IFNα2b augments immune responses of cisplatin+5-fluorouracil treated tongue squamous cell carcinoma patients - a preliminary study

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Background & objectives: Interferon alpha 2b (IFNα2b) has been reported to regulate several immune functions efficiently to enhance the cytotoxic activity of NK and T cells towards various forms of tumours. The objective of the present study was to evaluate the efficacy of IFNα2b in overcoming disease induced and/or treatment associated immunosuppression of tongue squamous cell carcinoma (TSCC) patients undergoing chemotherapy for better clinical outcome.

Methods: Seven TSCC patients under cisplatin + 5-fluorouracil chemotherapy in combination with IFNα2b were assessed for various immunohaematological parameters before treatment, after chemotherapy and after IFNα2b therapy.

Results: Deterioration of the haematological and immune responses was detected in immunosuppressed TSCC patients after chemotherapy. IFNα2b treatment led to a recovery in these parameters in most of the patients. Greater number of T/NK cells and enhanced secretion of type 1 cytokines were also noted. Haematological complications were reduced after completion of the therapy. Immune- and haematostimulation were also observed in patients with partial response. No positive clinical response was detected in one patient.

Interpretation & conclusions: IFNα2b appears to be an effective immunostimulator having clinical impact to combat the immunosuppression in TSCC patients. Successful immunostimulation by IFNα2b may help TSCC patients in clinical improvement. The findings of this preliminary study need to be confirmed on a large number of patients with TSCC.

Key words Cytokine - IFNα2b - NK cells - T cells - tongue cancer

Tongue is a common site affected during the development of carcinoma within the head and neck region1,2. Surgery and radiotherapy alone or in combination is the standard approach for the treatment of early tongue squamous cell carcinoma (TSCC) and chemotherapy is considered today as neoadjuvant or concomitant with radiotherapy in advanced inoperable TSCC patients. Irrespective of the mode of treatment, successful treatment in TSCC is occasionally hindered by severe immunosuppression3. We have also reported significant immunosuppression in head and neck squamous cell carcinoma (HNSCC) patients, as
reflected in impaired cellular and secretory functions. Interferon is widely used as the most effective agent in patients with various forms of cancer, including renal cell carcinoma and melanoma. The reason of this selection probably is immunogenecity and least immunosuppression in these two forms of cancer and variable extent of success is reported. In addition, bladder cancer, hepatocellular carcinoma, leukemia, etc. are also treated with interferon alpha 2b (IFNα2b). On the other hand, little effort has been made to evaluate the prognostic response of IFNα2b treatment with or without chemotherapy in HNSCC/TSCC patients. An initial trial evaluated the result of addition of IFNα2b to chemotherapy in head and neck cancer, and suggested improved progression free and overall survival, but not examined the immune response. In another study, Vlock et al. treated 14 patients of recurrent HNSCC, and one patient showed complete response.

We have earlier reported that IFNα2b regulates various immune functions efficiently to enhance the cytotoxic activity of NK and T cells towards various forms of tumours. Here, we attempted to utilize this immunostimulatory property of IFNα2b along with cisplatin+5-fluorouracil (5-FU) treatment for inoperable stage IV TSCC (anterior two third of the tongue) patients to enhance the therapeutic efficacy of chemotherapy in connection with immune cell mediated cancer cell killing.

### Material & Methods

#### Clinical study

**TSCC patients:** Patients (n=7) with histopathologically confirmed inoperable tongue (primary site anterior 2/3 of tongue) squamous cell carcinoma (TNM stage IV) were included in this preliminary study. All these patients attended the out patient department of Chittaranjan National Cancer Institute (CNCI), Kolkata, during 2006-2008. These patients were treated with cisplatin (70 mg/m² i.v. on day 1) + 5-FU (500 mg/m² i.v. on day 1-3) in two cycles in 21 days interval and IFNα2b (3 mIU twice wkly for 3 wk) was administered in between two cycles of chemotherapy. Treatment protocol was modified in some cases according to the demand of clinical situation. For example, IFNα2b cycle was divided into two due to leukopenia/neutropenia. Haematological and immune parameters were checked before initiation of the treatment, after first cycle of chemotherapy and after IFNα2b therapy. Six of these patients had tobacco habits either in one form or more. Blood specimens were collected after obtaining written informed consent from each patient. Study protocol was approved by Institutional Review Board (IRB) of CNCI, Kolkata.

