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Efficacy of *ex vivo* activated and expanded GMP grade Natural killer cell (NK cell) for patient with over expression of cancer marker CA19.9

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Abstract
Some benefits of tumour markers are screening and diagnosing cancer among patients with high risk, assessing cancer treatment response, as well as identifying cancer recurrence. The significance of natural killer (NK) cells has been emphasised for regulating adaptive and innate immune response. With its function as antigen-nonspecific lymphocytes, NK cells are crucial to detect and eliminate malignant, stressed, and virally-infected cells. This study reports the safety and the efficacy of NK cellsin patients with highly-elevatedCA19-9.Two patients with over expression of cancer marker were administered NK cells intravenously. About 30 ml of peripheral blood was collected from the patients, while NK cells were isolated and expanded for 14 days. On average, 2×10^9cells were harvested and infused to the patients for 30 minutes. The intravenous infusion of the expanded NK cells did not display adverse reaction, but enhanced the patients' health and a substantial decrease was noted in cancer markers. To sum, autologous NK cellsappear to be a viable approach for prevention ofcancer or early-stage cancer.

Key words:*Natural Killer cell; Immunotherapy; Adoptive Immunotherapy CA19.9, Active NK*

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I. INTRODUCTION

Healthy individualsexamine their health condition on regular basis toensure their wellbeing. Cancer prone individuals use tumour markers topredict the risk of early-stage cancer. The most common and the most highly-sensitive tumour marker refers to carbohydrate antigen 19-9 (CA 19-9) with higherspecificity for pancreatic biliary tract tumours, salivary glands, as well as epithelial cells of gastric, colonic, and uterine mucosa (Tong & Song 2013;Galli et al. 2013).

Non-malignant conditions (e.g., diabetes,thyroid, gynaecology complications, &pulmonary diseases) generate high CA 19-9 levels (Kim et al. 2013). Those with high-level CA 19-9 shouldperform abdominal computed tomography (CT) scan to detect malignant ailments (Tong et al. 2013).Although high-level CA 19-9 is accepted among non-cancerous individuals, they seek medical attention due to fear of cancer. Pathogen invasion in our body is countered by adaptive and innate immune cells. Innate immune system, which hasNK cells, epithelial barriers, dendritic cells, and phagocytes, are integral for initiation and subsequent activation of adaptive immune system (Mandal & Viswanathan 2015).

The NK cellsare vital for regulating adaptive and innate immune response. As antigen-nonspecific lymphocytes, NK cellsrecognise and eliminate malignant, stressed, and virally-infected cells (Trinchieri, 1989). Despite their highest level of cytotoxic in the human immune system, adjustment of NK cells functionality or number can strongly affect one's immune system. This notion is relevant for the ageing population as they are more susceptible to neurodegenerative and autoimmune diseases, infections, and cancer (Camous et al. 2012). Notably, physiological ageing is linked with alteration of the function, composition, and phenotype of NK cells

in circulation – also known as NK cell immunesenescence (Solana et al. 1999;Spörri et al. 2006).As low NK cell activity in the elderly can be related to infectious diseases, active NK cells are crucial to maintain one's wellbeing (Castle2000; Ogata et al. 2001). The higher the amount of NK cells, the better the health of cancer patients (Subramani et al. 2013). The two deficient mechanisms suffered mostly by the ageing population, which NK cells offer protection, are (1) secretion of immunoregulatory cytokines and chemokines, as well as (2) direct cytotoxicity. In fact, inactive NK cells among the elderly seemed to increase viral infection severity and incidence; thus signifying the crucial impact of NK cells on maintaining the wellbeing of the ageing segment. Therefore, active NK cells were deployed as a therapeutic tool to minimise high-level CA19.9 in patients above 40 years old.

II. MATERIALS AND METHODS

Case details

The procedures adhered to the ethical standards outlined in Helsinki Declaration. Two patients (male & female) with median age of 41-58 years and high CA19-9 levels were involved in this study.

