ABSTRACT

Lung cancer is known as the most common malignancy in the world in terms of incidence and death rate. GLOBOCAN data in 2018 showed that its prevalence reaches 11.6% with mortality reaching 1.7 million annually. The prevalence of lung cancer in Indonesia is also considerably high; there were 25,332 cases in men and 9,374 cases in women with mortality reaching 308,660 people. In general, lung cancer is classified as non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Although it is less common, SCLC has a worse prognosis with a 5 year survival rate of 6.4%. In addition, SCLC is also often diagnosed when it has metastasized or already reached extensive stage (ES-SCLC). Although SCLC is classified as chemo-responsive cancer, the overall outcome of first-line and second-line therapy is still unsatisfactory with an ORR of 10-25%. One of characteristic of SCLC is genomic instability which relates to high level of mutations especially EGFR mutation that strongly correlate with therapeutic outcome. Therefore, targeting EGFR mutation is a sensible and potential field in developing SCLC therapy. EGFR-CpG-ODN nanovaccine is one of the potential therapeutic choices that exploit this mutation. EGFR CpG ODN nanovaccine could inhibit resistance to EGFR TKI, increased dendritic cell maturation, inhibit cancer cell proliferation and apoptosis, as well as enhance the anticancer immune response. Therefore, this therapeutic approach is a promising future therapy for SCLC patients that could improve patient’s survivability.

INTRODUCTION

Lung cancer ranks as the most common and detrimental malignancy worldwide. GLOBOCAN data in 2018 showed that the prevalence of lung cancer in the world reached 11.6%. In 2018 alone, there were 2 million cases of newly diagnosed lung cancer. Lung cancer mortality is considerably high in both men and women, reaching 18.4% or 1.7 million annually. In Indonesia, the prevalence of lung cancer in men reached 25,332 cases while in women it reached 9,374 cases with mortality rate reached 308,660 cases.

In general, lung cancer is classified as non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC comprised the majority of lung cancer cases (85%), while SCLC only contributes to 15% of cases. Although it is less common, SCLC has a worse prognosis compared to NSCLC with a 5 year survival rate only of 6.4%. In 70-80% of cases of SCLC, the diagnosis is established when the disease already metastasized or extensive stage (ES-SCLC). The main factors that contribute to this phenomenon are high vascular density within the tumor, genomic instability, and rapid tumor cell growth which result in very low survival rate of SCLC patient, averaging only between 2 to 10 months even with first-line treatment.

However, SCLC is still classified as chemo-responsive cancer. Etoposide-platinum therapy is the first line treatment in SCLC, but its outcome is considered as unsatisfactory because the number of patients who managed to survive for 2 years is still very low (10-20%). Second-line therapy is also highly dependent on the condition of patients who are sensitive or not to platinum based therapy with an ORR of only 10-25%. Clinical trials also show that chemotherapeutic agents and targeted therapies have not been able to improve patient outcomes.

Epidermal growth factor receptor (EGFR) gene mutations are rare in SCLC, but their occurrence often related to unfavourable therapeutic response. However, the mutations can be targeted by immunotherapy that could potentially overcome the poor response of SCLC therapy. Immunotherapy targets neoantigens within the tumor, which in this case, mutated EGFR. Neoantigen originates from the accumulation of non-synonymous somatic mutations and presented on the surface of tumor cells by MHC class 2 as a different peptide, making it difficult for immune cells to recognize it.

Because of the absence of immune responses against EGFR due to central tolerance and peripheral tolerance, substances that are capable of inducing immune cell maturation against EGFR are needed. One of the approaches is by using EGFR vaccine. The EGFR vaccine will induce immune response of T cells against the EGFR antigen.
cell maturation against EGFR through the stimulation of dendritic cells that have been induced by the EGFR vaccine.\textsuperscript{5,6} To increase its effectiveness, adjuvant is needed in the form of CpG (Cytosine-phosphorothioate-Guanine) containing oligodeoxynucleotides (ODNs). CpG-ODN is known to improve Th1-immune response by inducing the production of IL-12 and IFN-γ.\textsuperscript{10} In addition, nanoencapsulation with chitosan improve vaccine delivery to dendritic cells and lymph nodi cells.\textsuperscript{11} The potential of EGFR-chitosan nanovaccine will be discussed further in this mini review.

