Enhancing the immune stimulatory effects of cetuximab therapy through TLR3 signalling in Epstein-Barr virus (EBV) positive nasopharyngeal carcinoma

Louise Soo Yee Tan, Benjamin Wong, Nagaraja Rao Gangodu, Andrea Zhe Ern Lee, Anthony Kian Fong Liou, Kwok Seng Loh, Hao Li, Ming Yann Lim, Andres M. Salazar, and Chwee Ming Lim

*Department of Otolaryngology, National University of Singapore-Head and Neck Surgery, **Department of Otolaryngology-Head and Neck Surgery, National University Health System Singapore, Singapore; †Department of Pathology, National University Health System Singapore, Singapore; ‡Department of Otorhinolaryngology, Tan Tock Seng Hospital, Singapore; ‡Oncovir, Inc., Washington, DC, USA; ‡Department of Otolaryngology, National University of Singapore, Singapore

ABSTRACT

Cetuximab immunotherapy targeting the epidermal growth factor receptor (EGFR) has been used to treat nasopharyngeal cancer (NPC) with some success. Therefore, combining an immune adjuvant to boost the immune microenvironment may improve its clinical efficacy. Herein, we investigate the immune-stimulatory effects of Poly-ICLC (a TLR3 agonist) in enhancing cetuximab-based immunotherapy and correlate these responses with FcγRIIlla (V158F) or TLR3 single nucleotide polymorphisms (SNPs—L412F and C829T) expressed on immune effector cells. We observed high levels of TLR3 mRNA in NPC cells; and both TLR3 and EGFR expression were unaffected by Poly-ICLC treatment. Cetuximab plus Poly-ICLC significantly enhanced NK-mediated ADCC through up-regulation of CD107a and Granzyme B expression. This effect was independent of FcγRIIlla-V158F and TLR3-L412F or TLR3-C829T polymorphisms expressed on NK cells. Additionally, IFN-γ expression and secretion were doubled following cetuximab plus poly-ICLC treatment; compared to either treatment alone. This effect was independent of TLR3 polymorphisms. Consequentially, adaptive immune responses were also seen with increased DC maturation (CD83), co-stimulatory molecules expression (CD80 and CD86) and increased frequency of EGFR-specific CD8+ T cells following Poly-ICLC treatment. The percentage of CD80+ CD83+ and CD83+ CD86+ DC was highest in the Poly-ICLC plus cetuximab group, compared to either treatment alone. These results demonstrate the effectiveness of Poly-ICLC in enhancing both cetuximab-mediated innate and adaptive anti-tumor immunity against NPC, which is independent of FcγRIIlla-158, TLR3-L412F or TLR3-C829T polymorphisms. Additionally, Poly-ICLC does not downregulate EGFR expression on NPC cells and hence, will not dampen cetuximab anti-tumor activity.

Introduction

Undifferentiated nasopharyngeal carcinoma (NPC) is an endemic cancer affecting patients in Southern China, Southeast Asia, Africa and Borneo. In certain geographic areas, it reached a high incidence of 80 per 100,000 populations and contributed directly to disease-specific mortality among these patients.1,2 Even though there is excellent clinical response to radiotherapy and chemotherapy, up to 20% of patients develop refractory loco-regional recurrences which are not salvageable surgically or with re-irradiation; and these patients are often treated with palliative chemotherapy with a short median survival of 9–15 months.3–5 Additionally, patients with metastatic disease, either at presentation or who subsequently developed distant metastasis, often succumb to this cancer.4–9 Therefore, there is a pressing need to develop newer treatment strategies beyond chemo-radiation in order to improve the clinical outcome of these patients.

One treatment approach is to target the epidermal growth factor receptor (EGFR) which is highly expressed in NPC.10 The immunotherapeutic mechanisms targeting tumor-associated antigens including EGFR are increasingly understood.11,12 Briefly, cetuximab binds to EGFR-positive tumor cells and undergo antibody-dependent cellular cytotoxicity (ADCC) upon cross-linkage with Natural Killer (NK) cells via the FcγRIIIa receptor.11 Tumor lysis following ADCC will increase presentation of tumor antigens to dendritic cells in order to enhance further downstream immune adaptive responses.11,12 However, immunosuppressive tumour microenvironment factors such as regulatory T cells (Treg), increased transforming growth factor beta (TGF-β) and enrichment of M2 macrophages through Galectin-9/Tim-3 pathways may suppress these anti-tumor immune responses and dampen the clinical efficacy.13,14 Therefore, Poly-ICLC may repolarize the M2 macrophage to the M1 phenotype by providing the Th1 cytokine milieu through IFN-γ secretion, and hence restore the anti-tumor signals accorded by the M2 macrophages.14,15

In this study, we sought to identify an immune adjuvant which may overcome these immune evasions; and hence boost the anti-tumor responses of cetuximab therapy. We have previously reported the feasibility of using Poly-ICLC to enhance the immune response in cetuximab therapy in head and neck....
squamous cell cancer (HNSCC).\textsuperscript{12} We believe this strategy of stimulating the innate anti-tumor response in NPC via TLR3 signaling is uniquely poised since there is typically an intense population of tumor infiltrating lymphocytes seen among NPC tumor cells which is classically associated with the Epstein-Barr virus (EBV) in the endemic variant.\textsuperscript{13} Moreover, TLR3 is reported to recognize EBV-encoded small RNAs (EBER) which can further stimulate the tumor environment via this mechanism for tumor clearance.\textsuperscript{16} Therefore, the possibility of recruiting more tumor-infiltrating lymphocytes and enhancing Th1-polarizing cytokines, such as interleukin-12 (IL-12) and type-1 IFN to the tumor microenvironment through TLR3 therapy is mechanistically feasible.\textsuperscript{17-19} Due to the existence of TLR3 polymorphisms, these immune stimulatory functions may be different among different patients. Therefore, we aim to investigate the immune-stimulatory effects of TLR3 signaling with cetuximab in NPC and identify the effects of FcγRIIIa (rs396991-158 Valine (V)/Phenylalanine (F)), or TLR3 (rs3775291-412 Leucine (L)/Phenylalanine (F); rs5743312-829 Cytosine (C)/Thymine (T)) polymorphisms in influencing the extent of immunostimulatory effects of cellular cytotoxicity and Th1 cytokines secretion.

