

# The Comparison of Exosome RNA Concentration and its LMP-1 and p53 content Among Patients with Nasopharyngeal Cancer before and after Chemoradiation

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## Research note

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# Abstract

## Objective

Nasopharyngeal cancer (NPC) remains the highest head and neck malignancy in Asia, especially in Southeast Asia. The exosome is a nanocarrier that has a role in inhibit or promote the progression of cancer as well as the marker to knows the response of therapy depending on its carrier inside. NPC-exosome carriers including LMP-1 and p53. LMP-1 is a main oncogene of Epstein Barr Virus (EBV) the predominant cause of NPC. Wild type p53 known as the main inhibitor of cancer growth. The study aims to compare the concentration of exosomes and the characteristic of LMP-1 and p53 inside exosomes among patients with NPC

## Result

Eight patients fulfilled the study criteria. The mean age was 37,5 years old (SD = 9,9), most of them were stage III. The mean exosome RNA concentration before and after chemoradiation was 14,39 and 3,61 ng/ml with a p-value of 0,04. LMP-1 expression was documented inside the exosome. Expression of p53 in exon 4-5 and 5-6 are express well, except on exon 7-8. Furthermore, there was a significant increase of p53 expression relative to the housekeeping gene in exon 4-5 and 5-6 after chemoradiation with a p-value of 0,001 and 0,002 consecutively.

# Introduction

The exosome is a small vesicle produced by the cell through the enocytic pathway, where the material endocytosis is sending to degradation after fusion with lysosome or entering the secretory pathway through the multi-vesicle apparatus (MVA). The exosome will release by MVA and going to fusion with the plasma membrane.[1],[2] Normally, the exosome has a role in cell-cell communication with several genetic materials inside.

NPC remains the highest head and neck malignancy in Asia, especially in Southeast Asia. Its incidence varies between 6,2-12 per 100.000 population in Asia. The progression of this cancer is strongly correlated to the infection of EBV. The major oncogene of EBV is latent membrane protein-1 (LMP-1).[3]

In a cancer cell, wild-type (WT) p53 plays a role to inhibit the progression of cell growth through activation of the apoptosis pathway. WT-p53 managing the important process of cell cycle arrest, DNA repair, apoptosis, and senescence.[4] However, in cancer cases, several WT-p53 was mutated which promotes cancer growth. The majority of this mutation is a missense of mutant protein with the complete length.[5]

Exosomes in cancer could participate in the interchanges of genetic material between tumor cells and stroma. As a result, it will promote angiogenesis and lead to growth, migration, invasion, and distance metastasis.[6]

Since the exosome as a transporter can carry genetic material to the other cell, it is important to explore the characteristics and content of exosomes before and after chemoradiation. There is a possibility to use exosomes as a marker to develop diagnostic tools or monitoring the therapy response, and estimate the prognosis in the future. In advance, there is a potency to insert genetic material such as WT-p53 to exosome to influence the result of therapy since WT-p53 is a main apoptotic cytokine that inhibits tumor growth. The study aims to compare the exosome concentration and its content especially LMP-1 and p53 before and after chemoradiation among patients with NPC.

## Methods

This study was prospective, observational, before-after treatment.

### *Setting and recruitment*

The subject was recruited consecutively, voluntarily, and should include the criteria NPC stage III or IV according to AJCC 2018, more than 14 years old, and Karnofsky index more than 70. The subject was recruited at the Wahidin Sudirohusodo General Hospital, Makassar, and the West Nusa Tenggara General Hospital, Mataram, Indonesia from January to October 2020.

Pre-treatment data on staging and clinical characteristics were collected. The concentration of exosome RNA, expression of LMP-1, and p53 pre-post chemoradiation were documented. Clinical therapy response post-treatment was evaluated with the head CT-Scan. Good response if there was no mass either in nasopharynx or neck nodes. Fair response if there was progress but remains found a mass on nasopharynx and/or neck. No response if the CT-Scan was similar to the initial condition. The treatment consists of induction 2 sequential chemotherapy with cisplatin and paclitaxel, continued by 7,000 centi-Gray IMRT.

