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Research Article

Relative Expression of mRNA BARF1 Epstein-Barr Virus from Tumor Tissue Biopsy in Formalin-Fixed Paraffin-Embedded in Nasopharyngeal Carcinoma

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Abstract

Nasopharyngeal carcinoma (NPC) is a malignant tumor originating from nasopharyngeal epithelial cells. NPC is endemic in some regions, especially in Southeast Asia countries. NPC is also a multifactorial disease involving environmental factors, genetic factors, and infection from Epstein-Barr virus (EBV). According to WHO classification, Undifferentiated NPC is histopathologically associated with EBV infection and categorized into WHO subtype 3. EBV has two phases in its infection cycle: the lytic and latent phases. The BARF1 gene is a mediator of the transition from the latent phase to the lytic phase. Previous studies suggest measurement of EBV mRNA activity at the primary tumor site in the nasopharyngeal reflects the pathogenesis of NPC rather than measuring circulating EBV DNA or serological diagnosis. This study aimed to determine the relative expression potential of BARF1 mRNA at different tumor stages in NPC patients as a predictor of NPC pathogenesis. This research design was a descriptive research method in the form of a cross-sectional study. The samples used were 22 patients diagnosed as NPC WHO class III at the Anatomical Pathology Section of Prof. Dr. Margono Soekarjo, Purwokerto, who met the inclusion criteria. The relative expression of BARF1 mRNA was carried out using the one-step real-time RT-PCR technique and then calculated using a formula of 2-ΔCt. The T-test was used to compare the relative expression of early and late-stage BARF1 mRNA. The relative expression of BARF1 mRNA in the late-stage advanced stage (n = 6; 0.708292-0.840177; med = 0.7164655) was increased compared to the early stage (n = 2; 0.708841-0.712423; med = 0. 710632).

Keywords: Relative expression of BARF1 mRNA, EBV, predictive factor, the pathogenesis of NPC

1. Introduction

Nasopharyngeal carcinoma (NPC) is a unique epithelial malignancy arising from the superior region of the pharyngeal mucosa, which is commonly associated with latent infection with the Epstein-Barr virus or *Epstein-Barr virus* (EBV) (Ariwibowo, 2013). In general, the tumor's location is the lateral wall of the nasopharynx, especially the Rossenmuelleri fossa, which is a transitional area of the cuboidal epithelium to the squamous epithelium, and continues to the upper posterior wall of the nasopharynx (Roezin & Adham, 2007). NPC is endemic in Asia, especially South China, Southeast Asia, and Japan, and is rare in other populations worldwide. The highest incidence of NPC in the world is found in the Cantonese ethnic population in the Guang Dong and Guangxi Provinces, South China, with an incidence rate of more than 50 cases per 100,000 population per year(Chang & Adham, 2006). The incidence of NPC is 24 cases per year per 100,000 population in Hong Kong and around 20 cases per 100,000 population in

Southern China. However, the incidence of NPC in Europe or North America is only 1 per 100,000 population per year (Meilany & Sofyan, 2013). NPC has a conspicuous racial and geographic distribution variable (Zhang, 2002). The prevalence of NPC in Indonesia, with 225 million people, occurs in indigenous people of ethnic Chinese descent. The average incidence in Indonesia is 6.2 per 100,000 population, or 12,000 new cases yearly. (Adham et al., 2012).

Based on the WHO classification, NPC is divided into three histopathological types, namely type I (keratinized squamous cell carcinoma) with an incidence of about 10%, type II (nonkeratinizing, differentiated carcinoma) with about 15%, and type III (nonkeratinizing, undifferentiated carcinoma) was the most frequent (75%) (Ariwibowo, 2013). Another study showed that WHO-II and WHO-III NPC had a higher association with EBV infection than WHO-I NPC, especially in non-endemic areas. Subsequent studies showed that almost all cases of NPC in endemic areas have comorbidities with EBV infection, proving that EBV is the etiology for NPC (Wahyono, 2012).