Results

\textbf{Poly-ICLC has no effect on the TLR3 and EGFR expression; but induces modest apoptosis on NPC cells}

Both NPC cells (HONE1 and C666-1) expressed extracellular EGFR and intracellular TLR3 (Figure 1A) with majority of these cells expressing both molecules (Figure 1B). To examine the effect of Poly-ICLC on TLR3 and EGFR expression, NPC cells were incubated with varying concentrations of Poly-ICLC (0.1-100µg/ml). After 24hrs treatment with Poly-ICLC, the mean fluorescence intensity (MFI) of EGFR and TLR3 remained unchanged for both cell lines (Figure 1C). Poly-ICLC induced modest apoptosis of these cells (Figure 1D and E). The untreated HONE1 and C666-1 had 12.6% and 12.3% apoptotic cells, respectively, whereas stimulation with Poly-ICLC increased the percentage of apoptotic cells to 14.4%-18.0% for HONE1 and 14.9%-19.7% for C666-1. For subsequent analysis, 10µg/ml Poly-ICLC was used as the standard concentration as it showed the highest expression of TLR3 and EGFR in both NPC cell lines.

Quantitative PCR analysis of TLR3 mRNA expression performed on both NPC and healthy control (Figure 1F). NPC biopsies were found to express significantly higher TLR3 mRNA by 1.5-fold ($p = 0.002$) compared to controls.

\textbf{Poly-ICLC enhances NK cell mediated cetuximab-dependent cytotoxicity through up-regulation of activated NK cells expressing CD107a+ granzyme B + perforin in NPC}

To investigate whether TLR3 stimulation affects NK cell-mediated cetuximab-dependent ADC, NK cells were negatively isolated from both the healthy controls and NPC patients using immuno-magnetic beads. These NK cells were stimulated with 10µg/ml Poly-ICLC for 18hrs, followed by incubation with BATDA-labeled C666-1 cells at the ratio of 10:1 (Effector: Target) in the presence of 10µg/ml cetuximab or IgG1 isotype (negative control). Our results showed that Poly-ICLC-stimulated NK cells demonstrated robust cetuximab-dependent cytotoxicity in healthy controls ($p < 0.05$) and NPC patients ($p = 0.006$) independent of FcγRIIIa-158 polymorphisms (Figure 2A) (Kruskal-Wallis test, $p = 0.30$ and $p = 0.68$, respectively).

To demonstrate the functional activity of Poly-ICLC-treated NK cells, NK cell degranulation and cytotoxic markers-CD107a and granzyme B were analyzed (Figure 2C). After incubation of NK cells with NPC cell lines for 4hr, the cells were stained with antibodies against CD56, CD107a, granzyme B and perforin. NK cells were positively selected through CD56+ and analyzed for the interested markers. The percentage of NK cells expressing high CD107a+ granzyme B+ in cetuximab or Poly-ICLC alone treatment increased compared to untreated (Figure 2C). Indeed, further enhancement of the percentage of NK cells expressing high CD107a+ granzyme B+ were observed in the combination treatment as compared to cetuximab alone. Furthermore, these NK cells demonstrated a similar profile as those stimulated by IL-2. NK cells of both healthy controls and NPC patients were found to demonstrate comparable enhancement of NK cell degranulation (Figure 2C). Percentage of perforin + cells was found to be relatively unchanged (data not shown).

\textbf{Poly-ICLC plus cetuximab enhances IFN-\gamma production than either cetuximab or poly-ICLC alone}

To determine whether Poly-ICLC has any effect on the adaptive response, we compared the percentage of NK cells expressing IFN-\gamma. Cetuximab treatment induced IFN-\gamma expression by NK cells (Figure 3A and B), but not in Poly-ICLC treatment. However, when used in combination, a higher percentage of NK cells expressing IFN-\gamma was observed as compared to either Poly-ICLC or cetuximab treatment alone. IFN-\gamma+ NK cells were increased by 2.3-fold and 1.6-fold for healthy controls and NPC patients, respectively.

To corroborate the findings, the concentration of IFN-\gamma of each co-culture was quantified using ELISA assay (Figure 3C). In concordance with the flow cytometry analysis (Figure 3B), combination therapy significantly increased IFN-\gamma secretion in healthy controls and NPC by 2.4-fold and 2.9-fold, respectively, as compared to cetuximab alone (healthy control-72.0 pg/ml vs 29.8 pg/ml; NPC – 85.2 pg/ml vs 29.4 pg/ml).

\textbf{Poly-ICLC treatment directly matures DC, and enhances EGFR-specific CD8 + T cells}

Monocytes-derived DCs are known to express TLR3.\textsuperscript{20} Therefore, we sought to investigate the direct effect of TLR3 stimulation using Poly-ICLC on DCs. Monocytes were isolated from PBMC of NPC patients; and monocyte-derived DCs were generated as previously described.\textsuperscript{12} DCs were cultured with cetuximab (10µg/ml) (either with or without Poly-ICLC (50µg/ml)) for 48hr in the presence of C666-1 cells. These treated DCs were examined for maturation marker CD83 and co-stimulatory markers CD80 and CD86. Poly-ICLC alone induced both DC maturation and differentiation; the percentages of mature CD80+ CD83

Figure 1A

Figure 1B

Figure 1C

Figure 1D

Figure 1E

Figure 1F

Figure 2A

Figure 2B

Figure 2C

Figure 3A

Figure 3B

Figure 3C

Figure 3D
Poly-ICLC treatment alone does not affect the expression of TLR3 or EGFR expression; but induces modest apoptotic effect in NPC cells. (A) Representative flow cytometry histogram and (B) dot plot analysis of TLR3 and EGFR on NPC cells (solid line: isotype control and dotted line: TLR3/EGFR). NPC cells were cultured in RPMI supplemented with L-glutamine and streptomycin/penicillin. Cells were stained for extracellular EGFR, fixed and permeabilized before staining for intracellular TLR3. (B) Majority of the NPC cells express both TLR3 and EGFR. (C) Poly-ICLC does not affect the expression of TLR3 and EGFR on NPC cells. NPC cells were stimulated with different concentrations of Poly-ICLC (0.1-100µg/ml) for 24hr. Cells were harvested and stained for TLR3 and EGFR. The median fluorescence intensity (MFI) levels were determined. Data were collected from triplicate and expressed as the mean ± SEM. (D and E) Poly-ICLC treatment alone induces modest cell death on the NPC cells. NPC cells were treated for 24hrs with different Poly-ICLC concentrations. Cells were subsequently harvested and incubated with recombinant FITC-annexin V and propidium iodide. (D) Representative dot plot demonstrating the cell death of NPC cells following treatment with Poly-ICLC. (E) Data were collected from triplicate and expressed as the mean ± SEM. (F) NPC express significantly higher level of TLR3 mRNA compared to healthy controls. Tissues mRNAs were extracted and the TLR3 mRNA expression was analyzed through real-time PCR. Data were collected from 33 controls and 41 NPCs.
as compared to untreated. Furthermore, Poly-ICLC alone or combination therapy induced significantly higher proportion of mature and differentiated DCs compared to cetuximab alone.