**Response assessment and follow up:** Clinical features of patients were assessed regularly by monitoring tumour size, ankyloglossia, node involvement and general health. The objective responses, evaluation of time to progression, duration of response, time to treatment failure and overall survival on this treatment were recorded. Computer tomography (CT) scan was done on the face and neck region.

**Leucocyte, platelet and haemoglobin status:** Blood (6 ml) was collected from TSCC patients and used for white blood cell (WBC), platelet count and determination of haemoglobin percentage. These parameters were assessed by Hematological Autoanalyzer, Sysmax 21, Japan. WBC differential count was performed by microscopic examination on Leishman’s stained blood smear on slides.

#### Study

**Reagents and antibodies:** Recombinant human IFNα2b was gifted by Shanferon, Santha-Biotech; India. CD4, CD8, CD56 monoclonal antibodies, OptEIA™ IFNα, interleukin (IL)-12, IL-10, IL-4 estimation kits and TMB substrate solution were procured from BD-Pharmingen (San Diego, CA, USA).

**Tumour cell lines:** HEp-2 (epidermoid carcinoma of larynx) cells were maintained in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, NY, USA), supplemented with 10 per cent (v/v) heat inactivated foetal bovine serum (FBS), 2mM L-glutamine and gentamycin (0.052 mg/ml) at 37°C without the supply

<table>
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of 5 per cent CO₂. The NK sensitive K562 (erythro-leukemic) cell line was maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Life Technologies, NY, USA), supplemented with FBS, penicillin (50 units/ml), streptomycin (50 µg/ml) and gentamycin (0.052 mg/ml) at 37°C in a humidified atmosphere with 5 per cent CO₂.

**PBMC culture:** Peripheral blood mononuclear cells (PBMC) from TSCC patients were isolated from heparinized venous blood by density gradient centrifugation over ficoll hypaque. Isolated PBMC were cultured in RPMI 1640, supplemented with 10 per cent FBS, penicillin (50 units/ml), streptomycin (50 µg/ml) and gentamycin (0.052 mg/ml) at 37°C in a humidified atmosphere with 5 per cent CO₂. Cells and supernatants (stored at -80°C) were used in different assays mentioned below.

**Extracellular secretion of cytokines:** Type 1 (IFNγ and IL-12) and type 2 (IL-4 and IL-10) cytokines were measured in PBMC culture supernatant by ELISA using commercially available kits (BD Pharmingen, San Diego, USA). In brief, 96 well microtitre plates were coated with capture antibody (anti-IFNγ/anti-IL-12/anti-IL-4/anti-IL-10), incubated overnight at 4°C and blocked with 5 per cent BSA for 1 h. After washing, 100 µl of cell free supernatant was added into each well to incubate for 2 h. Bound cytokines were detected by using biotinylated mouse anti-human IFNγ/IL-12/IL-4/IL-10 and avidin-horse radish peroxidase subsequently. Colour was developed with TMB substrate solution. Reaction was stopped with 2N H₂SO₄ and absorbance was measured at 450 nm using microplate reader (Tecan Spectra, Grodig, Austria).

**Flow cytometric analysis of immune cellular markers:** Blood (100 µl) was labelled with 20 µl of different anti-human fluorescence labelled antibodies (CD4-FITC, CD8-PE and CD56-PE) for 30 min as per manufacturer’s recommendation (BD Pharmingen, San Diego, USA). After labelling, RBC was lysed by FACS lysis solution (BD Pharmingen, USA), washed, fixed in 1 per cent paraformaldehyde in PBS and cytometry was performed by using Cell Quest software on a FACScan flow cytometer (Becton Dickinson, Mountainview, USA). Suitable negative isotype controls were used to rule out the background fluorescence. The data were generated by cytofluorometric analyses of 10,000 events. Percentage of each positive population was determined using quadrant statistics.