The causes of NPC are multifactorial, with several risk factors including EBV infection, excessive consumption of salted fish, lack of consumption of fresh fruit and vegetables, exposure to tobacco smoke and other smokes, the habit of consuming alcohol and herbal medicines, occupational and other exposures, *familial clustering*, *human leukocyte antigen genes,* and other genetic factors. EBV infection is a significant risk factor for NPC. EBV infects and persists latently in 90% of the world's population. In Hong Kong, 80% of children are infected by 6 years, and nearly 100% have seroconversion by ten years of age. Primary EBV infection is usually subclinical, with direct transmission via saliva, which usually occurs in developing countries with crowded communities and poor sanitation (Ariwibowo, 2013). The close association between EBV infection and NPC has been known in the last three decades. This is reflected in abnormal EBV antibody activity, increased levels of EBV DNA concentration in the blood, and EBV gene expression in tumour cells. In general, NPC is considered to be a latent expression form of type 2 EBV with the expression of *EBV-encoded small RNAs 1* and *2* (EBER1,2), latent membrane proteins (LMP1 and LMP2), noncoding sequences of *BamHI-A rightward transcripts* (BARTs), and *BamHI-A rightward reading frame 1* (BARF1). LMP 1 has been considered more heterogeneous in its expression activity on infected cells. However, BARF1 can also be oncogenic because BARF1 mRNA is exclusively expressed in both EBV-positive NPC and EBV-induced gastrointestinal carcinomas (Stevens et al., 2006).

Of the 90 proteins encoded by the EBV gene, two genes are known to be highly oncogenic, namely LMP1 and BARF1. So far, LMP1 is considered to be more influential in infection, transformation, and host cell malignancy. However, previous studies have shown that LMP1 is only found in 50% of cases of NPC caused by EBV infection. Meanwhile, the expression of the BARF1 gene was found in the range of >90%, both in cases of NPC and gastric carcinoma caused by EBV infection (Jiang, 2009). High expression of the BARF1 gene can be found in EBV-infected nasopharyngeal epithelial cells and become NPC tumor cells in fresh NPC biopsy tissue. The oncogenic activity of BARF1 was

first discovered in rodentic fibroblasts. BARF1 also induced the immortality of monkey kidney epithelial cells in vitro and induced uncontrolled malignancy of other epithelial types. Therefore, BARF1 can intervene in two oncogenic processes, namely the induction of immortality and, simultaneously, the malignant transformation of cells. Several cellular genes, such as CD21, CD23, and CD71 will be transactivated in cells transfected with BARF1 caused by EBV infection. (Tarbouriech, 2006).

BARF1 is located in the 40 kb *open reading frame* (ORF) of the EBV genome. These ORF locations range from *BamHI* D to *BamHI* A, and are the site of the EBV genome capable of inducing cell malignancy and mortality (Hausen, 2000). The BamHI, A fragment of the EBV genome, encodes two different genes, BARTs and BARF1. BARTs were located at 150,000-161,000 bp of the B95-8 EBV DNA cell culture. BARF1 lies *downstream* against the BARTs. BARF1 encodes 221 amino acids and is translated into 31-33 kDA protein. BARF1 can also inhibit apoptosis by activating the NH2-terminal moiety of Bcl -2 (Takada, 2012). The BARF1 protein structure consists of two large immunoglobulin family domains. The first superfamily, the N-terminal domain, is located at amino acid residues 21 to 123. The second superfamily, the C-terminal, is located at amino acid residues 125 to 220 (Tarbouriech, 2006). Previous studies have also shown that BARF1 is expressed in EBV-infected epithelial tissue, not in malignant lymphocytic cells (Takada, 2012).

The BARF1 promoter segment was found to be highly methylated in both lymphoma and carcinoma, indicating that the BARF1 transcriptional activator must be capable of overcoming methylation. The BARF1 transcription results in NPC can be detected using *nucleic acid sequence-based amplification* (NASBA) and RT-PCR techniques (Tarbouriech, 2006). suggested performing *soft brushing* of the nasopharynx in the non-invasive diagnosis of NPC. Previously BARF1 was considered an EBV lytic phase gene in NPC tissue whose expression was induced by host cell rupture due to the EBV lytic cycle. Therefore, BARF1 is included in the latent gene, and the expression level and quantity of the genetic material of the gene can be one of the biological markers for the examination of NPC complement. BARF1 activity indicates NPC tumour development.

Most patients with NPC at the time of diagnosis were in an advanced stage (stages III and IV) (Faiza et al., 2016). A CT scan of the head and neck area can be used to find visible and hidden primary tumours. Serological tests of IgA anti-EA (*early antigen*) and IgA anti-VCA (*viral capsid antigen*) can detect EBV in NPC. Early diagnosis determines the prognosis of NPC patients. The prognostic parameter of NPC is the 5-year survival rate of NPC patients from an early stage to an advanced stage. The difference in stage shows a significant difference in 5-year survival rates, namely stage I 76.9 per cent, stage II 56.0 per cent, stage III 38.4 per cent, and stage IV 16.4 per cent. The UICC and AJCC classifications are generally the same for all malignancies except for malignancies of the salivary glands and thyroid (Munir, 2007). Currently, the staging is carried out using the TNM system (UICC - 2002) as follows (Roezin & Adham, 2007).