Further investigation on the functional significance of Poly-ICLC on DC maturation and cetuximab-activated NK cells activity were analyzed by in vitro stimulation (IVS) of autologous CD8 + T cells. DC, NK and C666-1 cells were co-cultured with cetuximab or Poly-ICLC alone or both for 48hr to allow for DC maturation and cross-presentation, followed by incubation with autologous CD8 + T cells. After a 7-day IVS, Poly-ICLC or cetuximab alone did not significantly increase the frequency of EGFR-specific CD8 + T cells (Figure 4D) as compared to untreated. However, the Poly-ICLC plus cetuximab combination therapy increased the frequency of EGFR-specific CD8 + T cells as compared to untreated (p = 0.005) or cetuximab treatment alone (p = 0.027) by 1.5–1.8-fold.

Poly-ICLC enhances ADCC independent of L412F and C829T TLR3 SNPs

We investigated the distribution of the TLR3-rs3775291 (codon 412 L/F) and rs5743312 (intron 829 C/T) using Taqman genotyping assay. In this cohort, we found no correlation between these TLR3 SNPs and NPC; TLR3 L412F and C829T distributions are similar between healthy controls and NPC patients (p = 0.60 and p = 0.85) (Table 2).

Further study was done to investigate the functional effect of Poly-ICLC on DC maturation and ADCC in both healthy controls and NPC patients. In the NPC cohort, a 158 SNP genotype assay was performed. Data were collected from 10 samples for each group and presented as mean ± SEM.

Figure 2. Poly-ICLC significantly enhances NK cell-mediated cetuximab-dependent cytotoxicity in a FcγRIIIa (158) genotype-independent manner by up-regulating the percentage of NK cells expressing high CD107a+ granzyme B+. NK cells were isolated from healthy controls' (A) and NPC patients' (B) PBMC. The NK cells were treated with or without 10μg/mL Poly-ICLC for 18hrs, followed by co-culturing for 4hrs with BATDA-labeled C666-1 at a ratio of 10:1 in the presence or absence of 10μg/mL cetuximab. IL-2-treated NK cells were used as a positive control and IgG1 as a negative control. Genomic DNA was extracted from PBMCs and FcγRIIIa 158 SNP genotype assay was performed. Data were collected from 5 V/V, 17 V/F and 9 F/F from healthy controls; 3 V/V, 15 F/V and 12 F/F from NPC patients, healthy control p = 0.30 and NPC p = 0.68. (C) Poly-ICLC treated and untreated CD56+ CD16+ NK cells from healthy controls and NPC patients were analyzed for CD107a+ granzyme B+ high. Data were collected from 10 samples for each group and presented as mean ± SEM.
C829 SNPs, Poly-ICLC enhanced the cytotoxicity by 8.6%-23.1% (C/C-23.1% \( p < 0.0001 \), C/T- 18.3% \( p = 0.004 \) and T/T 8.6%).

Comparing the enhancement of Poly-ICLC-induced NK cell-mediated cytotoxicity, we observed constant functional improvement across all the alleles for both SNPs (Kruskal-Wallis test for NPC TLR3 L412F and C829T was \( p = 0.66 \) and \( p = 0.77 \), respectively) and similar phenomenon was observed in the healthy controls (TLR3 L412F and C829T was \( p = 0.80 \) and \( p = 0.14 \), respectively).

**Poly-ICLC enhances IFN-\( \gamma \) secretion in cetuximab-activated NK cells independent of L412F and C829T TLR3 SNPs**

To elucidate whether TLR3 SNPs has any effect on IFN-\( \gamma \) secretion, we analyzed the IFN-\( \gamma \) secretion in both TLR3 L412F and C829T cohorts (Figure 6). In this study, we combined healthy controls and NPC data since no correlation of TLR3 SNPs was found between these 2 groups. Poly-ICLC treatment alone showed no effect on IFN-\( \gamma \) secretion irrespective of TLR3 SNPs while combined Poly-ICLC and cetuximab treatment significantly increases IFN-\( \gamma \) secretion irrespective of TLR3 L412F (Figure 6A) and C829T (Figure 6B) SNPs. Similarly in C666-1 cells, Poly-ICLC treatment enhanced IFN-\( \gamma \) secretion in both TLR3 L412F and C829T genotypes by 2.8–5.7 fold and 5.4–9.8 fold, respectively.

Comparing the enhancement of Poly-ICLC-induced IFN-\( \gamma \) secretion, we observed constant functional improvement across all the alleles for both SNPs (Kruskal-Wallis one-way analysis showed \( p = 0.60 \) (HONE1) and \( p = 0.65 \) (C666-1) for TLR3 L412F; \( p = 0.13 \) (HONE1) and \( p = 0.86 \) (C666.1) for C829T).
Discussion

TLR3 agonists are potent immune adjuvants in inducing innate immunity and subsequently amplifying the adaptive immune responses. As such, Poly-ICLC has been developed to boost anti-tumor effects; primarily as a vaccine adjuvant in both brain and ovarian malignancies. Clinical trials using Poly-ICLC have shown it to be safe and well-tolerated in these patients, without significant adverse side effects. Although initially thought to induce primarily the innate immune-stimulatory effects via polarizing the Th1-cytokine secretion such as IFN-γ, subsequent studies have also shown further consequential increase in adaptive anti-tumor immune responses. The use of Poly-ICLC has been
investigated in combination with monoclonal antibody such as cetuximab, a IgG1 antibody targeting the EGFR, in head and neck squamous cell carcinoma (HNSCC). This study demonstrated enhanced NK-mediated antibody dependent cellular cytotoxicity (ADCC) with Poly-ICLC in combination; which resulted in increased in EGFR-specific CD8 + T cells. In EBV induced NPC, there is intense inflammatory infiltrates demonstrated in the tumor microenvironment. This observation; coupled with the high expression of EGFR seen in NPC; and with a phase II clinical trial demonstrating good clinical results with cetuximab in NPC; motivated this study to investigate the immune-stimulatory effects of Poly-ICLC in combination with cetuximab in NPC.