Isolation activation and expansion of activeNK cells

Upon gaining informed consent from the patients, 30 ml of peripheral blood (PB) was collected after ensuring that their blood parameters and haemoglobinlevel fell within the standard physiological ranges. Thecollected samples were transported within 5 hours to HITV Laboratory Sdn Bhd (cGMP Lab) at ambient temperature and unexposed to X-ray screening. After isolating the lymphocytes from PB mononuclear cells (PBMCs) using lymphoprep density gradient medium (AXIS-SHIELD PoC AS, Oslo, Norway),the isolated PBMCs were centrifuged for 20 minutes at 2000 rpm. Using phosphate buffered saline (PBS), the PBMCs were washed twice. Next, the cell pellet was resuspended in culture media that had IL2 and 10% autologous plasma to yield active NK©, as prescribed in Subramani (2014).

Cell count and Immunophenotyping analysis

Cell expansion was recorded based on quantity by using Trypan blue manual count at different intervals. Next, immunophenotypinganalysis was executed to quantify and quality NK cell population in PB and cultured cells with NK-specific antibodies (CD56 & CD3-) (Beckman Coulter Inc., USA).

Cell infusion

Upon reaching optimal expansion ($2x10^9$)based on the cell population, the cells were harvested via centrifugation and washed thrice using PBS. The cell number yield was measured with Trypan blue dye exclusion test. Next, the cells were resuspended in 100ml of normal saline with 25% human albumin for intravenous infusion on day 14.

III. RESULTS

Patient characteristics and *ex vivo* cell expansion

Table 1 presents the details of initial cell isolation and expansion retrieved from the two patients. The average quantity of PBMCs in 30 ml of PB for Cases I and II is35.2 $x10^6$ ±03 and41.3$x10^6$±02cells, respectively. After 2 hours of culture, microscopic image revealed round shape and non-adherence cell characteristics (see Figure 1a). However, following subsequent culture, days 3, 7,9, and 12 displayed single cells loosely adherent aggregates (Figures1b-e). The maximum cell numbers(active NK©)obtained on day 14 for Cases I and II were 2060.6$x10^6$and 1830.7$x10^6$± 2 cells, respectively. The final harvested cells are as portrayed in Figure 1f.

Immune phenotype characterisation and cell fold change

The pre-and post-expansion immunophenotyping analysis of PBMCs and cultured cells were analysed using CD3- CD56+ markers. As illustrated in Figure 2, a steady increase was noted in the number of NK cell population after expansion. With near similar number of PBMCs seeded on day 0, the cells exhibited expansion throughout the 14-day period. After harvesting the cells, they were counted using Trypan blue exclusion technique.The average cell fold had been 58.8 ± 3 and 44.6±4 for Cases I and II, respectively (see Figure 3).

Effects of active NK©

Finished Product Quality Control (FPQC) test was performed to confirm the asepsis of the products. Thus, the expanded active NK© immune cells (50ml of cells in normal saline) were transfused into the patients after confirmation ofFPQC via intravenous drip for 45-60 minutes.Afterinfusing active NK© cells, the CA19-9 level decreased considerably from 89.9 to 51.5 U/ml in Case I, while 46.4 to33.1 U/mlfor Case -II (see Figure 4) within 3 months.

Characteristic	**Case I**	**Case 2**
Age (year)	41	58
Gender	F	M
PB qty (ml)	30	30
Average of PBMNC (10^6)	35	41
Max cell count (NK) (10^6)	2060	1830
Initial CD3-CD56+ %	22.1	15.6
Max CD3-CD56+ %	98.0	97.3