Therefore, early diagnosis of NPC is needed to increase knowledge and awareness of the clinical symptoms of NPC so that the management of NPC can be optimal. One of the complementary methods that can be used for diagnosis and prognosis is PCR-based molecular methods, such as *reverse transcriptase real-time* PCR (RT-qPCR). This method has been used to measure the relative mRNA expression of seven latent critical genes, including BARF1, *in vitro* (Bustin, 2002). The RT-qPCR technique was also used to measure the increase or decrease in mRNA expression, both from lytic genes and latent EBV genes with the GAPDH gene as a *housekeeping gene* for internal control of the PCR process, such as in BARF1 mRNA BRLF1, BZLF1 mRNA, and BLLF1 mRNA (gp350/ 220) (Chia, et al., 2008). Because the advanced stage of NPC reflects the occurrence of tumour progression in NPC, the relative expression of EBV BARF1 mRNA is expected to be used as a predictor of NPC pathogenesis. The use of NPC tumour tissue biopsy in paraffin block has not been widely used in the molecular biology analysis of tumors, such as the analysis of the relative expression of BARF1 EBV mRNA from NPC tumour tissue biopsies in formalin-fixed paraffin-embedded.

2. Materials and Methods

2.1 Materials

This research design is descriptive research in the form of a *cross-sectional* study. The variable observed was the expression of BARF1 Epstein-Barr Virus mRNA ($2^{-\Delta Ct}$ value) in WHO-3 NPC patients. *A consecutive sampling* technique was used to take samples (Susworo, 2004). This research was conducted at the Laboratory of Genetics and Molecular Biology, Faculty of Biology, and the Research Laboratory of Universitas Jenderal Sudirman. The study was conducted in September to December 2017. All NPC formalin-fixed paraffin-embedded (FFPE) tumour biopsies were collected from untreated patients histopathologically confirmed as NPC WHO-3 at the Department of Pathology Anatomy and tumour stage based on a definite diagnosis of the stage by clinicians at Radiotherapy Poly, Prof. dr Margono Soekarjo Hospital/Fakultas Kedokteran, Universitas Jenderal Sudirman, Purwokerto. The histological diagnosis was confirmed by the pathologist involved in the study. Histological classifications and grading of NPC were performed according to WHO classification into WHO-3 (undifferentiated carcinoma, UC). The total of research subjects was 27 people for NPC WHO-3. All research subjects were given informed consent before taking part in this research.

The 8-10 slices of NPC formalin-fixed paraffin-embedded (FFPE) tumour biopsies were performed by PureLink FFPE RNA isolation kit protocol (Invitrogen) to obtain 50-100 L RNA solution. By 10 L RNA solution could be directly used for the analysis of Reverse Transcriptase PCR (RT-PCR) or stored for long periods at a temperature of -80°C. One-step RT-qPCR technique was used to measure the relative expression of BARF1 EBV mRNA in the study samples. The primers used in BARF1 EBV mRNA amplification will produce cDNA fragment amplicons. The *left/forward primer* is 5'-CGGGATCCATGGCCAGGTTCATC-3' and the right-side primer (*right/reverse primer*) is 5'-