Figure 5. Poly-ICLC significantly enhances NK-mediated ADCC across all TLR3 (L412F or C829T) genotypes in healthy and NPC. NK cells were isolated and stimulated with Poly-ICLC (10μg/mL) for 18hr, followed by co-culture with BATDA-labeled C666-1 at a E:T ratio of 10:1; with or without cetuximab. IL-2-treated NK cells were used as a positive control and IgG1 isotype as a negative control. Genomic DNA was extracted from these PBMCs for TLR3 SNPs identification. (A-D) NK cells from both healthy controls (A-L412F & C-C829T) and NPC patients (B-L412F & D-C829T) enhances cetuximab dependent ADCC with Poly-ICLC treatment. Cetuximab-dependent cytotoxicity was significantly enhanced by Poly-ICLC pre-treatment as compared to cetuximab treatment alone. Poly-ICLC stimulation showed no functional correlation effect on the TLR3 genotypes. For TLR3 L412F, data were collected from 15 L/L, 12 L/F and 6 F/F healthy controls; and 11 L/L, 13 L/F and 6 F/F NPC patients. For TLR3 C829T, data were collected from 20 C/C, 9 C/T and 1 T/T healthy controls; and 20 C/C, 9 C/T and 1 T/T NPC patients. Kruskal-Wallis one-way analysis showed healthy p = 0.80 (L412F) and p = 0.14 (C829T), and NPC p = 0.66 (L412F) and p = 0.77 (C829T).
Our preclinical study confirmed both TLR3 and EGFR expression on NPC cell lines, which were unaffected by treatment with Poly-ICLC. Poly-ICLC treatment alone induced modest apoptosis and did not confer any survival advantage on these cells. This finding was similarly reported among previous publications on HNSCC cell lines and other NPC cell lines (5-8F, HONE1, C666-1 and CNE1) with Poly-ICLC treatment in vitro. Additionally, we demonstrated that Poly-ICLC treated NPC cells did not down-regulate EGFR expression on these cells, which would invariably dampen the cetuximab anti-tumor responses.

In this study, the TLR3 mRNA level was significantly higher in NPC biopsy compared to healthy nasopharyngeal tissue and this finding is congruent with previous studies where the presence of TLR3 may confer a protective effect on tumour cells against viral infection. In EBV-induced NPC, EBER may play a role in inducing the expression of TLR3 in NPC cell lines. These findings support investigating the role of leveraging on TLR3 signaling pathway to modulate the immune tumor environment of NPC, so as to tip it towards a Th1 cytokine milieu which has been shown to enhance anti-tumor immune responses. In a NPC mouse model, TLR3 activation has also shown inhibition of chemokine receptor CXCR4 expression on NPC cells; which suppresses NPC metastasis.

The immunotherapy mechanisms of tumor antigen specific monoclonal antibody based therapy have been well reported. Therefore, in this study, we have sought to evaluate if the anti-tumor immune responses with cetuximab can be further enhanced with TLR3 signaling using Poly-ICLC. Indeed, Poly-ICLC treated NK cells demonstrated increased lytic activity on NK-mediated cetuximab dependent ADCC against EGFR+ NPC cells. This effect is secondary to activation of NK cells through increased expression of cytotoxic-related molecules such as CD107a and Granzyme B. This was demonstrated by the increase in the percentage of NK cells expressing higher level of both CD107a and Granzyme B than those NK cells that were not treated with Poly-ICLC.

Contrary to previous study, our study did not establish the differential stimulatory effects of Poly-ICLC across the three genotypes of FcγRIIIa (158) expressed on NK cells. The FcγRIIIa polymorphisms have been shown to result in differential lytic activity on NK mediated cetuximab dependent ADCC. Nevertheless, Poly-ICLC treated NK cells with these different FcγRIIIa (158) genotypes showed similar enhancement of lytic activity, making Poly-ICLC a convenient means of stimulating the immune responses, independent of the FcγRIIIa (158) genotypes. The allelic frequency of FcγRIIIa (158) genotypes in our NPC cohort is similar to the healthy controls; and as well as those reported in HNSCC and EBV-related classical Hodgkin’s lymphoma cohorts. This finding suggests that FcγRIIIa polymorphism is not related to the risk of developing NPC.

In many cancer models including NPC, the tumor microenvironments are primarily immunosuppressive in nature, with the presence of immune-suppressive tumor-
infiltrating lymphocytes such as regulatory T-lymphocytes (Tregs). Our hypothesis is that Poly-ICLC may modify the tumor immune milieu through augmenting the cross-talk between innate and adaptive immunity; and this is mediated via cytokines such as IFN-γ which promote an anti-tumour microenvironment. Poly-ICLC may up-regulate IFN-γ secretion by NK cells in the presence of IL-12, and the increased IFN-γ secretion can further induce NK cells chemo-attractant through a positive feedback loop. Indeed, this enhancement of IFN-γ production by NK cells was demonstrated following Poly-ICLC treatment in our study, which was dependent on cetuximab in combination, since Poly-ICLC treatment alone had no significant increase of IFN-γ compared to controls.

In the absence of NK cells, cetuximab treatment alone did not induce maturation of DCs. This NK-DC cross talk has been reported previously, where treatment with cetuximab with co-culture with NK cells showed significant increase maturation of DCs. This observation is again reproduced in this study where NK-DC cross talk is important for DC maturation in the presence of a monoclonal antibody such as cetuximab. This phenomenon is due to the fact that DC lack of FcγRIIIa receptor and needed the NK interaction following cetuximab to proliferate and mature. Our study showed that Poly-ICLC is a potent inducer of DC maturation alone since a significant increase of matured DCs characterized by the increased expression of CD80 and CD86 on DCs; as well as an increase in the percentage of both CD80+ CD83+ and CD83+ CD86+ DCs following Poly-ICLC treatment when compared to no treatment or cetuximab-treated DCs. This is not surprising since Poly-ICLC induces a type 1 interferon immune milieu that is crucial for DC maturation.