Pathogenesis of Lung Cancer
In the pathogenesis of lung cancer, it has been suggested that immune system also plays a role, especially when immune surveillance fail. Cancer cells are able to escape from the immune system through a process known as “immunoediting”. This process is influenced by intrinsic and extrinsic factors which lead to changes in the cell microenvironment. The intrinsic factors include increased expression of programmed cell death ligand (PD-L), Fas ligand (Fas-L), indoleamine-2-3-dioxigenase (IDO) enzyme, and Vascular Endothelial Growth Factor (VEGF).\textsuperscript{12-14}

In general, VEGF induces angiogenesis but it also plays important role in immunosupression by inhibiting dendritic cell maturation in the peripheral blood, lymph nodes and spleen due to inhibition of NFKB expression produced by immature dendritic cells, increase Treg cells differentiation and suppress proliferation and function of CD4 +, CD8 + and NK cells. Decrease NK and T-cell activation would result in lower level of Th1 cytokines such as gamma Interferon (INF-γ), Tumor Necrosis Factor alpha (TNF-α), and TNF-Related Apoptosis-Inducing Ligand (TRAIL).

With the reduction in cytokines expressed, signaling induction for apoptosis in cancer cell fails to occur.\textsuperscript{15} In carcinogenesis, IFN-γ, a cytokine that has the potential to inhibit carcinogenesis unable to induce MHC expression which alters antigen presentation and T helper-1 (Th1) response.\textsuperscript{12-16} On the other hand, Myeloid-derived suppressor cells (MDSCs) induce Treg cells, Tumor-Associated Macrophages or TAM and stromal cells to produce suppressor cytokines such as IL-10 and Transforming Growth Factor-B (TGF-β) which able to inhibit the adaptive immune response (Figure 1).\textsuperscript{13,14}

Other than immune response suppression, the mutation of Endothelial Growth Factor Receptor (EGFR) play additional crucial role in lung cancer progression and the majority of these mutations caused an increase in EGFR expression and activation of signaling pathways which enhanced proliferation, angiogenesis, as well as metastasis.\textsuperscript{20} The cytoplasmic part of EGFR is particularly important in this process especially the ligand domain of Tyrosin Kinase (TIK) such as HER1, HER2, HER3, and HER4.\textsuperscript{21}

Signal binding or auto-activation would lead to receptor dimerization which then induces the EGFR-TIK signaling pathway and tyrosine kinase phosphorylation. In the initial step, activated tyrosine kinase stimulate mTOR-serine/threonine protein kinase signaling pathway, leading to phosphorylation cascade that involves Phosphatidylinositol-3 Kinase (PI3K/AKT), Signaling Transducer and Activator Of Transcription 3 (STAT3), and Mitogen Activated Protein Kinase (MAPK).\textsuperscript{22} Abnormal growth and metastasis are the result of MAPK activation by its binding to the Cyclin–Dependent Kinase (CDKs) kinase domain and subsequent upregulation of Cyclin D1.

In addition, PI3K induction leads to increased proliferation and anti-apoptosis effect, mainly by PIP3 mediated pathway. The signal is forwarded to Protein Kinase B (AKT) which then translocated into the nucleus and alters the transcription of several oncogenes.\textsuperscript{23} Furthermore, EGFR activation also leads to extra cellular matrix (MES) remodeling in lung tissue and increases the blood supply to cancer cells through angiogenesis.\textsuperscript{24}

Overview of Chitosan Nanoparticle
Chitosan is a natural polysaccharide molecule that is non-toxic with a molecular size between 3800-20000 Dalton, biodegradable, and biocompatible. Due to its nature, chitosan is suitable as a drug-carrying molecule that enhances the drug targeting and prolongs the half-time of the drug.\textsuperscript{24} Chitosan is generally derived from crustacean chitin (Figure 2).