**Cytotoxicity assay in vitro:** Cytotoxicity of PBMC, after removal of adherent fraction by plastic adherence technique, against different cancer cells, was tested by lactate dehydrogenase (LDH) release assay using commercially available cytotoxicity detection kit (Roche Diagnostics, Mannheim, Germany). HEp-2 and K562 cells (1X10⁴ of each) were plated overnight as target. PBMC (1X10⁶) were added as effector in three effector: target (E:T) ratios (10:1, 50:1, 100:1) in each well and co-cultured for 4 h. Cell free supernatant was used to measure the level of released LDH. Data obtained from E:T, 10:1 are presented. Cytotoxicity was calculated by following formula:

\[
\% \text{ cytotoxicity} = \left( \frac{(\text{lysis from effector} - \text{target mixture} - \text{lysis from effector only} - \text{spontaneous lysis})}{(\text{maximum lysis} - \text{spontaneous lysis})} \right) \times 100
\]

**Results**

**Clinical data- Response and toxicity**

Patients included in this study presented with ankyloglossia and N1/N2 regional nodes. Among seven patients, two (Nos. 3 & 6) responded completely [complete response (CR)-no growth in tongue on palpation, no ankyloglossia, no node palpable, no growth seen in CT scan of face and neck], four patients (Nos. 1, 2, 5 & 7) responded partially [partial response (PR)-downgrading of ankyloglossia grade III to grade I, <50% response to tumour growth]. No response (NR) was found in patient no. 4. No such changes were noticed in the tongue of patient without response (Table I). Patients with complete and partial responses have shown significant changes in overall clinical conditions. These patients were followed for 6 to 12 months with median follow up of 9 months.

Total leucocyte count was decreased after cisplatin + 5-FU therapy in all seven patients studied (Table II). Higher grade of leukepenia was controlled by GCSF. Chemotherapy induced leukepenia was recovered in six patients after completion of IFNα2b treatment. The extent of recovery was much higher in two patients with complete response. In case of platelet count chemotherapy induced decrease was observed in four patients among five patients examined for platelet. IFNα2b treatment helped to recover this count in three cases within four. In patient 3, where complete response was detected, significant recovery of platelet count from 72, 000 to 1, 80, 000 was noticed. However, reflection of chemo-immunotherapy was not found on.
haemoglobin percentage. Mucositis was not detected in any of seven patients.

**Experimental data**

Cisplatin + 5-FU therapy along with IFNα2b treatment increased the number of CD8\(^+\) T cells in five patients among seven patients studied. Two patients have not shown much change before and after treatment. In case of CD4\(^+\) T cells, increase in the number was observed in single case who responded completely to chemo-immunotherapy. No change was reflected in CD4:CD8 ratio, however, this ratio was slightly decreased after chemotherapy and it was increased after IFN\(^{a2b}\) treatment. In addition to T cells, another important cytotoxic cells, CD56\(^+\) NK cells, also demonstrated similar profile of changes as observed in case of CD8\(^+\) T cells. Number of CD56\(^+\) cells was increased in five of the seven patients. Among patients with higher expression of CD8 and CD56 markers, complete response was noticed in patient nos. 3 and 6, with longest survival.

Cytotoxic ability of PBMC obtained from IFNα2b exposed TSCC patients against tumour cells was studied (Table II). Larynx cancer HEp-2 cells were used to get reflection of T cell cytotoxicity and NK sensitive erythroleukemic cells K562 were used to assess NK cell cytotoxicity. Four among six patients studied have demonstrated increase in the cytotoxicity towards HEp-2 cells, in comparison to their pretreatment values. It was noticed that per cent of cytotoxicity reduced after Cis+5-FU treatment was recovered in all patients after IFNα2b treatment. The patient (No. 3) with complete response has showed increase in HEp-2 cell cytotoxicity in maximum extent (post-chemotherapy, 7.2% to post-IFNα2b, 21.3%). Cytotoxicity of NK sensitive K562 cells demonstrated similar pattern, as observed in case of HEp-2 cells.

Type 1 cytokine status of TSCC patients, undergoing chemo-immunotherapy was studied by monitoring IFN\(_{\gamma}\) and IL-12, secreted from PBMC at different points of the treatment. Cisplatin+5-FU therapy resulted in no change in IFN\(_{\gamma}\) level. Treatment with IFNα2b increased IFN\(_{\gamma}\) level in six among seven patients studied. The patient no. 3 with complete response maintained high secretary level of IFN\(_{\gamma}\) till day 20 after radiotherapy. In case of IL-12, chemo-immunotherapy increased the IL-12 level in five among seven patients studied. This increment was not detected in patient no. 4, showing no response.