Additionally, Poly-ICLC further enhanced the NK-DC cross talk with cetuximab in combination, which resulted in the amplification of ensuring adaptive anti-tumour immunity. Therefore, the enhancement of matured DCs with Poly-ICLC treatment eventually resulted in the significant increase of EGFR-specific CD8 + T cells (CTLs) observed in this study. This finding is important because increase of tumour-specific CTL has been associated with better prognosis in cancer.

Finally, we evaluated the impact of TLR3 polymorphisms (TLR3 L412F and C829T SNPs) in influencing the immune-stimulatory effects demonstrated in this study. We have chosen these TLR3 SNPs because these two SNPs have been associated with an increased risk of development of NPC and HNSCC and could serve as biomarker to select patients for Poly-ICLC treatment. Of note is that the TLR3 allele frequencies of these 2 SNPs were similar across previous reported studies. Our study demonstrated no differences among the increase in ADCC and IFN-γ secretion across these 2 SNPs. These immune-stimulatory effects are independent of these two SNPs and support the universal use of Poly-ICLC to stimulate the immune responses against NPC. Therefore, these findings provided a rationale for using Poly-ICLC as an immune-stimulatory adjuvant in improving the modest advantage of adding cetuximab with standard concurrent chemo-radiotherapy in nasopharyngeal cancer.

**Conclusion**

Poly-ICLC is a potent immune adjuvant in enhancing the immune anti-tumor responses of cetuximab against NPC cells. Specifically, the lytic activity of NK cell mediated cetuximab-dependent ADCC was enhanced independent of FcyRIIIa (codon 158) and TLR3 L412F and C829T SNPs expressed on NK cells. Additionally, enhancement of NK: DC cross talk was facilitated with cetuximab plus Poly-ICLC treatment which eventually resulted in significant increase in EGFR-specific CD8 + T cells. These findings support the use of adding Poly-ICLC with cetuximab in NPC to boost the anti-tumor immune responses.

**Materials and methods**

**Cells and cell lines**

Blood samples were collected from healthy donors and newly diagnosed NPC patients from the National University Hospital, Singapore, in accordance to the approved Institutional Review Board protocol (2012/02230). Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by density gradient centrifugation on Lymphoprep” (Axis Shield). In brief, the blood sample was diluted with 1X Dulbecco’s Phosphate-Buffered Saline (DPBS) (Biowest) at a ratio of 1:1 and layered over the Lymphoprep (Axis Shield) and the suspension was centrifuged at 400g for 30mins. The interphase layer (PBMCs) was collected and diluted with equal volume of DPBS, followed by repeat centrifugation at 400g for 30mins.

NPC cell lines (C666-1 and HONE1) were used in these experiments. NPC cell lines C666-1 cells were kindly provided by Dr Tan Min Han’s laboratory from Institute of Bioengineering and Nanotechnology, Singapore; and HONE1 cells were provided by Dr Goh Boon Cher’s laboratory from National University Hospital, Singapore. The C666-1 cell was authenticated by short tandem repeat (STR) profiling, but no authentication was done for the HONE1 cells. All the cells were mycoplasma-free, as tested using the MycoFluor Mycoplasma Detection Kit (Invitrogen). HONE1 and C666-1 were cultured in RPMI-1640 media (PAAP-GE Healthcare) supplemented with 1% penicillin/streptomycin, 1% L-glutamine and 10% fetal bovine serum (FBS, PAA-GE Healthcare) at 37°C with 5% CO2.

**NK-cell degranulation**

Purified NK cells were isolated from PBMCs using the EasySep NK negative isolation kit (StemCell Technologies) in accordance to the manufacturer’s protocol. NK purity of more than 90% was confirmed on flow cytometry using CD56 positive immunostaining. NK cells were treated with or without Poly-ICLC (10μg/mL) (Hiltonol, Oncovir) for 18hrs. The NK cells were then co-cultured with NPC cells (10: 1 ratio), with either 10μg/mL of cetuximab or a human IgG1 isotype for 4hrs. These co-cultures were subsequently fixed with brefeldin A (e Biosciences). NK cells were harvested and stained with anti-CD56, CD16 and NKG2D monoclonal antibodies (MiltenyiBiotec) at room temperature for 20mins. Cells were then fixed and permeabilized using an intracellular fixation & permeabilization buffer kit.
(eBioscience), and finally stained for intracellular anti-CD107a, granzyme B, perforin and IFN-γ monoclonal antibodies (MiltenyiBiotec) at room temperature for 20mins. Cells were analyzed with a Becton Dickinson (BD) LSR II flow cytometer (BD Biosciences). NK cells analysis was performed using Flowjo version V10 (FlowJo, L.CC).

**Antibody-dependent cellular cytotoxicity (ADCC)**

BATDA europium release assay was used to determine the extent of ADCC. Purified NK cells were pre-treated with or without Poly-ICLC (10 µg/mL) for 18hrs. NPC cells were labeled with BATDA (PerkinElmer) for 30mins at 37°C and the cells were washed twice with 1XDPBS; before being co-cultured with NK cells at an E: T ratio of 10:1 plus cetuximab (10µg/mL). After incubation for 4hrs at 37°C, supernatants were harvested and counted on a Perkin-Elmer 96-well plate gamma counter. Percentage of cytotoxicity was calculated using the formula: [(experimental-spontaneous release)/(maximum-spontaneous release)] × 100%; where spontaneous = release from targets incubated with medium alone and maximum = release from targets incubated by 5% Triton X-100 (Bio-rad).

**Apoptosis assay**

NPC cells (2.5 × 10⁶ cells/well) were plated onto a 12-well plate for 24hrs and were treated with varying concentrations of Poly-ICLC (0.1-100µg/ml) for 24hrs. Cells were harvested and stained with Annexin V and Propidium Iodide followed by analysis using BD LSR II flow cytometer (Becton, Dickson and Company). Annexin V is a marker for apoptosis and Propidium Iodide a marker for dead cells.

**Dendritic cell culture**

Immature dendritic cells (DCs) were generated from the PBMC using the protocol published previously. In brief, monocytes were isolated from the PBMC by plastic adherence for 2hrs at 37°C. The non-adherent cells were removed leaving the adherent monocytes which were washed with 1XDPBS twice. The adherent cells were then incubated with AIM-V medium at 37°C. The non-adherent cells were removed leaving the adherent cells. The adherent cells were then incubated with 1XDPBS; before being co-cultured with NK cells at an E: T ratio of 10:1 plus cetuximab (10µg/mL). After incubation for 4hrs at 37°C, supernatants were harvested and counted on a Perkin-Elmer 96-well plate gamma counter. Percentage of cytotoxicity was calculated using the formula: [(experimental-spontaneous release)/(maximum-spontaneous release)] × 100%; where spontaneous = release from targets incubated with medium alone and maximum = release from targets incubated by 5% Triton X-100 (Bio-rad).

