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Detection of 30bp deletion LMP1 oncogene as potential diagnostic and prognostic Biomarker among nasopharyngeal carcinoma patients

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ABSTRACT

Nasopharyngeal carcinoma (NPC) is endemic to several regions of North Africa and Southeast Asia and has a striking geographic distribution. It is closely linked to the Epstein-Barr virus (EBV) and is still found at an advanced stage that permits metastasis. However, only a limited number of EBV genes, including latent membrane protein 1 (LMP1), have been identified. The 30bp deletion variant of LMP1, which is thought to be the primary EBV oncoprotein, was more prominent in NPC biopsies. The aim of this study was to employ a 30bp deletion in LMP1 as a biomarker to help clinicians diagnose NPC early, improve prognosis, and reduce mortality rate. Polymerase chain reaction (PCR) was used to detect the prevalence of EBV in patients with NPC by amplification of the EBNA-1 and LMP1 genes. β-actin was amplified in all samples as a positive PCR control. The incidence and correlation of the 30bp deletion of LMP1 in 71 paraffin-embedded NPC tissue samples from Jordanian patients (51 males and 16 females) were also evaluated. The results were confirmed by Sanger sequencing and statistically analyzed using SPSS software. EBV was detected in all 71 NPC tissue samples, with positive results for EBNA-1 gene in 58 cases (81.7%) and in combination with LMP1 with or without 30bp deletion in 69 cases (97.18%) whereas, LMP1 30bp deletion was detected in 39 samples (54.9% of all samples). Heterogeneity of mutation was observed in 14.08% of samples. No significant differences were found between the stages with regard to the LMP1 30bp deletion. Statistical analysis showed a significant association between age and LMP1 30bp deletion with p-value = 0.015, and between the source of sample with LMP1 30bp deletion p-value = 0.032, whereas there was no association between LMP1 30bp deletions and patient sex or histological NPC type. A 30bp deletion of LMP1 was found in 54.9% of NPC tissues. This is within the range reported in other studies. There was no significant association between the 30bp deletion of LMP1 and histological type or disease phase. Further research is required to determine how this mutation in LMP1 affects the course and results of patients with NPC in Jordan.

Keywords: genetics, oncology, oncogene, mutations

INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a type of head and neck tumor that originates from nasopharynx epithelial cells. It is a rare malignancy with an incidence of < 1 per 100,000 people per year in North America and Western countries. The most endemic region is southern China, with an annual incidence rate of 15-50 people per 100.000 per year. NPC is the third most common cancer in men in South China [1, 2]. Asia accounts for 85.2% of the newly registered NPC patient's worldwide [3]. In Jordan, NPC occurs in both adults and children, and males are more commonly affected than females, with an incidence rate of 1 per 100.000 of the population [4]. Despite progress in radiotherapy and chemotherapy, NPC is characterized by its resistance to conventional treatments and a high risk of recurrence, emphasizing the need for innovative therapeutic approaches [5].

Although it is a rare cancer, it is aggressive and presents with varying degrees of differentiation. Undifferentiated NPC is

the most common type found in endemic regions. NPC has been found in a family cluster in a diverse population, indicating that genetic factors may play a role in the development of this type of cancer. It has been noted that environmental factors such as; fish and alcohol consumption, smoking, and Epstein-Barr virus (EBV) infection may play an important role in the development of NPC [6]. NPC is characterized by a strong etiological association with EBV infection, a relationship that is particularly pronounced in endemic regions such as Southeast Asia and North Africa [5, 7]. e The association between NPC and EBV begins when elevated anti-EBV IgA is observed in NPC patients [6].

EBV is a DNA virus that affects the Rosen Muller's fossa, which is a layer of epithelial cells on the superior side of the nasopharynx. It has a high prevalence that can cause latent infections in approximately 95% of the world's population. Physiological changes and the appearance of tumors can be observed at the site of EBV infection in both epithelial and B cells and occur as a result of the expression of latent genes, mainly EB-encoded early RNAs (EBER), EBV nuclear antigens (EBNA1/2/3 a, b, c), and latent membrane protein 1 (LMP1) or latent membrane protein 2 (LMP2) [8].