CCGCTCGAGTCATTGCGACAAGTAT-3' (Stevens, et al., 2006). Amplifying the GAPDH gene (glyceraldehyde-3-phosphate dehydrogenase) as a housekeeping gene that normalizes the Ct value will produce a cDNA amplicon measuring 226 bp. The left-hand primer for GAPDH is 5'GAAGGTGAAGGTCGGAGT-3', while the right-side primer is 5'-GAAGATGGTGATGGGATTTC-3' (Chia, et al., 2008). One-step RT qPCR mixture (qPCR mix) was made in a volume of 25 L, which consisted of Verso Enzyme MIx, 2X 1-step qPCR SYBR Mix, RT Enhancer, BARF1 gene primer (*forward and reverse*), *nuclease-free water* and RNA *template* (100 ng). Amplification was performed on a real-time PCR machine (Bioer – LineGene K FQD 48A) for 40 cycles with the following PCR conditions. cDNA synthesized 50 °C for 15 minutes with 1 cycle, *thermostat inactivation* 95 °C for 15 minutes with 1 cycle, denaturation 95 °C for 15 seconds with 40 cycles, *annealing* 50-60 °C for 30 seconds with 40 cycles, extension 72 °C for 30 seconds with 40 cycles. The relative expression of BARF1 mRNA was calculated according to the formula of $2^{-\Delta Ct} = [Ct_{(BARF1)} - Ct_{(GAPDH)}]$ (Livak & Schiimtgen, 2001). *Housekeeping genes* are genes whose expression does not change under experimental conditions. The GAPDH gene (*glyceraldeyde-3-phosphate dehydrogenase*) is a *housekeeping gene* with the most stable expression so it is used as an internal control in RT-qPCR (Barber, et al., 2005)(Wahyono, et al., 2016). The research data were statistically analyzed using Box Plot analysis and Mann Whitney test (SPSS version 17) to determine the differences in the relative expression of BARF1 mRNA from each group of NPC tumor stages (Wahyono et al., 2016).

3. Results and Discussion

3.1 Results

Table 1 shows the relative expression values of BARF1 mRNA and staging data for each sample. The eight amplified samples had a median value of BARF1 mRNA relative expression value of 0.714412 (0.708292 - 0.840177).

Table 1. Relative expression value of BARF1 mRNA and NPC stage.

No	Number of Sampel	Ct BARF1	ΔCt	$2^{-\Delta Ct}$	Stadium
1.	3208	38,68	9,05	0,708292	IV A
2	2558	36,19	6,56	0,715628	-
3	4090	38,85	9,.22	0,70844	IV C
4	1784	31,91	2,28	0,840177	III
5	2465	35,87	6,24	0,716401	III
6	2822	36,68	7,05	0,712423	II
7	3274	33,44	3,.81	0,755842	IV A
8	3738	35,.85	6,22	0,71653	IV
9	2142	38,3	8,67	0,708841	II

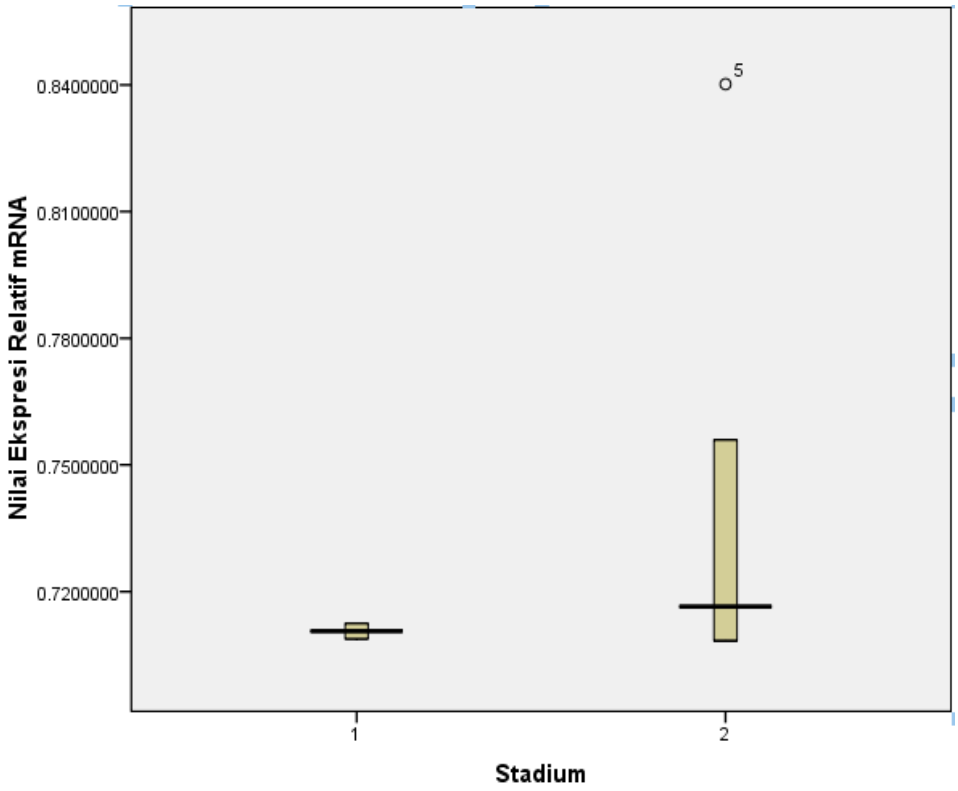

Figure 1. Box-plot analysis of the relative expression of BRLF1 EBV mRNA in patients with early-stage (1) median value = 0.710632 (0.708841 - 0.712423) and advanced-stage (2) median value = 0.7164655 (0.708292 - 0, 840177).