**TLR3 mRNA expression**

Real-time PCR was used to analyze TLR3 mRNA expression level in the healthy nasopharynx and NPC. Total RNA of NPC proven and healthy control samples was isolated using AllPrep RNA mini kit (Qiagen) and 500ng of RNA was converted to cDNA using Bioline Tetroc DNA synthesis kit (Bioline) with Bio-Rad iCycler (Bio-Rad Laboratories). TLR3 gene expression was performed using Taqman assay (FAM-Hs01551078_m1) and Taqman assay VIC-ACTB (Applied Biosystems) was used as a house keeping gene with Taqman Universal PCR master mix (Applied Biosystems), according to the manufacturer’s instruction. Real-time PCR was performed using the Step One Plus detection system by running the samples at the following cycling conditions: 95°C for 10mins (1 cycle), 95°C for 15s and 60 for 1min (40 cycles).

**CD8 + T cells in vitro stimulation**

Autologous CD8 + T cells were isolated using Miltenyi Biotec CD8 T cell isolation kit, according to manufacturer’s instructions. Dendritic cells and NK cells were incubated with combination treatment or single treatment- Poly-ICLC or cetuximab alone for 48hr and then used for CD8 + T cells stimulation *in vitro* for 7days. Samples were harvested and stained for CD11c, HLA-A02, HLA-A11 and HLA-A24 (common HLA-A allele in Singapore population) or CD3, CD8, 7-AAD and EGFR853-861 tetramers, and at the end analyzed by flow cytometry. CD3 + and CD8+ cells were gated out and analyzed for EGFR853-861 tetramers. The corresponding tetramers were kindly provided by NIH Tetramer Facility (Emory University).

**FcγRIIa and TLR3 genotyping**

Genomic DNA was extracted from PBMC using the QIAamp DNA Kit (Qiagen). FcγRIIa-158 and two previous TLR3 polymorphisms (TLR3-412 and ~829) which had been reported to be associated with head and neck cancer, including NPC were investigated in our study. FcγRIIa and TLR3 genotyping were carried out on healthy controls and NPC patients using a Taqman allelic discrimination assay (Life Technologies). In brief, 10-20ng of genomic DNA was mixed with 20XPCR containing primers and FAM/VIC -labeled probe specific for FcγRIIa or TLR3 (Table 1) along with 2XTaqman genotyping master mix (Applied Biosystems). Amplification and allelic discrimination were performed using ABI Prism 7500 Fast real-time PCR system (Applied Biosystems). The PCR amplification profile was as follows: initial denaturation at 92°C for 10mins, and then 40 cycles of denaturation at 92°C for 15s and annealing/ extension at 60°C for 1min.

### Table 1. FcγRIIa and TLR3 polymorphisms information.

| Gene      | Mutation | Transcript | Sequence |
|-----------|----------|------------|----------|
| FcγRIIa   | Val158Phe| rs396991   | TGCTAAAGGACACATTTTTTACTCCCAA(C/A) AAGCCCGCTGGAAAGGTAGAGGGCCG |
| TLR3      | Leu412Phe| rs3775291  | ACTGCTGCTACCCGCGCTACATAAT(T/C) TCAACCTAACAACGAAATATAAAATCTC |
| TLR3      | C829T    | rs5743312  | ATAGCTCTTCTTCTGTGCTACATAAC(T/T) AGGAAATGAAAGCCTCCTAGGCTCCT |

**Dendritic cell culture**

Immature dendritic cells (DCs) were generated from the PBMC using the protocol published previously. In brief, monocytes were isolated from the PBMC by plastic adherence for 2hrs at 37°C. The non-adherent cells were removed leaving the adherent monocytes which were washed with 1XDPBS twice. The adherent monocytes were then incubated with AIM-V medium (Invitrogen) supplemented with 1% penicillin/streptomycin, 1000IU/mL granulocyte macrophage colony-stimulating factor (GM-CSF, MiltenyiBiotec) and 400IU/mL interleukin-4 (IL-4, MiltenyiBiotec) at 37°C in a 5% CO2 atmosphere. After an incubation of 3 days, the media was replenished. The cells were harvested on day 6 using TrypLE Select (GIBCO) according to manufacturer’s protocol. DCs were then co-cultured with NPC cells plus NK cells at a ratio of 1:1 for 48hrs incubated with media alone, 10µg/mL cetuximab, 50µg/mL poly-ICLC, or both. Cells were then harvested and stained with FITC-CD11c, APC-CD80, PE-Vio-CD86, PE-CD83, APC-Vio 770 MICA/B antibodies (MiltenyiBiotec) and then analyzed using flow cytometry. CD11c+ phenotype were gated out and analyzed for the percentage of CD80+ CD83+, CD86+ CD83+ and MICA/B+ CD83+ as well as the mean fluorescence intensity (MFI).
Statistical analysis

A post-hoc Mann-Whitney non-parametric t-test was performed to analyze the differences between groups and Kruskal-Wallis one-way test was applied to analyze the differences for FcγRIIIα or TLR3 polymorphisms. All statistical tests were performed with Graphpad Prism 5 (GraphPad Software, Inc.) and p values < 0.05 were considered as statistically significant.

Disclosure of Potential Conflicts of Interest

The authors declare no potential conflicts of interest.

Funding

This work was supported by the National Medical Research Council Singapore, Transitional Award. (NMRC/TA/0035/2015). The tetramers HLA-A2, HLA-A11 and HLA-A24 were obtained through the NIH Tetramer Core Facility.