Recent work by [5] has further illuminated the role of EBV-encoded oncoproteins, including EBNA1, LMP1, and LMP2, as critical molecular drivers in NPC development and persistence. Drug discovery efforts are increasingly focusing on inhibitors targeting specific functional regions of these viral proteins due to their pivotal involvement in tumor cell survival and immune evasion [5].

Recent advancements underline the growing recognition of EBV oncoproteins, including EBNA1, LMP1, and LMP2, as promising therapeutic targets in NPC, with inhibitors for these proteins now moving toward clinical development. Additionally, molecular diagnostic approaches for secreted LMP1 and related viral markers in serum and saliva show great utility for early detection and management of NPC [5, 9]. These advances suggest that therapies directed against EBV oncoproteins represent a promising avenue for improving outcomes in EBV-associated NPC, with several candidates now advancing through preclinical and clinical testing [5]. LMP1 of EBV is a 356 amino acid integral membrane protein that has three domains:

(1) a short cytoplasmic N-terminal tail,

(2) six transmembrane alpha-helices of hydrophobic nature, and

(3) a long cytoplasmic C-terminal tail.

This tail has the highest activity region and consists of three functional domains: C-terminal activation regions 1, 2, and 3 CTAR (1, 2, and 3) [10].

LMP1 was the first EBV protein to exhibit oncogenic features [11]. Once expressed on the cell surface, it aggregates and forms an activated receptor that acts as a member of the tumor necrosis factor receptor (TNF-R) family, leading to different cellular signaling cascades [12, 13]. Some studies have considered LMP1 to be a causative factor of NPC [14]. This was explained by the fact that LMP1 acts in several ways: it causes upregulation of the A-20 gene and bcl-2 gene that lead to the inhibition of apoptosis in the infected cell [15], changes in the morphology of the epithelial cell, downregulation of the suppressor of metastasis, progression of angiogenesis, and induction of cytokines.

A 30bp deletion in the C-terminal tail, specifically in CTAR2 at the end of the tail, was found to be a prominent polymorphism of LMP1 in EBV that increases oncogenic features compared to prototype B95-8 LMP1 [16]. The goal of this study was to detect the presence of EBV and LMP1 30bp deletion genetic biomarkers in paraffin tissue samples from Jordanian NPC patients, and to assess the efficacy of utilizing LMP1 30bp deletion genetic biomarkers as diagnostic and prognostic tools for NPC in Jordan.

MATERIAL AND METHODS

Sample Collection

A total of 71 tissue samples from confirmed NPC patients at King Abdulla University Hospital and King Hussein Medical Center, Jordan, were investigated in this study. Formalin-fixed Paraffin-embedded tissue samples were used for DNA extraction. Using a microtome (Manual Rotary microtome cut 4060, SLEE Medical GmbH, Germany), approximately eight sections were obtained from each tissue block with 7 µm thickness and placed in a 2.0 ml microcentrifuge tube. Clinical data were collected for each patient, including the type of NPC, sex, age, stage of the disease, and the last follow-up. Ethical approval was obtained from the Institutional Review Board (IRB) were obtained from Zarqa University before the study began (IRB 33/166/2024).