Chitosan is synthesized from chitin using deacetylation method using sodium hydroxide reagent and dissolved in water.\textsuperscript{25} The degree of deactilisation (%) DD) usually ranged between 60% - 100%.\textsuperscript{26} In addition, chitosan can also be made from trimethyl chitosan derivatives, zwitterionic chitosan, and glycated chitosan. Glycated chitosan is a new development of nanotechnology chitosan with its advantages as being able to dissolve in water which is not the case of other types of chitosan derivatives.\textsuperscript{27,28} Glycated chitosan (GC) is also found to be able to increase cytokine secretion ie TNF-α and INF-γ according to animal studies.\textsuperscript{27,29}

Currently, the development of the chitosan does not only reach drug delivery, but evidences showed that it can also be used as an adjuvant in immunotherapy. Therefore, chitosan is often combined
with vaccines in the treatment of cancer. Based on research conducted by several researchers such as McNeela et al and L. illum et al demonstrated the ability of chitosan as a promising adjuvant through intranasal vaccination. Several administration routes that are recommended for chitosan-based therapeutic delivery are intraperitoneal (i.p), intravenous (i.v), subcutaneous (s.c), and intratumoral (i.t). On i.p administration, it was found that single dose of chitosan was able to induce humoral immune response, but s.c. was looked more promising due to the activational effect of both humoral and cell-mediated immune response. The same finding was also reported by Zaharoff et al by inducing β-galactosidase, a protein antigen injected in mice and found to increase the antibody titers up to fivefold and increased the proliferation of specific CD4+ T cells antigen in the spleen more than sixfold.

**Anti-EGFR Vaccine in Lung Cancer**

The EGFR signaling pathway mediated by EGF ligands is associated with increased cell proliferation, apoptosis, angiogenesis, and metastasis in lung cancer cells. Thus an anticancer therapeutic vaccine was developed consisting of P64K protein derivative EGF protein Neisseria meningitides and Escherichia coli, which was combined with immunoadjuvant Montanide ISA 51. Cancer vaccines targeting EGF is the third strategy against the EGFR pathway. The first EGFR-based vaccine developed was CIMAvax-EGF which consists of human EGF which was conjugated with P64K carrier protein originating from Meningitis B and Montanide ISA 51 bacteria as adjuvants.

CIMAvax-EGF was reported to be able to induce immune responses against EGFR positive cells and blocking cancer cell proliferation. The anti-EGF vaccine could efficiently reverse the effects of EGF and potentiates the EGFR-TKI antitumor activity, which then inhibits Erk1/2 phosphorylation, increase cell cycle arrest and apoptosis. In addition, the anti-EGF vaccine significantly downregulated AXL and delayed the emergence of resistance to afatinib and gefitinib.

**Adjuvant Cytosine-phosphorothioate-guanine (CpG)**

Cytosine-phosphorothioate-guanine (CpG) containing Oligodeoxynucleotides (CpG-ODN) is a Toll Like Receptor-9 ligand (TLR9). If CpG ODN interacts with TLR-9, the signaling pathway activation will be triggered through the recruitment of the myeloid 88 (MyD88) differentiation factor, IL-1R-associated kinase (IRAK) and Tumor Necrosis Factor Receptor-Associated Factor 6 (TRAF6). This cascade of signaling then leads to the activation of several Mitogen-Activated Kinases (MAPK) and transcription factors (such as NF-κB and AP-1).

CpG-ODN could increase humoral and cellular immune responses (Th1 and CTLs) induced by vaccines against pathogens, allergens, and/or tumors. Clinical trials of CpG-ODN as immunotherapy agents in cancer patients such as melanoma and NSCLC, suggest a combination with chemotherapy or CpG ODN monotherapy could induce a Th1-type immune response, therefore, many scientists consider CpG-ODN as a potential adjuvant for cancer vaccine. Interaction between CpG-ODN and APC will accelerate immune induction, optimizing the efficiency of antigenic presentation by DC and extend the duration of the induced immune response.