Of the 22 target samples used in this study, only 9 samples had tumor stage which would be analyzed for relative expression of BARF1 mRNA with real-time PCR machine. These nine samples were then grouped into two groups. Group 1 is an early-stage group consisting of stage I and II samples. Group 2 consisted of samples that were in stage III and IV. Table 4.1 shows the relative expression value of BARF1 mRNA and stage data for each sample. The number of early-stage samples was 2 samples, the number of advanced-stage samples was 6 samples, and 1 sample had no known stage. Samples with unknown staging were not included in the statistical analysis.

Based on the calculation of the derivative formula of $2^{-\Delta ct}$, mRNA expression is said to be low if the value is 1. A value of 1 is obtained from the calculation $2^0 = 1$(Livak & Schiimtgen, 2001) . The relative expression is said to be high if its value = 2. The value of 2 is obtained from the calculation of the derivative of the formula 2^1. The relative expression value is called overexpression if the value is >2. Meanwhile, Nolan (2006) argues that the relative expression value of mRNA is considered low if the results of $2^{-\Delta ct}$ are at 1 $\pm$ 0,3 range. The value of the expression is called high/optimal if the value of $2^{-\Delta ct}$ are at 2 $\pm$0,3 range. Based on this argument, the relative expression value of BARF1 mRNA in this study is included in the low category because the value ranges from 0.708292 to 0.840177 or less than 1.

Figure 1 shows the results of the box plot analysis of the relative expression of BARF1 mRNA. The early-stage group had a relative expression of BARF1 mRNA with a median value of 0.710632 (0.708841 - 0.712423). The relative expression value of the advanced BARF1 mRNA median was 0.7164655 (0.708292 - 0.840177). This study showed a tendency to increase the relative expression of BARF1 mRNA in the late stage compared to the early stage. However, the increase in the relative expression of BARF1 mRNA was not statistically significant.

3.2 Discussion

The data generated in this study showed a tendency to increase the relative expression of BARF1 mRNA from the early stage to the late stage. However, the increase in the relative expression of BARF1 mRNA was insignificant. Previous researchers, especially lytic genes, have analyzed the relative expression of NPC genes. Previous research conducted by Wahyono (2012) on the BRLF1 gene showed a similar trend, namely an increase in the relative expression value of the BRLF1 gene mRNA from the early stage to the late stage with fresh biopsy tissue. The increase was significant between staging groups. A significant increase occurred in the advanced-stage group, between distant metastatic status (M_1) and before distant metastases (M_0). However, different results were obtained by Estania (2017) with samples derived from paraffin block tissue. The results of Estania's research (2017) show the opposite. The relative expression value of BRLF1 mRNA in the early stage was 1.8 times greater than the relative expression value in the late stage.

A previous study using the EBV latent gene type (with FFPE samples) by Afriansya (2016) also showed statistically similar things. Afriansya's research showed that 6 out of 22 samples (27.3%) were amplified and included in moderate positivity (Jasaputra & Santosa, 2008). Meanwhile, a study by Wahyono (2012) and Herkilini (2017) using the lytic gene (BRLF1) showed different results. The results of research by Wahyono (2012) and Herkilini (2017) show the results of a progressive increase in the relative expression level of BRLF1 mRNA.

The difference in results with previous studies is thought to be caused by several factors. The first factor is the condition of the sample preparation. Different sample conditions between Wahyono's research (2016) compared to this study and Estania (2017) are thought to affect the results of the relative expression value of mRNA. Wahyono (2012) used samples from fresh biopsy tissue, while Estania (2017) and this study used samples in the form of tissue biopsies in paraffin blocks or formalin-fixed paraffin-embedded (FFPE). The use of fresh biopsy tissue directly affects the actual condition of the sample when in vitro analysis is performed. The progression of the biological condition of the tumour can be better described (Gouviea & Ferreira, 2014). The use of tissue in paraffin blocks or *formalin-fixed paraffin-embedded* (FFPE) has several negative effects, namely the degradation of genetic material (DNA and RNA) and cross-

linking degradation of proteins and their derivatives, and inhibition and termination of certain biological reactions (Gouviea & Ferreira, 2014). This affects the quality of the genetic material in the samples used.