DNA Extraction

DNA was extracted from the tissue samples using a GENE ALL® Exgene™ FFPE tissue DNA extraction kit (Gene All Biotechnology, Korea). DNA was extracted according to the manufacturer's instructions. First, the deparaffinization step was performed using the deparaffinizing buffer provided with the kit. The lysis step was performed by adding 180 µL of FBL and 20 µl of proteinase K (20 mg/ml, prepared previously by adding 1.2 ml of storage buffer to the lyophilized PK provided in the kit and stored at -20 °C for further use) and incubated overnight at 56 °C for complete lysis. After that, the translucent lysate was transferred to 90 °C for 1 h to allow the DNA to decrosslinking from the protein. Subsequently, 200 µL of FPB and 200 µL of absolute ethanol were added to the sample, and the mixture was transferred to the SV column and centrifuged at 6,000 × g for 1 min. Two washing steps were then performed. Then, 50 µL of elution buffer was added and the column was incubated at room temperature for 1 min before the final centrifugation at 14,000 × g for 1 min. Total DNA was quantified using the Eppendorf SupaLiq SnapFuge Micro Tube (PorLab Scientific Co. Ltd, China) and stored at -20 °C until use.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was first applied to confirm the DNA yield from extraction and then to study the Prevalence of LMP1 30bp deletion of EBV among NPC patients. PCR was performed using GoTaq ® Green Master Mix 2X (Promega, USA) which contains 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl2 and Taq DNA polymerase. The reaction mix was prepared in a total volume of 25 µL by adding 12.5 µl of master mix with 1X final concentration, 2 µl of 10 µM primer for LMP 30bp deletion amplification, and the primers used were L30F (primer sequence: 5' GTCATAGTAGCTTAGCTGAAC3') and L30R (primer sequence:5' GAAGAGGTTGAAAACAAAGGA 3') [17] with

annealing temperature. 50.5°C for 1min. For EBNA amplification, the primers used were (forward sequence: 5'-GCC GGT GTG TTC GTA TAT GG 3') and reverse primer sequence (R: 5'-CAA AAC CTC AGC AAA TA TATGAG 3') [18] with an annealing temperature of 58°C for 30 s. FOR beta β-actin gene analysis, the primers used were (F: 5-TTC CTT CCT GGG CAT GGA GT 3') and the reverse primer sequence was (R: 5-'GCA ATG ATC TTG ATC TTC ATT G 3') with an annealing temperature of 60 °C for 30 s. 2-4 µL of the DNA sample, depending on the beta-actin results (housekeeping gene). Nuclease-free water (NFW) was added to a final volume of 25 µl. For each PCR run, the negative control samples containing NFW instead of DNA were processed. A positive EBV sample was used as a positive control for the EBNA and LMP1 30bp deletion.

The program started with an initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation, annealing, and extension, and a final extension at 72 °C for 5 min, using a Veriti thermal cycler (Applied Biosystems, USA). The 10 µM primers was prepared by adding 10 µl of 100 µM stock primer to 90µl of NFW. The primer was lyophilized from integrated DNA technologies and then diluted with NFW according to the manufacturer's instructions to become 100 µM stock primer (stored at -80 °C). Gradient PCR was performed for each primer at different annealing temperatures and times, and the most suitable results were obtained.

Gel Electrophoresis

The product of PCR was analyzed by gel electrophoresis on 3% LE agarose gel (Thistle Scientific Ltd., Uddingston, Scotland). Electrophoresis was performed using an electrophoresis set (Thistle Scientific Ltd., Uddingston, Scotland) at 100 voltages for 50 minutes.

in 1X Tris/Borate/EDTA (TBE) buffer. A DNA ladder with 50bp and 100bp (GeneDireX, Taiwan) was used as a standard DNA molecular weight marker. The gel was stained with 3µl of ethidium bromide and visualized using an Ultraviolet Transilluminator Imaging system (Thistle Scientific Ltd., Uddingston, Scotland).

DNA Sequencing

The PCR amplicons of the LMP1 30bp deletion were chosen randomly and sent to the Enzyme Company (Irbid, Jordan) for confirmation by DNA Sanger sequencing. The results were compared with B95.8 EBV strains using DNASTAR software.

Before beginning the sequencing procedure, the PCR product was quantified using a Qubit fluorimeter, and the ExoSAP-IT™ kit (Applied Biosystems, USA) was used in the first cleaning step to remove unwanted reagents, such as free primers.