ORCID

Andrea Zhe Ern Lee http://orcid.org/0000-0002-8519-4478
Hao Li http://orcid.org/0000-0001-7191-5118
Ming Yann Lim http://orcid.org/0000-0001-5038-7037
Chwee Ming Lim http://orcid.org/0000-0001-9528-4365

References

1. Ma BB, Chan AT. Recent perspectives in the role of chemotherapy in the management of advanced nasopharyngeal carcinoma. Cancer. 2005;103:22–31. doi:10.1002/cncr.20768.
2. Meng R, Wei K, Xia L, Xu Y, Chen W, Zheng R, Lin L. Cancer incidence and mortality in Guangdong province, 2012. Chin J Cancer Res. 2016;28:311–320. doi:10.11147/j.issn.1000-9604.2016.03.05.
3. Chee J, Ting Y, Ong YK, Choo SS, Loh KS, Lim CM. Relapse status as a prognostic factor in patients receiving salvage surgery for recurrent or residual nasopharyngeal cancer after definitive treatment. Head & Neck. 2016;38:193–1400. doi:10.1002/hed.38.9.
4. Lee N, Harris J, Garden AS, Straube W, Glisson B, Xia P, Bosch W, Morrison WH, Quivey J, Thorstad W, et al. Immunosuppression, radiation therapy with or without chemotherapy for nasopharyngeal carcinoma: radiation therapy oncology group phase II trial 0225. J Clin Oncol. 2009;27:3684–3690. doi:10.1200/JCO.2008.19.9109.
5. Tham IW, Hee SW, Yeo RM, Salleh PB, Lee J, Tan TW, Fong KW, Chua ET, Wee JT. Treatment of nasopharyngeal carcinoma using intensity-modulated radiotherapy-the national cancer centre singapore experience. Int J Radiat Oncol Biol Phys. 2009;75:1481–1486. doi:10.1016/j.ijrobp.2009.01.018.
6. Mak HW, Lee SH, Chee J, Tham I, Goh BC, Choo SS, Ong YK, Loh KS, Lim CM, Gao C-Q. Clinical outcome among nasopharyngeal cancer patients in a multi-ethnic society in Singapore. PLoS ONE. 2015;10:e0216108. doi:10.1371/journal.pone.0216108.
7. Blanchard P, Lee A, Marguet S, Leclercq J, Ng WT, Ma J, Chan ATC, Huang P-Y, Benhamou E, Zhu G, et al. Chemotherapy and radiotherapy in nasopharyngeal carcinoma: an update of the MAC-NPC meta-analysis. Lancet Oncol. 2015;16:645–655. doi:10.1016/S1470-2045(15)70126-9.
8. Leong YH, Soon YY, Lee KM, WONG LC, Tham IWK, Ho FCH. Long-term outcomes after reirradiation in nasopharyngeal carcinoma with intensity-modulated radiotherapy: A meta-analysis. Head & Neck. 2018;40:622–631. doi:10.1002/hed.24993.
9. Au KH, Ngan RKC, Ng AWY, Poon DMC, Ng WT, Yuen KT, Lee VHF, Tung SY, Chan ATC, Sze HCK, et al. Treatment outcomes of nasopharyngeal carcinoma in modern era after intensity modulated radiotherapy (IMRT) in Hong Kong: A report of 3328 patients (HKNPCSG 1301 study). Oral Oncol. 2018;77:16–21. doi:10.1016/j.oraloncology.2017.12.004.
10. Chan AT, Hsu MM, Goh BC, Hui EP, Liu TW, Millward MJ, Hong R-L, Whang-Peng J, Ma BY, To KP, et al. Multicenter, phase II study of cetuximab in combination with carboplatin in patients with recurrent or metastatic nasopharyngeal carcinoma. J Clin Oncol. 2005;23:3568–3576. doi:10.1200/JCO.2005.02.147.
11. Lee SC, Srivastava RM, Lopez-Albaitero A, Ferrone S, Ferris RL. Natural killer (NK): dendritic cell (DC) cross talk induced by therapeutic monoclonal antibody triggers tumor antigen-specific T cell immunity. Immunol Res. 2011;50:248–254. doi:10.1007/s12026-011-8231-0.
12. Ming Lim C, Stephenson R, Salazar AM, Ferris RL. TLR3 agonists improve the immunostimulatory potential of cetuximab against EGFR head and neck cancers. Oncol Immunol. 2013;3:e24677. doi:10.4161/onci.24677.
13. Gourzones C, Barjon C, Busson P. Host-tumor interactions in nasopharyngeal carcinomas. Semin Cancer Biol. 2012;22:127–136. doi:10.1016/j.semcancer.2012.01.002.
14. Wang N, Liang H, Zen K. Molecular mechanisms that influence the macrophage m1-m2 polarization balance. Front Immunol. 2014;5:614. doi:10.3389/fimmu.2014.00614.
15. Eningia EAL, Chatzopoulos K, Butterfield JT, Sutor SL, Leonovich AA, Nevala WK, Flotte TJ, Malovic SN. CD206-positive myeloid cells bind galectin-9 and promote a tumor-supportive microenvironment. J Pathol. 2018. doi:10.1002/path.5093.
16. Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, Seya T, Imai S, Fujiyda M, Kawa K, Takada K. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med. 2009;206:2091–2099. doi:10.1084/jem.20081761.
17. Smits EL, Ponsaerts P, Berneman ZN, Van Tendeloo GM, Fields M. The use of TLR7 and TLR8 ligands for the enhancement of cancer immunotherapy. Oncologist. 2008;13:859–875. doi:10.1634/theoncologist.2008-0097.
18. Lombardi V, Van Overveldt L, Horiot S, Moingeon P. Human dendritic cells stimulated via TLR7 and/or TLR8 induce the sequential production of IL-10, IFN-gamma, and IL-17A by naive CD4+ T cells. J Immunol. 2009;182:3372–3379. doi:10.4049/jimmunol.0801969.
19. Girart MV, Fuertes MB, Domaico CI, Rossi LE, Zwirner NW. Engagement of TLR3, TLR7, and NKG2D regulate IFN-gamma secretion but not NKGD2-mediated cytotoxicity by human NK cells stimulated with suboptimal doses of IL-12. J Immunol. 2007;179:3472–3479. doi:10.4049/jimmunol.179.6.3472.
20. Matsumoto M, Funami K, Tanabe M, Oshiumi H, Shingai M, Seto Y, Yamamoto A, Seya T. Subcellular localization of Toll-like receptor 3 in human dendritic cells. J Immunol. 2003;171:3154–3162. doi:10.4049/jimmunol.171.6.3154.
21. Okada H, Kalinski P, Ueda R, Hoji A, Kohanbash G, Donegan TE, Mintz AH, Engh JA, Bartlett DL, Brown CK, et al. Induction of CD8+ T-cell responses against novel glioma-associated antigen peptides and clinical activity by vaccinations with [alpha]-type 1 polarized dendritic cells and polynucleosinic-polyricydilic acid stabilized by lysine and carboxymethylcellulose in patients with recurrent malignant glioma. J Clin Oncol. 2011;29:330–336. doi:10.1200/JCO.2010.30.7744.
22. Butowski N, Lamborn KR, Lee BL, Frados MD, Cloughesy T, DeAngelis LM, Abrey LE, Fink K, Lieberman F, Mehta M, et al. A North American brain tumor consortium phase II study of poly-ICLC for adult patients with recurrent anaplastic gliomas. J Neurooncol. 2009;91:183–189. doi:10.1007/s11060-008-9705-3.
23. Sabbatini P, Tsuji T, Ferran L, Ritter E, Sedrak C, Tuballes K, Jhundhla A, Ritter G, Aghajanian C, Bell-McGuinn K, et al. Phase I trial of overlapping long peptides from a tumor self-antigen and poly-ICLC shows rapid induction of integrated immune response in ovarian cancer patients. Clin Cancer Res. 2012;18:6497–6508. doi:10.1158/1078-0432.CCR-12-2189.
24. Ammi R, De Waele J, Willemen Y, Van Brussel I, Schrijvers DM, Lion E, Smits EL. Poly-ICLC as cancer vaccine adjuvant: knocking on the door of medical breakthroughs. Pharmacol Ther. 2015;146:120–131. doi:10.1016/j.pharmthera.2014.09.010.
25. Li Z, Duan Y, Cheng S, Chen Y, Hu Y, Zhang L, He J, Liao Q, Yang L, Sun LQ. EBV-encoded RNA via TLR3 induces inflammation in nasopharyngeal carcinoma. Oncotarget. 2015;6:24291–24303.

