RESEARCH ARTICLE

Comparison of The Means of Argyrophilic Nucleolar Organizer Region (mAgNOR) Pre- and Post-Therapy in Nasopharyngeal Carcinoma Patients at Wahidin Sudirohusodo General Hospital Makassar

Freddy George Kuhuwael, Muhammad Fadjar Perkasa, Upik Anderiani Miskad, Abdul Qadar Punagi, Fatmawati Arsyad Said*

Department of Otolaryngology, Faculty of Medicine, Hasanuddin University, Jl. Perintis Kemerdekaan Km.10, Makassar, Indonesia

*Corresponding author. E-mail: fatmaummugaza@gmail.com

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Abstract

BACKGROUND: Nasopharyngeal carcinoma (NPC) is malignant tumor growing in nasopharynx with a predilection in fossa Rossenmuller and nasopharyngeal roof. This research aimed to prove whether the means of argyrophilic nucleolar organizer region (mAgNOR) can predict the success of treatment in nasopharyngeal carcinoma patients.

METHODS: We used diagnostic test method with longitudinal design and purposive sampling technique. Endoscopic biopsy examination was performed on 15 nasopharyngeal carcinoma patients before and after therapy, 13 patients underwent chemotherapy and other two underwent chemoradiotherapy. Tumor tissues were stained and AgNOR was calculated.

RESULTS: Based on the tumor stage, sample characteristic showed 3 patients (20%) were in stage II, 3 patients (20%) in stage III, and 9 patients (60%) in stage IV, with pre- and post-therapy mAgNOR were 1.610±0.988 and 1.000±0.000, respectively in stage II, 1.100±0.092 and 1.000±0.000, respectively in stage III, 1.226±0.265 and 1.107±0.164, respectively in stage IV patients. Based on histopathology type, 4 patients (26.7%) had non keratinizing squamous cell carcinoma with pre- and post-therapy mAgNOR were 1.117±0.134 and 1.060±0.120, respectively, while 11 patients (73.3%) had undifferentiated squamous cell carcinoma with pre- and post-therapy mAgNOR were 1.335±0.528 and 1.065±0.146, respectively. Overall the pre-therapy were significantly higher than post-therapy mAgNOR. In subgroups there are significant differences in stage IV and type 3.

CONCLUSION: The values of AgNOR were decreased in all NPC stages and significantly decreased in undifferentiated squamous cell carcinoma. AgNOR can be used to predict the successfulness of therapy in NPC.

KEYWORDS: nasopharyngeal carcinoma, therapy, proliferation, mAgNOR

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Introduction

Nasopharyngeal carcinoma (NPC) is a nonlymphomatous squamous cell carcinoma that occurs in the epithelial lining of the nasopharynx. This neoplasm shows varying degrees of differentiation and is frequently seen in pharyngeal recess (Rossenmuller's fossa), posteromedial to the medial crura of the Eustachian tube opening in the nasopharynx.(1,2)

Epidemiologically of NPC indicated, the highest rate was found in the Province of South China which are 40-50 cases among 100,000 residents. In Indonesia, NPC ranks 4th after cancer of uterus, breast and skin, with an incidence of 4.7 per 100,000 population. In Makassar, South Sulawesi Province, Kuhuwael reported in Dadi Hospital and Dr. Wahidin Sudirohusodo General Hospital over a period of 10 years (1990-1999), there were 274 (47.98%) cases of the malignant tumors over head and neck with a comparison

between men and women was 2.6:1. Bastiana reported in Dr.Wahidin Sudirohusodo General Hospital within the period of 10 years (2000-2009), there were 362 cases (57.28%) of the malignant tumors over head and neck.(3,4)

Radiotherapy has been the primary treatment for NPC for years. This is because the nasopharynx adjacent to critical structures and properties of NPC infiltration, resulting in surgery of the primary tumor has been difficult. NPC is generally inoperable, more responsive to radiotherapy and chemotherapy than other head and neck malignant tumors.(5,6)

Management of an advanced NPC stage is chemotherapy combined with radiotherapy. Chemotherapy is usually used in cases of recurrent or metastatic NPCs. The working mechanism functions as an anti-metabolite chemotherapy, disrupting the structure and function of DNA and as mitotic inhibitor. Antimetabolites act by inhibiting the biosynthesis of purine or pyrimidine, thus changing the structure of DNA and holding cell replication.(7)

