about the administration of cyclophosphamide (CP) therapy significantly revealed that this chemotherapy could impair the primary and advanced metastasis tumor formation of Lung adenocarcinoma [7]. After the selective injection of a low dose of CP, the decreased amount of circulating Tregs in animal models could enhance the antitumor responses to the immunotherapy as well. Besides, the expression of FOXP3 is also inhibited in tumors by a low dosage of CP therapy, consequently with the suppression of the plasticity of Tregs. The overall response rate has reached 76.5% for SCLC patients within the early stage [7]. Moreover, the treatment of repetitive and low doses of administered CP for one month to the patients with the late stage of cancer shows that, a selectively decreased amount of Treg cells could also be observed in advanced metastasis tumors. In this case, the proliferation of peripheral T cells and the activities to damage innate tumor cells has been restored in treated patients, with a stabilization of the lung cancer.

5.2. Immunotherapy

Since Tregs have a pathological role in damaging desirable anti-tumor activity by dysregulating the amount, immunotherapy to deplete the intra-tumoral Tregs has become the most promising strategy for anti-tumor treatment. So far, anti-immune checkpoint therapy, such as anti-PD1 and anti-CTLA-4 treatment, has been proposed with apparent therapeutic effects in treating several types of cancer [8,9].

Since the number of checkpoints in immune system is positively correlated with the abundant amount of Tregs, blocking these checkpoints could therefore downregulate and suppress the Tregs function and related synergistic effects to FOXP3. In this approach, the conversion process of TBET+Th1 cells into FOXP3+ positive Tregs could be inhibited by the anti-PD-1 antibodies in vivo, which consequently maintain the anti-tumor immunity [8]. For example, pembrolizumab and atezolizumab are Food and Drug Administration (FDA) approved anti-PD1-drugs for treating metastasis NSCLC patients with stimulated PD-L1 expression, acting as the first line therapy. In cases of PD-L1+NSCLC patients, the dosage of 10mg/kg pembrolizumab results in an overall response rate (ORR) of 27% and a median overall survival rate (OS) of 12.7 months, with a long-term safety profile [9]. Furthermore, the anti-PD-L1/TGF-β inhibitor bifunctional fusion peptide, M7824, is under clinical investigation nowadays to damage the function of TGF-β and PD-L1 simultaneously for NSCLC patients [8].

In contrast, the phase II clinical trial (NCT02607631) of patients in advanced stage of NSCLC treating anti-PD-1 immunotherapy with either pembrolizumab (200mg per 3 weeks) or nivolumab (2mg/kg per 2 weeks) revealed favorable responses by the occurrence of a high TGF-β and FOXP3 level [8]. The high level of TGF-β and FOXP3 could result in a higher overall survival rate, better prognosis, and responses for anti-PD1 therapy. This treatment effect conflicts with the previous finding that high FOXP3 expression is detrimental for NSCLC patients. The possible explanations from several aspects are the different genotypes of SD NSCLC patients and the varied density of FOXP3 density [9]. Besides, this experiment is a retrospective one, which means the level of FOXP3 and TGF-β were measured only after the experiment.

5.3. Combination therapy

Since 80% of the patients are resistant to immune checkpoint therapy alone in treating advanced-stage NSCLC, the combination therapy of FOXP3 antisense inhibitor and immune checkpoint therapy has shown effective synergistic effects in mutant tumors by relieving tumor immunosuppressive

microenvironment and drug resistance. For example, the KEYNOTE-407 experiment indicated that the combination of pembrolizumab and another chemotherapy reveal higher efficacy with better clinical responses [9]. The improvement in responses and prognosis from NSCLC patients after treatment of the combination therapy of pembrolizumab (10mg/kg) plus carboplatin pemetrexed was sharply viewed due to the raised ORR of 57% and twofold increase in 2-year OS after injection.

To shed light on the combination treatment of immune checkpoint blockade and the method targeting FOXP3 directly, the antisense oligonucleotide AZD8701 could selectively impair the immunosuppression function of Tregs by reducing the abundant FOXP3 expression [10]. After the injection of continuous doses of ASO in vitro and vivo, the experiment result indicated a 70% reduction in FOXP3 levels in tumor models. Consistent with this situation, the application of the combination therapy of AZD8701 and immune checkpoint blockades shows advantageous effects, as no symptoms of overt autoimmunity after long-term dosing existed during the trial [10].