A reaction mixture with 10 µl total volume was prepared using 4 µL of BigDye™ Terminator v3.1 (Big RR) and 2 µl of BigDye™ Terminator buffer (Thermo Fisher Scientific, USA), 1 µl of 3 ng from treated PCR product, 2 µl of 5M forward and reverse primer was prepared. The volume was completed to ten with 1 µl NFW. A sequencing cycle was applied, with an initial denaturation at 95 °C for 1 min, followed by 25 cycles of annealing at 50 °C for 5 s and extension at 60 °C for 4 min.

The BigDye™Xterminator kit (Biosystem, Thermo Fisher Scientific, USA) was used in the final cleaning step by adding 45µl of BigDye XTerminator and 10 beads in 10 µl of PCR product, which was then mixed for 30 min. The mixture was centrifuged for 2 min. Finally, the reaction mixture was placed in an Applied Biosystems DNA Analyzer SeqStudio (Thermo Fisher Scientific, USA).

Table 1. NPC patient's information, cancer origin, and classification

Characteristics		Frequency	
		N	%
Gender	Female	16	23.9
	Male	51	76.1
Age	Mean ± standard deviation	53.5 ± 15.41 years	
Source of sample (tissue)	Post-nasal space	26	40.0
	Nasopharyngeal	21	32.3
	Cervical lymph node	5	7.7
	Liver	2	3.1
	Nose	2	3.1
	Sinus	3	4.6
	Vocal cord polyp/pelvic bone	3	4.6
	Tongue or oral cavity	3	4.6
	Missing data	6	
Type of NPC	Undifferentiated cell carcinoma (type III)	42	80.8
	Non keratinizing squamous cell carcinoma (type II)	6	11.5
	Other	4	7.7
	Missing data	19	
Cancer stage	Stage I	4	8.2
	Stage II	8	16.3
	Stage III	19	38.8
	Stage IV	18	36.7
	Missing data	22	

Note. N: Number; %: Percentage; & Other types of NPC were basaloid squamous cell carcinoma in one case & keratinizing squamous cell carcinoma was isolated from other cases

Statistical Analysis

Data were assembled in Microsoft Excel and statistical analyses were conducted using the statistical package for the social sciences (IBM SPSS V.25). The level of statistical significance was set at p-values < 0.050. Chi-square test and Fisher's exact test were used to determine the association between categorical variables. In addition, descriptive tests (frequency, median, mean, and standard deviation) were used for continuous data such as age.

RESULTS

General Characteristics of the Studied Population

This study included 71 Jordanian patients with confirmed NPC, of whom 51 (76.1%) were male and 16 (23.9%) were female, with a mean average age of 53.5 years (**Table 1**). In 6 out of 71 samples, the tissue origins were unknown. Most NPC tissue samples were from post-nasal space 26 of 65 (40.0%), followed by nasopharyngeal 21/65 (32.3%). 19 out of 71 cases had an unknown NPC type. The NPC type III (undifferentiated type) comprised the majority of cases 42 of 52 (80.8%), followed by non-keratinizing carcinoma (NKC, WHO type II) 6/52 (11.5%) and 4 (7.7%) with keratinizing squamous cell carcinoma (SCC, WHO type I) 7.7 others. Other types included one patient with a rare subtype of NPC known as basaloid squamous cell carcinoma. Based on the patient's CT scan, most patients 19/49 (38.8%), 18/49 (36.7%) were classified as being in stage III and IV, whereas 8/49 (16.3) and 4/49 (8.2%) were in stage II and stage I, respectively. Only 49 patients were included in this study.