Cell proliferation can be studied by destroying tissues such as "flowcytometry" method or by maintaining the tissues such as labeling with a radioisotope, Ki-67, proliferating cell nuclear antigen (PCNA) and staining argyrophilic nucleolar organizer region (AgNOR) techniques. The advantage to study cell proliferation without destroying the tissues is the relationship between subpopulations of cells in the tumor tissues is retained.(8)

AgNOR is a proliferation assessment by calculating the "nucleolar organizer regions" (NOR) which is the curve ribosomal DNA (rDNA) transcribed into ribosomal RNA (rRNA) by polymerase assistance. NOR is located in the short arm of acrocentric chromosome (number 13, 14, 15, 21 and 22) in human and ultra-structurally seen to associate with fibrils in the phase of interphase. NOR contains genes of 18s and 28s rRNAs in shape, which is essential for protein synthesis.(8,9) Neoplastic changes at the nucleus that is the size of enlarged, irregular shape, marginality to the peripheral location of the nucleus. Neoplastic cell transformation characterized by an increase in protein synthesis. Thus the increase in the number of proteins associated with NORs describe the increase in the transcription of rRNA. These figures reflect an increase in cell proliferation activity. AgNOR staining technique is a technique in which the silver colloidal silver staining will bind to form a compound NOR-associated proteins (NORAP) and AgNOR (silver deposition). By AgNOR specks of light microscopy based on the nature argilliferous visualized form of grains of black in the nucleus.(10,11)

The relationship between radiosensitivity and cell proliferation is complex and the pattern depends on the phase and the cell cycle, mitosis frequency and the period of interphase. High frequency mitotic cells (short interphase phase) has a shorter recovery period prior the next mitosis phase after radiation exposure. Likewise high proliferating tumor cells are generally more radiosensitive than those with low proliferation rate, as they are more susceptible to die due to radiation effects. Other factors that influence the pattern are histological event, growth fractions and spontaneous dead tumor cells.(9)

Heber, *et al*., reported that mean differences between the AgNOR of pre-radiotherapy and after the first fraction of radiotherapy showed a positive correlation, healing the cancer within one year post-radiotherapy. A similar result was reported by Irawan showing that there were differences between proliferative activity (counted AgNOR) of pre- and post-therapy in patients with advanced stage carcinoma of cervix uteri in which the proliferative activity of pre-therapy is higher than those post-therapy.(12,13)

Methods

The method used in this experiment was diagnostic test with longitudinal design. The research was conducted in Otolaryngology and Anatomy Pathology Department of Dr. Wahidin Sudirohusodo General Hospital in cooperation with the Anatomy Pathology Department of Cipto Mangunkusumo General Hospital Jakarta, from November 2014 to May 2015 who signed the post-informed consent form to participate. Approval was released by the ethics committee of human protection in biomedical research, Medical Faculty of Hasanuddin University number 02271/H4.8.4.5.31/PP36-KOMETIK/2014.

Sampling was conducted by purposive sampling technique. Inclusion criteria were patients with NPC based on the results of histopathological examination included in the study that were willing to sign statement to participate in the study after receiving an explanation (informed consent). Exclusion criteria were patients with another primary tumor. The examination of endoscopic biopsy was performed on 15 nasopharynx carcinoma patients before and after therapy, 13 samples underwent chemotherapy and the other 2 samples underwent chemoradiotherapy. The silver-staining of NORs was applied to 30 paraffin sections of NPC biopsy specimen.

AgNORs Staining Procedure

Histological sections were stained with haematoxylin and eosin. Also, AgNORs by method of Crocker, *et al*., by taking 4µ thick sections were made for each case, deparaffinize in Xylol, hydrate through graded alcohol to water, incubate the sections in freshly prepared working solution i.e., silver colloid developer (containing one part by volume of 2% gelatin in 1% formic acid and two parts by volume of 50% aqueous silver nitrate solution) in a closed coplin jar for 30 to 45 minutes in dark room at room temperature. This silver colloidal solution was washed with three changes of deionized water for 2 minutes each and blot the section, and dehydrate through ascending grade of alcohol cleared in xylene mounted.(14)

AgNORs Count

For counting, most cellular representative areas were elected. In each case approx 100 nuclei were examined under X 100 oil immersion lens. Multiple regions were examined in each section by selecting microscopic fields at random and by taking 100 cells into account, the means of AgNOR (mAgNOR) per cell is then calculated simultaneously. We also observe the morphology of NOR dots to avoid biased results. The counts were done by two different observers. AgNORs dots are intranuclear black to brownish black against pale yellow to golden yellow nuclear background.