Table I. Characteristics of patients and *ex vivo* cell expansion

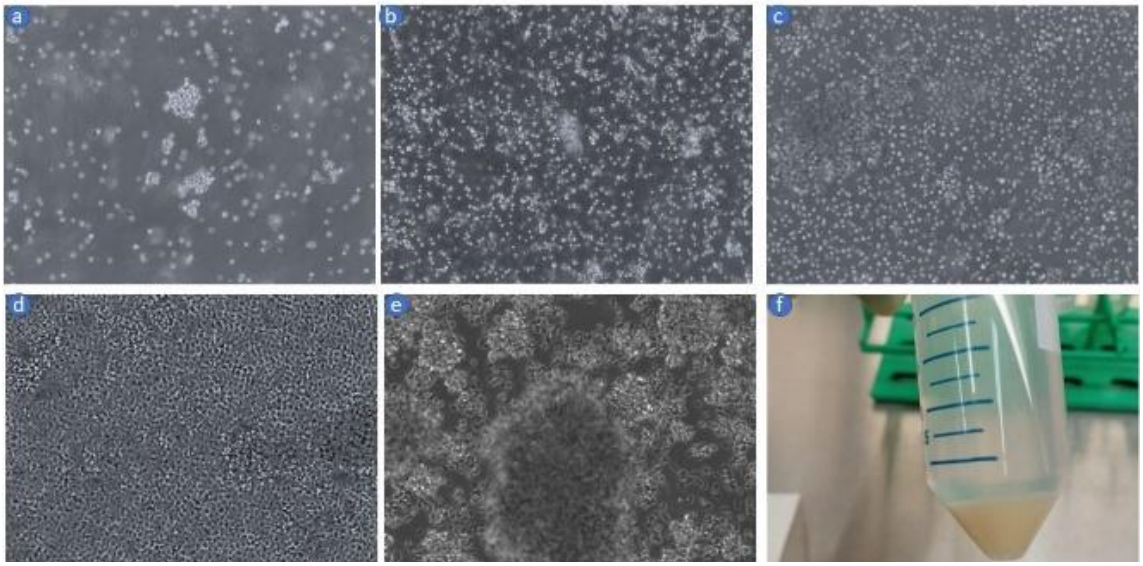

Figure 1. Active NK cell culture. Morphology of cell population at day (a) 0, (b) 3, (c) 7, (d) 9, (e) 12, and (f) 14 of harvest for Case I. Images were set at phase contrast microscope at 100× magnification. Note: Scale bar = 100 μm