### *Data collection tools*

Serum was taken from the forearm vein, then centrifuge with 3000 rpm for 5 minutes, then stores at  $-80^{\circ}\text{C}$ . The isolation of the exosome was done with Seramir Exoquick™ by following the manual instruction. The concentration of exosome RNA was obtained by nano drops (Thermoscientific™) and expression of LMP-1 and p53 were obtain using RT-PCR with SensiFAST SYBR No-ROX kit.

The primer for LMP-1 consist of forward 5'-CAGTCAGGCAAGCCTATGA-3' and reverse 5'-CTGGTTCCGGTGGAGATGA-3'. The primer for p53 consists of 2 different primers on exon 4-5, and 5-6 sequentially as follows forward and reverse 5'-GCCATCTACAAGCAGTCACAG-3' and 5'-TCATCCAAATACTCCACACGC-3', 5'-GAAGGAAATTTGCGTGTGGAG-3' and 5'-AGTGTGATGATGGTGAGGATG-3'.

### *Statistical analysis*

The different mean of exosome concentration and folding difference of gene expression ( $2^{-\Delta\Delta CT}$ ) of LMP-1 and p53 relative to a housekeeping gene (GAPDH) will analyze using pair t-test or Wilcoxon rank test depend on the distribution of the data with the significance level less than 0,05. Folding difference gene will be calculated by 3 sequential steps according to Fu et al.,(2006).[7]

## Results

Eight patients were fulfilled in this study, 6 males and 2 females. Most of the subjects were stage III. According to the Karnofsky index, most subjects were physically excellent. The response therapy sequentially good, fair, and no response were 5 (62,5%), 3 (37,5%), and (0 %) (Table 1).

The mean exosome RNA concentration before and after treatment was 14,39 and 3,61 ng/ml (figure 1). According to the paired t-test, the concentration of exosome RNA was significantly decreased post-treatment with a p-value of 0,04. The exosome concentration between the clinical characteristics was shown in table 1.

The mean LMP-1 CT value expression before treatment was 34,29 and increase to 35,74 after chemoradiation. According to the Wilcoxon rank test, the increase of LMP-1 expression was not significant ( $p=0,263$ ). In advance, with the folding LMP-1 gene expression relative to GAPDH (mean CT value before and after chemoradiation were 27,08 and 28,72) found that there was a significant reduction of LMP-1 gene expression with the mean 2,71 to 0,25 with  $p=0,036$  (figure 2A).

The mean p53 CT value expression in exon 4-5 before treatment was 26,84 and increases to 27,17 after treatment. On the other hand, on exon 5-6, the mean CT value was decreased from 30,15 to 29,98. Both differences were not significant. According to p53 folding gene expression relative to GAPDH found that there was a significant increase either in exon 4-5 and 5-6 with gene expression consecutively from 6,09 and 1,43 to 28,98 and 5,05 with p-value 0,001 and 0,002 (figure 2B-C).

## Discussion

Chemotherapy and radiotherapy are the main treatment modalities for combating NPC. Both will trigger the oxidative stress in the cell; hence, cell apoptosis occurs, especially on the cancer cells. Cisplatin-based chemotherapy is a drug of choice for HNC. The action mainly through breaking the DNA by attacking mitochondria and triggering the oxidative stress production, breaking the lysosome and reticulum endoplasm.[8]

There are several pathways activated by ionic radiation such as DNA damage, altering the gene expression, mutation, cell cycle arrested, and finally cell apoptosis.[9] The other effect of ionic radiation is the cell characteristics changes. An experimental study on HNC cell lines with radiotherapy found that the characteristics of exosomes and their content were changes. Radiotherapy could increase the number and uptake of exosomes and activate the AKT signalling pathways.[10]