Statistical Analysis

The statistical analysis was done using Wilcoxon test and the statistical significance was set at $p<0.05$.

Results

We performed this study on 15 NPC patients, 13 of them had underwent chemotherapy and the other 2 patients had underwent chemoradiotherapy. General distribution of the subjects is shown in Table 1.

Table 2 shows mAgNOR pre- and post-therapy in NPC patients. There is significant difference between the mAgNOR pre- and post-therapy based on statistical test (p=0.002).

Table 3 shows the mAgNOR pre- and post-therapy in NPC patients based on the stage of tumor. There is a significant difference between the means in stage IV with p=0.018, but no significant difference between the means in stage II and III.

Table 4 shows the mAgNOR pre- and post-therapy in NPC patients based on the histopathology type. The difference between the means in the WHO type 3 was significant with p=0.008, but no significant difference in WHO type 2 with p=0.102.

Table 1. Distribution of the samples by age, gender, NPC stage and histopathology type.

Variable	Subjects (n=15)	
	n	%
Age		
31-40 years	5	33.3
41-50 years	4	26.7
51-60 years	5	33.3
≥60 years	1	6.7
Gender		
Men	14	93.3
Women	1	6.7
NPC stage		
Stage II	3	20.0
Stage III	3	20.0
Stage IV	9	60.0
Histopathology type		
WHO type 1	4	26.7
WHO type 2	11	73.3

Discussion

In this study, the distribution of samples by gender were 14 (93.3%) females and 1 (6.7%) male. The ratio between female and male patients is in contrast with the literature which is 2.6:1 in ratio, caused by the analyzed samples are only those that meets the selection criterias, not all patients who came in that mean time.(15)

Based on the age, the subjects was included into two groups: 31-40 years old (33%) and 51-60 years old (33%). The youngest is 35 years and the oldest is 65 years.

There are three factors influencing occurrence of the malignancies which are individual, environment, and agents. Epstein Barr virus is an agent associated with the occurrence of nasopharyngeal carcinoma, therefore the infection may occur at youngs. Ebstein Barr virus infection, led to the transformation of individual DNA, whose expression is influenced by other factors, *i.e.,* environment and host, so that nasopharyngeal carcinoma is also found in the aged.

Table 2. The mAgNOR pre- and post-therapy in NPC patients.

mAgNOR of NPC patient	n	Median (Min-Max)	Mean±SD	*p*
Pre-therapy	15	1.00-2.75	1.277±0.461	0.002
Post-therapy	15	1.00-1.48	1.064±0.136	

Table 3. mAgNOR pre- and post-therapy in NPC patients based on the stage of tumor.

Stage	mAgNOR value	n	Min-Max	Mean±SD	*p*
Stage II	Pre-therapy	3	1.00-2.75	1.610±0.988	0.18
	Post-therapy	3	1.00-1.00	1.000±0.000	
Stage III	Pre-therapy	3	1.02-1.20	1.100±0.092	0.109
	Post-therapy	3	1.00-1.00	1.000±0.000	
Stage IV	Pre-therapy	9	1.00-1.66	1.226±0.265	0.018*
	Post-therapy	9	1.00-1.48	1.107±0.164	

Table 4. The mAgNOR pre- and post- therapy in NPC patients based on histopathology type.