Based on differential activation of immune cells, CpG ODN is classified into four classes, namely: (a) CpG-A or type D (b) CpG-B or type K, (c) CpG type C, and (d) CpG-P. In general, CpG type K ODN effectively triggers pDC differentiation and induces secretion of TNF-α and activate B-cells proliferation, IL-6 expression and IgM production. CpG type D ODN is known to induce pDC maturation and interacts with the MyD88 / IRF-7 complex, triggering a signaling cascade that stimulates the expression of interferon alpha (IFNa). On the other hand, CpG class C has the same properties as CpG ODN types K and D, while CpG ODN type P could only activate B cells and pDC as well as induces IFN-α production which is much greater when compared to C-type ODN.

**EGFR-Nanovaccine Pharmacokinetic**

EGFR nanovaccine will reach peak concentration in 24 hours after administration. Its nano-size facilitates the vaccine penetration from injection site to the nearest lymph node. It also facilitate uptake by dendritic cells that will present the antigen to naïve-Th cells via MHC-II. The EGFR neo-epitope within the vaccine is then broken down by the cathepsin enzyme, while at the same time class 2 MHC is also formed and bound by the CLIP protein. Then the MHC class 2 molecule will bind to the EGFR epitope in the class 2 MHC compartment to be carried toward the surface of the dendritic cell and presented to the naïve CD4+ T cell.

Meanwhile, CpG ODN is internalized by B cells and plasmacytoid dendritic cells and stimulates TLR 9 which helps accelerate humoral and specific immune responses. CD4+ T cells will recognize epitope-MHC-II complexes and release several types of cytokines such as IL-4, IL-5, IL-6, and IL-10. Activated CD4+ T cells also interact with

B-cells and then present the EGFR epitope in B cells followed by attachment of the CD40 molecule which induces B cell proliferation. In addition, interaction between CD28 and CD80 molecules will further amplify the anti-cancer immune responses. The secreted IL-4, IL-5, and IL-6 mediate the adaptive immune response by inducing B-cells differentiation into memory B cells and EGF specific antibody producing plasma cells. The remaining epitopes within the vaccine will naturally degraded, while CpG ODN will be metabolized in the liver and kidneys to be excreted.

The Effect of EGFR-Nanovaccine toward Lung Cancer Cells

**Countering Tyrosine Kinase Inhibitor Resistance**

Several antitumor therapies have been developed against epidermal growth factor receptor pathway (EGFR). Tyrosine kinase EGFR inhibitors (TKI) are standard treatment in lung cancer with the EGFR mutation. At present, the availability of vaccination (anti-EGF VacAbs) in the EGFR mutation has the potential to reverse the effects of EGF and potentiate the antitumor activity of EGFR TKI. In addition, the EGFR vaccine can inhibit Erk1/2 phosphorylation and increase cell cycle arrest and apoptosis.

The anti-EGFR vaccine can also block the formation of EGFR-AXL heterodimers and delays the emergence of resistance to afatinib and gefitinib in vitro. Increased serum levels of two EGFR ligands namely TGFα and amphiregulin (ARG) also correlated with a worse response to EGFR TKI. The anti-EGF vaccine consistently suppresses TKI-induced STAT3 activation thereby suppressing resistance to drugs in lung cancer.

**Inducing Dendritic Cells (DC) Maturation**

Antigen presenting cells (APC) play an important role in the tumor immune response and DC is the most efficient APC known today. Functional defects within DC would potentially lead to sub-optimal antigen presentation and inefficient immune response toward tumors. After encountering tumor antigens, the DC will travel through afferent lymph nodes where it will prime the naïve-T cells. Subsequent T-cells activation will follow and the activated T-cells actively migrate through efferent lymph nodes, thoracic ducts, and blood to reach tumor cells.