26. Verillaud B, Gressette M, Morel Y, Paturel C, Herman P, Lo KW, Tsao S, Wassef M, Jimenez-Pailhes A-S, Busson P. Toll-like receptor 3 in Epstein-Barr virus-associated nasopharyngeal carcinomas: consistent expression and cytotoxic effects of its synthetic ligand poly(A:U) combined to a Smac-mimetic. Infect Agent Cancer. 2012;7:36. doi: 10.1186/1750-9378-7-36.

27. Zhang Y, Sun R, Liu B, Deng M, Zhang W, Li Y, Zhou G, Xie P, Li G, Hu J. TLR3 activation inhibits nasopharyngeal carcinoma metastasis via downregulation of chemokine receptor CXCR4. Cancer Biol Ther. 2009;10:8047–8052. doi: 10.1073/pnas.0700664104.

28. Bondhopadhyay B, Moirangthem A, Basu A. Innate adjuvant receptor Toll-like receptor 3 can promote breast cancer through cell surface. Tumour Biol: J Int Soc Oncodevelop Biol Med. 2015;36:1261–1271. doi: 10.1007/s13277-014-2373-8.

29. Ferris RL. Immunology and Immunotherapy of Head and Neck Cancer. J Clin Oncol. 2015;33:3293–3304. doi: 10.1200/JCO.2015.61.1509.

30. Ghesquières H, Dogan A, Link BK, Maurer MJ, Cunningham JM, Novak AJ, Larrabee BR, Slager SL, Allmer C, Habermann TM, et al. FCGRA2A and FCGRA3A polymorphisms in classical Hodgkin lymphoma by Epstein–barr virus status. Leuk Lymphoma. 2013;54:2571–2573. doi: 10.3109/10428194.2013.796048.

31. Chow V, Tow C, Huang C, Bard-Chapeau E, Copeland NG, Jenkins NA, Weber A, Lim KH, Toh HC, Heikenwalder M, et al. Toll-like receptor 3 expressing tumor parenchyma and infiltrating natural killer cells in hepatocellular carcinoma patients. J Natl Cancer Inst. 2012;104:1796–1807. doi: 10.1093/jnci/djs436.

32. Sivori S, Falco M, Della Chiesa M, Carlomagno S, Vitale M, Moretta L, Moretta A. CpG and double-stranded RNA trigger human NK cells by Toll-like receptors: induction of cytokine release and cytotoxicity against tumors and dendritic cells. Proc Natl Acad Sci U S A. 2004;101:10116–10121. doi: 10.1073/pnas.0403744101.

33. Stephenson RM, Lim CM, Matthews M, Dietsch G, Hershberg R, Ferris RL. TLR8 stimulation enhances cetuximab-mediated natural killer cell lysis of head and neck cancer cells and dendritic cell cross-priming of EGFR-specific CD8+ T cells. Cancer Immunol Immunother: CII. 2013;62:1347–1357. doi: 10.1007/s00262-013-1437-3.

34. Zhou J, Dudley ME, Rosenberg SA, Robbins PF. Persistence of multiple tumor-specific T-cell clones is associated with complete tumor regression in a melanoma patient receiving adoptive cell transfer therapy. J Immunother. 2005;28:53–62.

35. Wang BG, Yi DH, Liu YF. TLR3 gene polymorphisms in cancer: a systematic review and meta-analysis. Chin J Cancer. 2015;34:19. doi: 10.1186/s40880-015-0020-z.

36. Cheng D, Hao Y, Zhou W, Ma Y. Association between Toll-like receptor 3 polymorphisms and cancer risk: a meta-analysis. Tumour Biol: J Int Soc Oncodevelop Biol Med. 2014;35:7837–7846. doi: 10.1534/g3.112.005371.

37. Moumad K, Lascorz J, Bevier M, Khyatti M, Ennaji MM, Benider A, Huhn S, Lu S, Chouchane L, Corbex M, et al. Genetic polymorphisms in host innate immune sensor genes and the risk of nasopharyngeal carcinoma in North Africa. G3 (Bethesda). 2013;3:971–977. doi: 10.1534/g3.112.005537.

38. Zeljic K, Supic G, Jovic N, Kozomara R, Brankovic-Magic M, Obrenovic M, Magic Z. Association of TLR2, TLR3, TLR4 and CD14 genes polymorphisms with oral cancer risk and survival. Oral Dis. 2014;20:416–424. doi: 10.1111/odi.12144.

39. You R, Hua YJ, Liu YP, Yang Q, Zhang YN, Li JB, Li C-F, Zou X, Yu T, Cao J-Y, et al. Concurrent chemoradiotherapy with or without anti-egfr-targeted treatment for stage II–IVb nasopharyngeal carcinoma: retrospective analysis with a large cohort and long follow-up. Theranostics. 2017;7:2314–2324. doi: 10.7150/thno.19710.