Histopathology Type	mAgNOR value	n	Min-Max	Mean±SD	*p*
WHO Type 2	Pre-therapy	4	1.00-1.31	1.117±0.134	0.102
	Post-therapy	4	1.00-1.24	1.060±0.120	
WHO Type 3	Pre-therapy	11	1.00-2.75	1.335±0.528	0.008*
	Post-therapy	11	1.00-1.48	1.065±0.146	

Mostly, the NPC patients were in the IV stage, which are 9 or 60%, similar with the previous research conducted at Dr. Wahidin Sudirohusodo General Hospital.(16) Another previous study conducted by Jeane and Kuhuwael in Dr. Wahidin Sudirohusodo General Hospital also found the same, where the majority of NPC patients was stage IV (56%).(17)

Based on the histopathology type, the majority found was undifferentiated carcinoma cells squamous (WHO type 3) which is 73.3%. It is similar with the findings by Jeane and Kuhuwael reported that WHO type 3 was the highest about 70%, Savitri found 80.9% of the same type.(17,18) Based on the literature the distribution of histopathology in South China is WHO type 1 (2%), WHO type 2 (3%) and the WHO type 3 (95%). While in North America, WHO type 1 (25%), WHO type 2 (12%) and the WHO type 3 (63%). The WHO type 3 is frequently found in endemic areas of NPC.(19)

This study showed that mAgNOR pre-therapy generally higher than post-therapy. There are decreases in the mAgNOR after treatment of the patients, and proven to be statistically significant. The high mAgNOR pre-therapy marks an increase in activity of the malignant cells proliferation. This is similar to the previous study in Malaysia Mohan and Hayati, reported that the mAgNOR past-therapy was lower, as respons on the radiation to the head and neck squamous cell carcinoma. Research at Dr. Kariadi Hospital performed by Irawan regarding cervical cancer had the same result. But there is no research that shows the mAgNOR pre- and post-therapy in NPC patients. In counting AgNOR, the higher the mean value the higher proliferation of cancer cells that would be more radiosensitive to radiation compared with cancer cells at lower proliferation rate. The higher proliferation of cells the longer encountered S phase (DNA synthesis phase) which is mostly sensitive to radiation. Kinoshita stated AgNOR

number reflects the activity of cancer cells and numbers of researchers agree that the proliferation of cancer cells is very sensitive to radiation and other anti cancer drugs.(13,20,21)

In malignancies including in nasopharyngeal carcinoma there are uncontrolled cell division, resulting in high activity of the cells to proliferate. After therapy however, the proliferation activity will decrease as a sign of successful therapy, that can be examined by counting AgNOR.(22)

The decreased of mAgNOR post-therapy in the presenting study happened in all stages (II, III and IV). This proves a decrease in cell proliferation activity which indicates the response of tumor cells to the treatment. A slight decrease of AgNOR in stage II which is not significant statistically, is caused by too little samples at this stage.

Several studies on various types of carcinomas (ovarian, cervix, bone, *etc.*) also showed the same effects. But less studies finding a significant relationship between the decrease in the mAgNOR post-therapy with stage.(13,23,24)

Chemoradiotherapy is a chemotherapy combined with radiotherapy concurrently used to control tumor lokoregional and improve the survival of patients in order to overcome cancer cells systemically through microcirculation. In this study most of the samples will undergo neoadjuvant chemotherapy intended to reduce the size of the tumor before radiotherapy. Neoadjuvant chemotherapy was based on considerations that the tumor vascular bed is still in intact, therefore the penetration of the drug through the tumor mass is still adapted. In addition, treated early chemotherapy could eradicate systemic micrometastases as early as possible. Neoadjuvant chemotherapy over head and neck of stage II-IV malignancies were reported with overall response rates reached 80%-90% and complete response approximately reached 50%. Neoadjuvant chemotherapy treated prior to definitive radiation therapy could retain the function of the preserving organ.(25,26)

The level of differentiation is generally associated with malignancy and proliferation rate of cells, thus undifferentiated cells type (typical in WHO type 3) have higher proliferation rate, reflected in higher AgNOR value. As found in this study that the mean AgNOR pre- and post-therapy for the WHO type 3 were statistically different in significant level, therefore, in accordance to the research conducted in 70 patients with NPC paraffin specimens, findings the mean AgNOR for WHO type 3 was higher than those in WHO type 2, our result is proven to be reliably supported.(27)

The weakness of this research is limited of time, so that only a few samples can be observed until chemoradiotherapy completion.

Conclusion

The values of AgNOR were decrease in the all NPC stages and significantly decrease in undifferentiated squamous cell carcinoma (WHO type 3). AgNOR could be used to predict the successfulness of therapy in NPC.

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