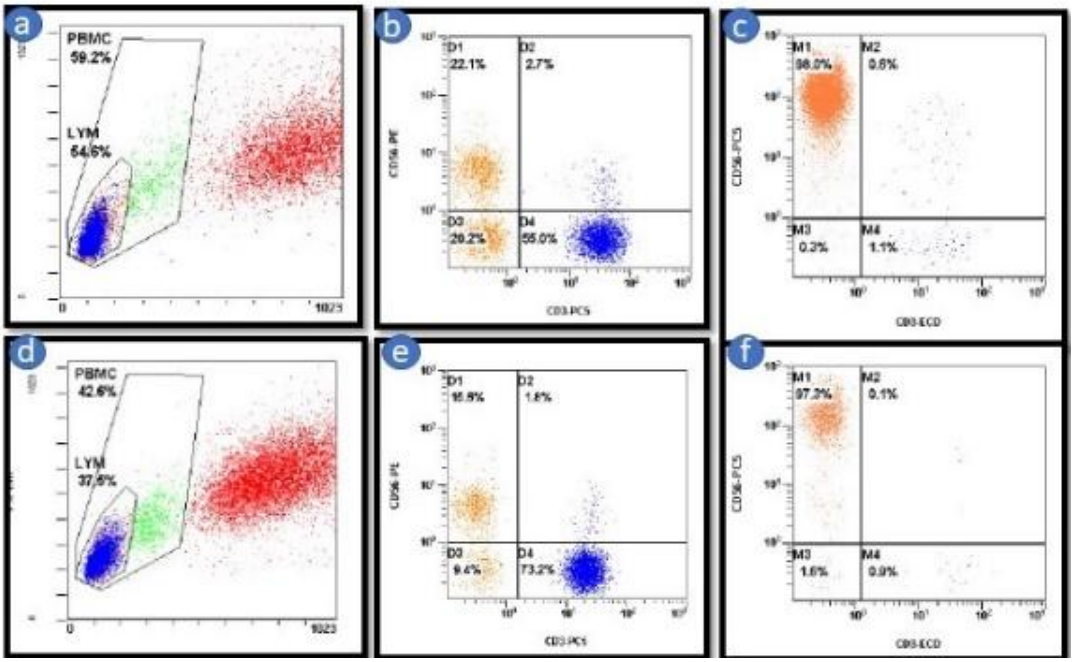

Figure 2. Characterisation of active NK derived from peripheral bloodflow cytometry analysis displaying the immunophenotype of NK cell before and after expansion for Case I (a-c) and Case II (d-f)

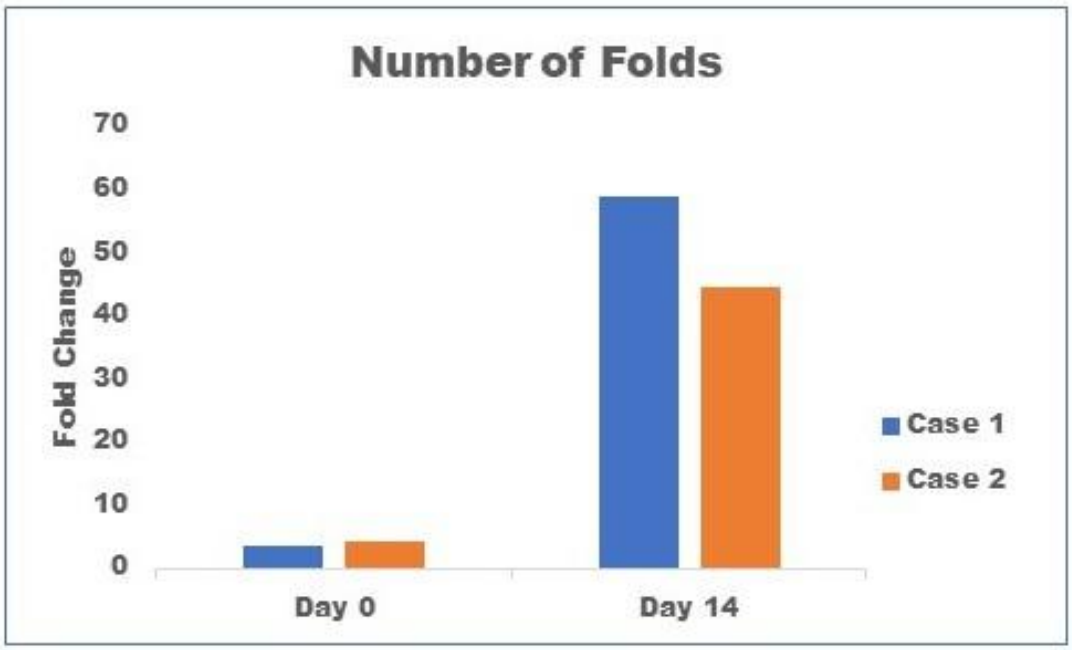

Figure 3. NK cell growth before and after expansion. Cell fold significantly increased in both cases after 14 days of culture.