The quality and quantity of RNA that was not optimally isolated from biopsy tissue in formalin-fixed paraffin-embedded were caused by (1) fixation of RNA with formalin and its derivatives making the RNA degraded more severely, (2) the number of contaminants due to the *formalin-fixed paraffin-embedded* (FFPE) manufacturing process. and (3) chemical reactions between formalin and its derivatives and RNA change conformity and tend to make RNA irreversibly change and become resistant to other treatments (Rezeki et al., 2014). To minimize the negative impact of formalin in FFPE, formalin substitution can be made with other fixative chemicals. The selected chemical must have a fixative ability, such as formalin, but its destructive effect on the sample must be lower than that of formalin. Other compounds commonly used to replace formalin include Bouin's solution, Carnoy's solution, acetone, alcohol, or *HEPES glutamic acid buffer mediated organic solvent* (HOPE) (Gouviea &Ferreira, 2014).

The second factor is the timeliness of sampling and the unique activity of the BARF1 gene and its ability to synchronize with the host cell cycle. In general, the BARF1 gene is considered one of the absolute latent genes in EBV (Fiorini & Tadamasa, 2008). Because the BARF1 gene is a methylated latent gene, the BARF1 mRNA activity level remains low. The activity of the BARF1 gene in cells that have been infected will be synchronized with the host cell through a complex mechanism that is not fully understood. The activity of this gene will increase before the lytic cycle and then stop entirely during the lytic phase (Seto et al., 2005).

The latent phase of EBV occurs as long as the host cell does not enter the mitotic or meiotic phase or stagnates in G0. However, several studies have shown that BARF1 is capable of being a lytic phase initiation gene in some cases (Takada, 2012). Together with the initiator function of the lytic phase of the cell genome, the ORF1 gene can stimulate host cells to enter the mitotic or meiotic phase, depending on the host cell type (Seto et al., 2005). Because of this unique ability, some experts and researchers consider BARF1 a latent and transitional gene (Seto et al., 2005). Several cases of NPC in specific epithelial cells and gastric carcinoma caused by EBV showed that the BARF1 gene could initiate the lytic phase of the EBV genome and simultaneously trigger the entry of host cells into the mitotic and meiotic phases. If the sampling were carried out when the host cell was in the G0 phase, the expressed BARF1 mRNA would be relatively low because the BARF1 gene was being methylated. On the other hand, if the sampling is carried out when the host cell is in the mitotic or meiotic phase, it is possible that the concentration of BARF1 mRNA will increase because the expression activity of the BARF1 gene is synchronized with the phases of the host cell (Seto et al., 2005).

The third factor is the sampling location. At the stage that has undergone distant metastases (stage IVC), there will be fluctuations in the relative expression level of latent and lytic gene mRNA compared to the initial site of tumour development (Tsao, 2012).

The efficiency and effectiveness of EBV infection on epithelial cells of oral organs is lower than the ability of infection on B lymphocytes and gastric cells. This less efficient and less effective infection ability is caused by the mechanism of infection in the oral epithelium, which involves a small number of host membrane integrin proteins with a complex mechanism. Therefore, the relative expression level of mRNA at the metastatic site will tend to be higher to increase the ability of infection (Tsao, 2012).

Based on the box plot test, further statistical analysis was carried out using a nonparametric test, namely the Mann-Whitney test. This test is carried out if the available data is not normally distributed. The Mann-Whitney test tested the data for the median value at the early and advanced stages that were not symmetrical (Dahlan, 2014). Table 4.2 shows the difference in the median value of the relative expression of BARF1 mRNA in the two staging categories. The difference between the median values for the early and late-stage groups was 0.0058335. This means there was an increase in the relative expression value of BARF1 mRNA from the early to the late stages of 0.0058335. However, the results of the Mann-Whitney analysis (Table 4.3.) show the significant value of Asymp. Sig. (2-tailed) of 0.505. Thus, statistically, this increase is not effective because the resulting significance value is greater than the relative probability (value Asymp. Sig. (0.505) > 0.05) (Jasaputra & Santosa, 2008). Therefore, an relative expression of EBV BRLF1 mRNA has low potential as a predictor of NPC tumour pathogenesis.

4. Conclusion

The relative expression value of BARF1 mRNA from tumour tissue biopsies in paraffin blocks tended to increase from early to advanced stages, although not significantly. The relative expression of EBV BRLF1 mRNA could not serve as a predictor of NPC tumour pathogenesis.

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