Dendritic cells consist of mature and immature forms. Immature dendritic cells have an active role in endocytosis and express low co-stimulator surface molecules. When an immature DC captures and processes the antigen, it will become mature and lose the antigen absorption capacity. According to previous research, CpG-ODN act in this phase to enhance the antigen presentation by mature DC. In addition, mature DCs have a very good ability to present Ag peptides to T cells with MHC class II and express co-stimulator molecules on the cell surface. Takahasahi et.al reported that administration of CpG ODN could stimulate cytokine production in mature DCs and, thereby, increasing...
cytotoxic activity of effector cells (CD8+ T-cells and NK-cells). Induction by CPG on mature DC could also increase IL-12 and IL-18 production as indicators to induce Th1 response.56

Anti-Proliferative and Pro-apoptotic Effect
Inhibition of proliferation and induction of tumor cell apoptosis are the most effective way to prevent cell growth and eliminate tumors in vivo and in vitro. Chitosan has prominent antitumor activity with low toxic effects on normal cells. Induction of tumor cell apoptosis can also be triggered by cell cycle arrest. Numerous studies have shown that many antitumor drugs can block the cell cycle at certain check points and thus induce cell apoptosis.57,58 Sub G1 represents the percentage of core tumor cell apoptosis and is used to detect the number of apoptotic cells.59 Chitosan can play a role in the S phase cell cycle which is regulated by Cyclin A and CDK2.60

Chitosan is known to downregulate cyclin A expression as well as phosphatidylserine translocation from the inside to the outer leaflets of plasma membranes which play an important role in the apoptosis.61 In addition, chitosan could also induce cytotoxic via ROS production as shown in A549 cells and inducing DNA damage, genome instability and cell apoptosis.

Chitosan is also involved in the downregulation of Bax and Bcl-2 in A549 lung cancer cells. The Bcl-2 family consists of important regulatory proteins involved in the mitochondrial apoptotic pathway. Members of this family such as Bax and Bcl-2 play important roles in complex interactions to determine cell apoptosis. Bcl-2 inhibits Cyt c release from mitochondria to the cytosol and enhances cell growth, while Bax work by opening the mitochondrial permeability transition (PTP) pores, which cause MMP disruption and, subsequently, Cyt c release. When the Bax/Bcl-2 ratio increases, the protective effect of Bcl-2 on the mitochondrial membrane will be blocked while the permeability of the mitochondrial membrane is increased, allowing Cyt c to enter the cytosol and trigger cell apoptosis.62 Cyt c release combined with cytosolic apoptotic activating factors proteases (Apaf-1) and caspase 9 would lead to cause caspase 3 cleavage and eventually lead to cell death.25

Activation of Anti-Tumor Immunity
EGFR-based vaccines can be used efficiently to target epithelial tumor cells from different locations. EGFR is expressed in many different hematopoietic cell types and its expression is very important for its function. These cell types include macrophages, monocytes, plasma cells and certain T cell subsets such as effector CD4 T cells and FoxP3 that express

Figure 4  Marked cytotoxic effect of CTL induced by CpG induced DC toward WEHI-164 and CT26 cells (A and B). CpG stimulation also enhanced IL-12 and IL-8 production which are the hallmark of Th1 immune response (C and D)56

Figure 5  Increased proportion of CD8+ (A) and CD4+ CD44+ CD62L (B) Lymphocytes in the spleen after stimulation by EGFR vaccine67

Figure 6  Cell cycle arrest induced by chitosan according to the dosage (A) and timing (B). Chitosan primarily affects Cyclin A expression without prominent effect in CDK2 (C). Chitosan also induced apoptosis which possibly the secondary effect of cell cycle arrest (D)25
regulatory CD4 T cells (Treg). In the paracrine loop, intra-tumoral macrophages secrete EGF, which binds to EGFR in tumor cells, enhancing tumor invasion. At the same time, tumor cells secrete colony stimulation factor-1 (CSF-1) which will increase the expression of EGF by macrophages. EGFR expression regulates the production of cytokine macrophages as the main driver of tumorigenesis.

Therefore, blocking the function of EGFR in macrophages using the EGFR vaccine would lead to cytokines supply disruption and decreased tumor growth. Besides macrophages, Treg also plays a role in maintaining immune homeostasis by negatively regulating other types of immune cells. In optimal function, human Treg is dependent on EGFR-mediated intrinsic signaling in Amphiregulin ligand binding (AREG). Treg also plays a role in protecting tumor cells from immune responses mediated by vaccination-induced CD8 + T cells.