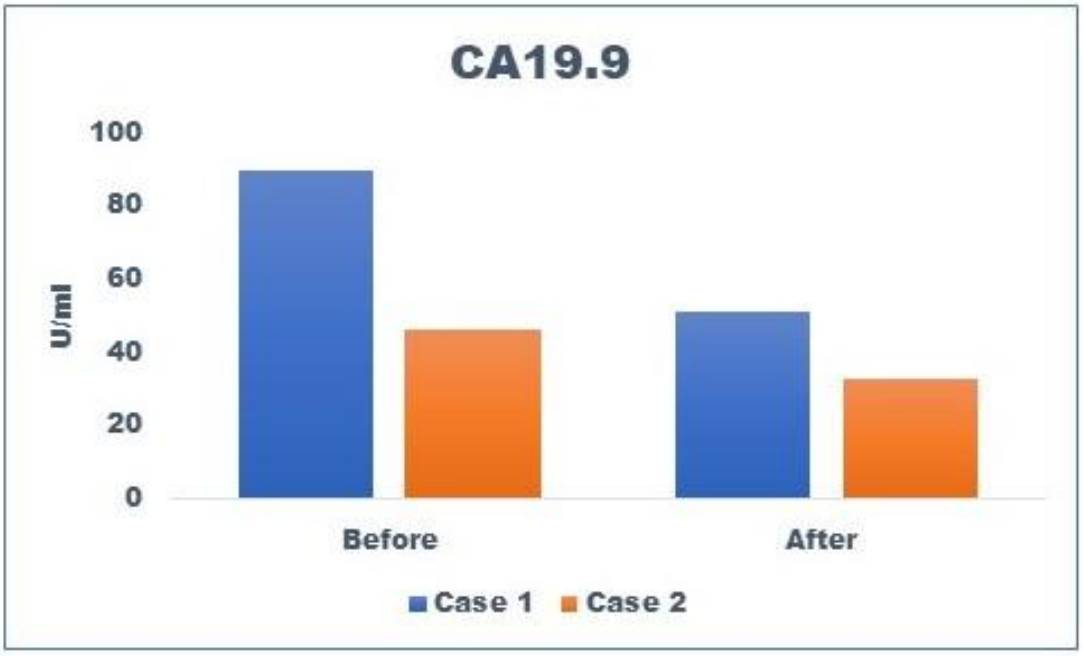

Figure 4. Comparison of CA19.9 levels before and after active NK cells

IV. DISCUSSION

This is the first study to have used active NK cellsin patients with high-level CA19-9. In this study, the safety and efficacy of *ex vivo* activated and expanded activeNK cells from patients' PB were assessed. Apparently, healthy individuals with low cytotoxic activity face higher cancer riskthan those with medium and high cytotoxic activity (Imai et al. 2000). On the contrary, other studies asserted that NK cells generated better cytotoxicity in healthy individuals (Sansoni et al. 1993; Kirkwood et al. 1992). Turning to this study, total lymphocyte count deteriorated with age (see Figs2a &2d). Similarly, Chidrawaret al.(2006) proved thedeclinein lymphocytes as one aged.

Standard optimised protocol was deployed in this study to expand active NK cells in order to yield the desired quantity within 14days. As a result, 60- and 45-fold had been recorded in Cases I and II, respectively.

Although the outcomes are similar to a prior study that reported reduction in proliferation ability upon ageing (Gounder et al. 2019), Terunuma et al. (2008)had proven that *ex vivo* expanded NK cells by 5- to 10-fold increment in cytotoxic ability, in comparison to purified blood cells without expansion.Besides, NK cells can improve the cytotoxic impact on cellular immune responses (Manjunath et al. 2012;Subramani et al 2014). Turning to this study, the use of *ex vivo* expanded NK cells was safe; signifying the potential immune reaction to deal with any malignant cell by supporting the viable first line of defence. Repeated follow-ups with the patients verified the wellbeing of the patients with better quality of life.Various other reports also (see Subramani et al. 2014) provide consent on the safe administration of *ex vivo* expanded NKcells. Substantial reduction in CA19-9 level indicated the use of NK cells as a therapeutic alternative for cancer patients or those prone to succumb to cancer after health check-up routine(Fig 4).

Further follow-ups on bigger cancer patient population for a lengthier duration may lead to an effective therapeutic alternative or routine treatment options for cancer patients to lead a cancer-free life.

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