Type 2 cytokine status of TSCC patients was studied by monitoring IL-10 and IL-4, secreted from PBMC at different points of the treatment (Table II). IFNα2b therapy reduced secretary IL-10 level in four of seven patients; including those two patients with complete response. Other three patients showed no change in comparison to their pre-treatment values. In case of IL-4 downregulation was noticed in most of the patients. IL-4 level was unchanged in patient no.4 with no response.

**Discussion**

We have reported earlier that PBMC isolated from immunosuppressed HNSCC patients appear immunoefficient after *in vitro* stimulation of these
cells with IFNα2b. Mechanism to overcome the immunosuppression was also elucidated. In this preliminary study, the detailed haematological and immune functions of patients with TSCC were assessed and all patients exhibited poor immune functions in terms of the poor cytotoxic function of PBMC, less number of cytotoxic T and NK cells and type1/type2 cytokine imbalance. These immunocompromised patients appeared more immunologically unstable, when immune parameters were examined after cisplatin+5-FU therapy. Parameters at this point were immunocompromised with leukepenia and low platelet count. In spite of the efficacy of cisplatin+5-FU treatment in lowering tumour burden of HNSCC patients, it is often associated with immune paralysis. Following cisplatin+5-FU treatment, IFNα2b therapy was initiated and patients were examined clinically and immunologically upon completion of the treatment. The study revealed a prominent recovery in the haematological and immunological functions.

Based on clinical examination, these seven TSCC patients were categorized into three groups. Two patients responded completely and their survival was recorded 28 months after initiation of the treatment. Robust immunostimulation by IFNα2b was demonstrated in these two patients, particularly in patient no. 3. This particular patient reported in clinic as tumor free and clinically fit 50 months after treatment initiation. Four patients with partial clinical response also demonstrated immune response following IFNα2b therapy and no difference was noted between patients with CR and PR. Improved prognosis and prolonged survival of head and neck cancer patients using different chemotherapeutic regimens, along with IFNα2b was reported from an initial multicentre open trial; however, no effort was done to know the immune status of these patients. Volck et al. treated 14 patients of recurrent HNSCC and reported clinical benefit, including complete response in one. They checked the NK cell activity of these patients and observed a superior correlation of NK cell function with clinical outcome. We also found enhancement of the NK cell activity and NK derived cytokine milieu after IFNα2b therapy. Frequency of T cells and their cytotoxic ability was also increased after IFNα2b treatment. It was noted that proportion of CD8+ cells decreased after chemotherapy, but was increased after completion of chemo-immunotherapy. In most of the cases these values exceeds the CD8 values observed in pre-treatment conditions. Increase in CD4/CD8 ratio following IFNα2b treatment indicated favourable prognosis of these patients. Shah et al. reported that in cancer cervix patients, survival rate was significantly higher in patients with a high CD4/CD8 ratio as compared to patients who had a low CD4/CD8 ratio. Unitt et al. observed that a high CD4/CD8 ratio was associated with a reduced risk of tumour recurrence after liver transplantation in hepatocellular carcinoma. IFNα2b activated T cells can kill cancer cells, possibly by the induction of cytotoxic T lymphocyte (CTL) response and antibody dependent cellular cytotoxicity (ADCC). IFNα2b mediated upregulation of the perforin, granzymeB synthesis and expression was associated with either antibody or CTL mediated tumour cell killing. Patients with CR were able to maintain the immune system in activated state, till the completion of radiotherapy, 20 days after the completion of the IFNα2b therapy. Such durable immune activation was not demonstrated in patients with PR or NR.

Neoadjuvant chemotherapy with platinum and taxens or concomitant chemo-radiotherapy in advanced inoperable TSCC patients is a usual clinical practice. Results obtained from this preliminary study with cisplatin+5-FU followed by IFNα2b suggest that inclusion of IFNα2b in the therapeutic protocol enhances the immune response of immunosuppressed patients, that may ultimately enhance the clinical outcome. No mucositis was experienced in any patient after IFNα2b therapy possibly due to the use of IFNα2b for a short duration (3 wk). Leukepenia and neutropenia were monitored on a regular basis and controlled by the use of GCSF as and when required. Similar studies with large number of TSCC patients are required to be conducted, where repeated IFNα2b therapy can be given to maintain long term immune activation and concomitant radiotherapy can be tested.

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References


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