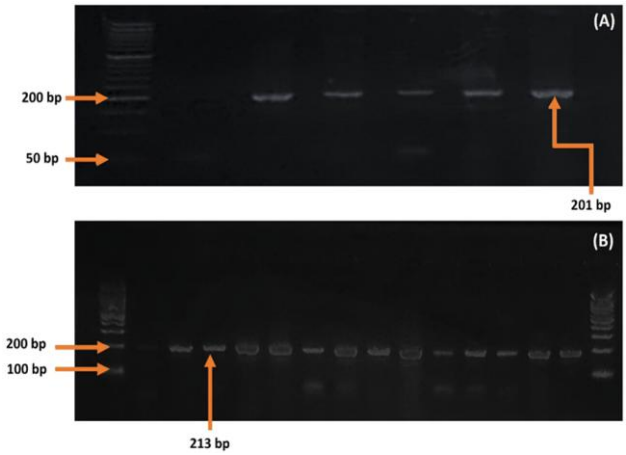

Figure 1. (A) Agarose gel electrophoresis of PCR product of beta-actin gene (201bp) (L: ladder marker 50bp; N: Negative control; P: Positive control [DNA extracted from whole blood sample]; DNA from NPC tissue samples [1, 2, 3, and 4] with product size 201bp) & (B) Agarose gel electrophoresis of PCR product of EBNA-1 (213bp) gene (L: Ladder 100bp; N: Negative control; P: Positive control; DNA of NPC tissue sample [1 to 12] with product size 213bp) (Source: Authors' own elaboration)

Control of DNA Extraction (DNA Yield)

To evaluate the quality of DNA extraction from tissue samples, the β-actin gene was amplified, and the intensity of the band in the gel after electrophoresis and under UV light was detected for 71 samples, each of which yielded positive results with different intensities and a product size of 201bp that was used as a positive control for PCR. The results for representative samples are shown in part A in **Figure 1**.

Molecular Detection of EBV Among NPC Patients

EBV DNA was detected in all tissue samples by amplification of the EBNA-1 gene with a product size of 213bp in combination with amplification of LMP1 in the C-terminal region with or without a 30bp deletion with a product size 156bp and 186bp, respectively. Fifty-eight samples (81.7%) produced amplification products of EBV using the EBNA-1, and 69 (97.18%) using the LMP1 gene. Part B in **Figure 1** shows the results of EBNA-1 (213bp) agarose gel electrophoresis for representative samples. Part A in **Figure 2** show the proportion of EBV in EBNA-1 cells.

Detection of LMP1 Gene Variants of the EBV

Of the 71 NPC patient samples, 69 samples (97.18%) were successfully amplified for the LMP1 gene. Different variants of LMP1 were detected in the NPC samples. mutant genotype (MT) with a 30bp deletion was detected in 29 patients (40.85%), whereas the wild-type genotype, which doesn't have 30bp deletion was amplified in 30 patients (42.25%) and a heterogeneous pattern in 10 (14.08%) (part B in **Figure 2**). **Figure 3** shows the results of the LMP1 amplicon with (156bp) and without 30bp deletion (186) in 3% agarose gel.

Association Between LMP1 30bp Deletion and General Characteristics and Histological Features of NPC Patients

Overall, the number of patients who were positive for LMP1 30bp deletion (MT, heterogeneous mutation) was 39 (54.90%), whereas 32 patients (45.10%) had a mutation (WT or negative for LMP1). Twenty-eight (54.90%) of males were reported with positive LMP1 mutations and nine (56.25%) of females were positive for it. In NPC type III, 30bp mutation was present in 20 patients of 42 patients (47.61%). LMP1 with 30bp deletion was found in stage III and IV of NPC cases (52.6% and 44.6%, respectively). Elderly patients aged 56 years and more were significantly reported with the highest percentage of LMP1 mutations, 24 of 35 (68.57%) (p-value = 0.015). In addition, the LMP1 mutation was found in 10 of 26 cases (38.46%) post-nasal space. A significant association was found between the source of the malignant tumor and the presence of a 30bp mutation (p value = 0.032). No statistical significance was found between the presence of a 30bp deletion and other clinical and histological features (stages, NPC types, and sex) (**Table 2**).