**Clinical Effect of EGFR-Nanovaccine in Lung Cancer Cases**

Inhibition of the expression of proteins related to Tyrosine Kinase Inhibitor resistance

Codony et al. showed the cellular effect of anti-EGF antibody to the cells that experience an EGFR mutation. In their experiment, several types of lung cancer cells were used and treated by anti-EGF vaccine, TKI, or their combination. Evaluation after 24-hour incubation showed that EGFR blockage was effectively blocked the phosphorilation of EGFR-associated signaling proteins such as pEGFR, pAKT, and pERK (Figure 3). The same experiment also showed decreased the level of protein that often associated with EGFR-based therapy resistance such as cleaved NOTCH-3, HES-1, Bmi1 and AXL in PC9 cells. The same result was consistently observed in the validation stage using other cell types.

Dendritic Cells Maturation Effects

As stated previously, the crucial step in immune activation by EGFR-nanovaccine is dendritic maturation. This effect is mainly derived from stimulatory nature of CpG as adjuvant mediator. According Yuan et al, CpG could increase the expression of p38, p53, Bax, p21, and decrease the expression of Bcl-2 which indicated that CpG activated p53-MAPK signaling through TLR9 stimulation.

Additionally, adjuvant CpG induced mature DC cytokine production. CpG stimulated DC also showed a considerable specific cytotoxicity level toward WEHI-164 and CT26 cell lines. This finding also consistent with a report from Arab et al. in 2006 that showed significant increase in T lymphocyte cytotoxicity (CTL) (P <0.01) in CpG-induced cells compared to CpG-induced mature DC control. This group also observed increased level of IL-12 and IL -8 as an indicator of Th1 immune activation.

**Anti-Tumor Immune System Induction in Clinical Setting**

Reactivity to EGFR vaccine peptides is very specific, as indicated by a lack of response in unvaccinated animals. An analysis of flow cytometry from cell surface markers in a subset of mice showed differences in the character of CD4+ and CD8+ lymphoid populations in the spleen. In particular, the overall percentage of CD4+ and CD8+ cells in the spleen of vaccinated animals were tend to increase (p = 0.16 and 0.056, respectively; Figure 5A). In the CD4+ population, other changes were also observed. The percentage of CD4+ lymphocytes expressing CD44 and CD62L increased significantly in vaccinated animals compared to controls (p = 0.0093; Figure 5B). In addition, the percentage of CD4+ cells expressing FoxP3 transcription factors was significantly attenuated in vaccinated animals compared to controls (p = 0.006).

Clinical Anti-Proliferative and Apoptotic Effect

Besides acting as an adjuvant, chitosan nanoparticles also has anti-proliferative effect through cell cycle arrest. In in-vitro studies conducted by Zhao Y, chitosan was given in different concentrations of 100, 200 and 400 µg / ml for 2 days (Figure 6A). They observed decreased number of G0/G1 cells from 77.95% to 46.27% (p <0.05), at concentration of 200 µg/ ml given for 24-72 hours (Figure 6B). The results (Figure 6C) showed that chitosan caused inhibition of S phase related to cyclin A downregulation, whereas CDK2 expression was not affected. In addition, chitosan is also capable of inducing apoptosis which can be seen through changes in cell morphology and cell nucleus chromatin condensation that occur on A549 line cells that had been given chitosan (Figure 6D), but not in the control group seen in blue as fluorescence.

**CONCLUSION**

CpG and chitosan based EGFR nanovaccine is a promising new candidate of cancer treatment agent that tackles lung cancer via intra-tumoral (cytotoxic) pathway as well as extra-tumoral factors by inducing anti-tumor immune response and inhibiting angiogenesis. However, further validation studies are needed to confirm the combination effect of those agents as well as its effect in primary tumor cell lines.
CONFLICT OF INTEREST
All authors declared that there is no conflict of interest regarding this publication.

AUTHOR CONTRIBUTION
All authors contributed equally in the writing of this article.

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