The response to 2 series of chemotherapy and 7000 centi-Gray radiotherapies in our study showed that most of the subjects were a good response. This result was interesting and proofed that radiotherapy was predominant in NPC management. However, in this study, all subjects were advised to continue their chemotherapy for six series after completing the radiotherapy as the National Board of Cancer Management recommendation.[11]

Exosomes are known to have a role in the progression of several diseases, depend on the material genetic that carried by exosome itself. For instance, exosomes can alter cancer and are related to the severe infection including covid-19.[12] The exosome is similar to a double-side sharp knife which could give a benefit on one side but the other could harmful the cells. Bach et al.,(2017) state that exosome could generate resistance to chemotherapy through the production of P-glycoprotein that causes the chemotherapy efflux from the cells. On the other hand, the exosomal-microRNA was acting conversely. [13] Micro RNA 34c-5p excreted by mesenchymal stem cell exosome could attenuate the NPC malignant behavior and reverse the radioresistance.[14]

Studies on the exosome concentration after chemoradiation in a human subject is limited. A study by Theodoraki et al.,(2019) found that the concentration of exosomes decreases significantly in 5 patients with a good response on the HNC who treated with cetuximab, ipilimumab, and IMRT. On the other hand, on the recurrent disease, the concentration was increased significantly.[15]

The research on HNC cell lines with the electron microscope found an increased number of exosomes after 6 Gray irradiation. However, if the radiotherapy increased to 9 Gray, the result was conversely. Additionally, the uptake of exosomes on radiotherapy was time-dependent.[16] According to the above studies, our result supports the hypothesis that exosome with EBV content will reduce after chemoradiation and its expression related to the therapy response.

Since the knowledge of EBV is one of the main causes of NPC, several studies explored the characteristics of EBV, including its LMP-1. On exosome released by EBV-NPC cells was well known carried the LMP-1.[17] In the present research, the presence of LMP-1 inside exosome was expressed and the relative LMP-1 gene expression showed a significant reduction after chemoradiation. LMP-1 could enter the exosome through C-terminal farnesylation from UHC-L1. The methods for blocking this pathway were found, hence, could be used as an alternative NPC treatment in the future.[18] The presence of LMP-1 could induced oxidative stress and lead to EBV reactivation and affect the radioresistant of NPC.[19]

Another study found that LMP-1 inside exosomes could increase the risk of radioresistant among patients with NPC through activation of P38 MAPK signalling pathways.[20] Hence, the presence of LMP-1 inside the exosome could be a predictor of NPC prognosis. Based on our result, the expression of LMP-1 inside the exosome was low. This result indicated that the prognosis in this study was good. Moreover, according to comparative analysis LMP-1 expression relative to GAPDH before and after chemoradiation, found a significant reduction after chemoradiation. The result was coherent with a good clinical response. Additionally, this result supports the hypothesis of LMP-1 as a predictor prognosis.

The p53 protein is well known for apoptotic cell regulation and controlled tumor growth. The folding gene expression of p53 found a significant increase in exon 4-5 and 5-6. Logically, p53 will increase and promote apoptosis after chemoradiation. According to Liu et al.,(2019), the most mutation location of the p53 were exon 5 (26%). The mutation of p53 was a significant prognostic factor on NPC survival.[21] The other study reported, a positive mutant p53 was associated with a low survival rate.[22] Meanwhile, radiation will activate the suppressor tumor protein, especially p53 which will induce apoptosis, cell cycle arrest, and maintain the DNA repair.[9]

Reported that p53 could be transferred through exosomes, and it could alter the physiology of recipient cells and lead to the repression of cell growth and proliferation.[24] Exosome, through several in vivo and in vitro studies, could transfer the drugs such as curcumin, paclitaxel, and doxorubicin as an anti-cancer metabolite. In advanced, exosomes could transfer microRNA-155 which could depress the cancer growth with a minimal side effect.[25]

Conventionally, response therapy monitoring was using a clinical examination, imaging, and histologic appearance.[26] Recently, exosomes could be utilized as a biomarker for these purposes. According to Konig et al.,(2018), if the exosome concentration was increased in neoadjuvant chemotherapy on breast cancer, it refers to a poorer prognosis.[27] In the present study, the exosome concentration decreased significantly and correlated to clinical response.