However, several developmental challenges are still unsolved regarding this Treg-targeted therapy. Compared with the experiments in the mouse model, the toxicities generated by immune-based NSCLC therapeutics in the clinical trials should also be evaluated. Besides, immune-related adverse events (irAEs) are still unsolved problems for immune checkpoint therapies, with fatigue being the most common one for Pembrolizumab at phase 2/3 of the therapy [8].

In aggregate, there are still many limitations in directly targeting FOXP3 due to the complicated and unclear mechanisms behind it. Regard with the therapeutic therapy to NSCLC treatment, researchers could consider targeting other signaling pathways that affect FOXP3 expression or even targeting T helper1 (Th1) cells related enzymes that act in opposition to T regulatory cells. It should be pointed that the combination treatment targeting these impact pathways and the immune checkpoint blockade is indeed a future approach to mitigating FOXP3 expression in the treatment of NSCLC.

5.4. Targetability of FOXP3 linked SHP2 signaling

Furthermore, SHP2 signaling is linked with the plasticity of Th1 cells by inactivating the STAT molecules [11]. This assumption has been verified by the experiment in a conditional knockout model, indicating that STAT3 phosphorylation has been increased due to the lack of SHP2. Similarly, STAT1 phosphorylation also exhibited a downregulation by the presence of SHP2, which further propagate the expression of FOXP3 conversely and activate Th1 cell differentiation constitutively. Besides, the downregulated STAT1 phosphorylation has been proved in animal models that the transmitted instructions from PD-L1 signaling indeed act as the dominant cause for it. Therefore, the drug aimed at inhibiting SHP2 might dampen the situation of reduced STAT1 phosphorylation, which consequently inhibits the Th 1cell plasticity and FOXP3 expression [11].

For example, the combination of FDA-approved anticancer drug JAB-3068 and PD-1 antibody spartalizumab can be used in the treatment of NSCLC, which reveals the effect of limiting the activity of SHP2 for the elevation of STAT1 phosphorylation. Consequently, FOXP3 expression is depressed in TME [11]. Regard with the combination of SHP2 inhibitors and immune checkpoint inhibitors, it is currently in the phase II clinical research stage with a requirement of more in-depth study in the future.

5.5. Targetability of FOXP3 linked CCL5-CCR5 signaling

The expression of FOXP3 in tumor malignancy has a positive link with one member of the chemokine family, CCL5, which is flexible in regulating the tumor progression and antitumor responses [12]. In this context, the direct transactivation of CCL5 caused by FOXP3 in cancer cells could promote the crosslinking of CCL5-CCR5 and further accumulate the Tregs progression in tumor tissues from peripheral blood both in vitro and in vivo. Since FOXP3 has been recognized as the biomarker for NSCLC prognosis, it is possible to suggest that the interaction between several CCL5-CCR5 also induces the Tregs migration in NSCLC. Therefore, the movement of Tregs to tumor areas is limit if the initiation of CCL5 transcription and the CCL5-CCR5 signaling are disrupted.

There are several mechanisms to inhibit the signaling of CCL5-CCR5, such as the application of CCR5 inhibitor TAK-99, suggesting that intervention to CCL5 and CCR5 is one of the possible methods for NSCLC treatment according to the function of FOXP3 [12].

6. Conclusion

In this study, the association of FOXP3 and Treg with immunosuppressive activity has been supported by many lines of evidence, which further resulted in advanced tumorigenesis in the TME. Besides, it should be emphasized that FOXP3 promote tumorigenesis by stimulating tumor progression, metastasis, and renewal of stem cells in NSCLC. Several drugs possessing the mechanism that target FOXP3 and related signaling pathways have been proved by FDA for treatment of advanced solid tumors with good efficacy, such as the chemotherapies of AZD8701 and CP administration. Clearly, the combination therapy of chemotherapy targeting FOXP3 and immune checkpoint block, especially anti-PD-1 therapy, offers a significant advantage in therapeutic application of treating NSCLC by enhanced anti-tumor responses. Besides, the combination therapy also opens an opportunity to target the FOXP3-related enzymes and signaling pathways that are controlled by Th cells. There are still certain challenges remained around FOXP3 from several aspects, such as the high variability of NSCLC patients, low efficacy, inevitable toxicity, and drug-induced resistance. Furthermore, the unsured influence of FOXP3 level on anti-PD1 therapy indicates that this field still requires more innovation and deeper understanding. In conclusion, the findings around the association of FOXP3 with the variation of TME highlight the significant future potential to target FOXP3 and the related signaling pathways for NSCLC treatment.

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