Several studies showed that exosomes have a role as a non-invasive diagnosis biomarker and prognosis prediction. Zhou et al.,(2018) explained that inside NPC exosome was found to express the EBV-BART-miRNA which has a role in tumorigenesis; hence, it could be used to determine the diagnosis and predict the prognosis.[6]

The exosomal-p53 derived from brain glioblastoma cancer cells has a similar expression on tumor and blood. This result indicated that p53 inside exosome which is taken from blood has potency as a marker on the non-invasive diagnosis of brain glioblastoma cancer.[28] Liu et al.,(2019) proposed the cyclophilin A exosome, a family of immunophilin, as a biomarker on EBV related NPC.[23] Regarding the present study, there is a potency to developed p53 related exosome as a biomarker on NPC diagnosis.

## **Limitation**

The present study uses a limited number of subjects, distributed un-normally, and the non-complete chemotherapy series. Due to those limitations, several advanced research could be done with larger sample size, matching variables, and complete chemoradiation treatment. On the other hand, with several significant findings on exosome role in NPC patients with chemoradiation, there are opportunities to develop advanced research on exosomes intervention as the future strategy on NPC management.

## **Abbreviations**

AJCC (American Joint Committee on Cancer), EBV (Epstein Barr Virus), NPC (nasopharyngeal carcinoma), LMP-1 (lethal membrane protein-1), GADPH (Glyceraldehyde 3-phosphate dehydrogenase), IMRT (Intensity Modulated Radiotherapy), AKT (Protein Kinase B), DNA (Deoxy-ribonucleic acid), RNA (Ribonucleic acid), HNC (head and neck cancer) UCH-L1 (Ubiquitin carboxy-terminal hydrolase-L1), WT (wild-type).

## Declarations

**Ethics approval and consent to participate:** This research was approved by the Hasanuddin University ethical board no 53/UN4.6.4.5.31/PP36/2020. Participants or parents/guardians (if the age under 17 years old) sign a research consent form.

**Consent to publish:** Not Applicable.

**Availability of data and material:** All data and materials were stored at the Centre for Bioscience and Biotechnology, Mataram University.

**Competing of interest:** Authors state that they have no competing of interest.

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**Author contribution:** HK prepare the research project; HK, ESP, KA prepare and running the laboratory testing; HK, NALP collecting the subject data; NALP, MG, AQP critical review the manuscript; all authors were read and approved the final manuscript.

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## Tables

**Table 1. Characteristics of the subject with mean exosome concentration**

Variable	Subject			Mean exosome concentration			
	N	%	Normality test	Pre-treatment	P-value (Anova)	Post-treatment	P-value (Anova)
Age (years)							
<41	5	62,5	0,004	10,74	0,02	3,64	0,77
41-50	2	25		16,35		4,05	
51-60	1	12,5		28,7		2,6	
>60	0	0		0		0	
Gender							
Male	6	79	0,001	13,18	0,45	3,7	0,69
Female	2	21		18,00		3,35	
Stage							
III	7	87,5	0,001	15,1	0,43	3,77	0,27
IV A	1	12,5		9,4		2,5	
IV B	0	0		0		0	
Histopathology							
Undifferentiated SCC	7	87,5	0,001	15,1	0,43	3,77	0,27
Moderate SCC	1	12,5		9,4		2,5	
Well diff SCC	0	0		0		0	
Karnofsky index							
Normal (100)	0	0	0,001	0	0,12	0	0,63
Normal with mild symptoms (90)	4	50		10,36		3,53	
Normal activity with the effort and several symptoms (80)	4	50		18,45		3,7	
Response to therapy							
Good response	5	62,5	0,001	12,36	0,37	3,94	0,25
Fair response	3	37,5		17,77		3,07	
No response	0	0		0		0	