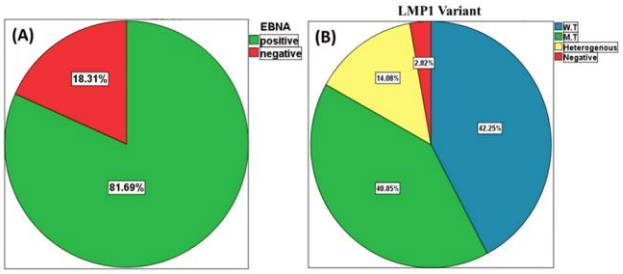

Figure 2.(A) Proportion of amplified EBNA-1 gene in NPC patient's samples (N = 71) & (B) Proportion of LMP1variants among EBV positive sample in all NPC cases (Source: Authors' own elaboration)

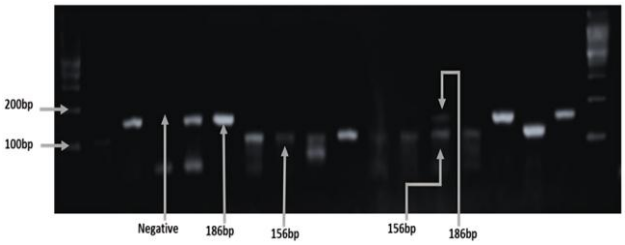

Figure 3. Agarose gel electrophoresis of LMP1 amplicon with 30bp deletion (156bp) and without 30bp deletion (186) (L: ladder 100bp; N: Negative control; P: Positive control [EBV positive sample with no 30bp deletion, wild type186bp]; NPC tissues sample with 30bp deletion 156bp [4, 5, 6, 7, 8, 9, 11, and 13]; NPC tissues sample without deletion186bp [2, 3, 12, and 14]; NPC tissue samples have negative result [1]; & NPC tissue sample with heterogeneous gene [10]) (Source: Authors' own elaboration)

DNA Sequencing

Four amplicons of LMP1 with and without a 30bp deletion were chosen randomly for sequencing to identify the region of the 30bp deletion and the results were aligned with B95.8 EBV GenBank accession no. V01555 from 168.141 to 168.200 using a DNASTAR Navigator. The results are shown in **Figure 4**. The sequencing results agreed with the conventional PCR results.

DISCUSSION

Although NPC is uncommon in Arab countries, it is becoming more common due to increased exposure to a larger variety of risk factors. Numerous studies have demonstrated that, in the majority of the population, the incidence of NPC in men is two or three times higher than that in women.

Table 2. The presence of 30bp deletion and other clinical and histological features (stages, NPC types, and gender)

Characteristics		Frequency: N (%) LMP1 30bp deletion		p-value
		Positive (N = 39)	Negative (N = 32)	
Gender	Female (N = 16)	9/16 (56.25)	7/16 (43.75)	0.355
	Male (N = 51)	28/51 (54.90)	23/51 (45.09)	
Age category 1	< 56 years (N = 31)	12/31 (38.70)	19/31 (61.29)	*0.015
	≥ 56 years (N = 35)	24/35 (68.57)	11/35(31.42)	
Sources of sample (tissue)	Post-nasal space (N = 26)	10/26 (38.46)	16/26 (61.53)	*0.032
	Nasopharyngeal (N = 21)	9/21 (42.85)	12/21 (57.14)	
	Cervical lymph node (N = 5)	5/5 (100)	0/5 (0.00)	
	Liver (N = 2)	1/2 (50.00)	1/2 (50.00)	
	Nose (N = 2)	1/2(50.00)	1/2 (50.00)	
	Sinus (N = 3)	3/3 (100.00)	0/3 (0.00)	
	Vocal cord polyp/pelvic bone (N = 3)	2/3 (66.66)	1/2 (33.33)	
	Tongue/oral cavity (N = 3)	3/3 (100)	0/3 (0.00)	
Type of NPC	Undifferentiated cell carcinoma (N = 42)	20/42 (47.61)	22/42 (52.38)	0.520
	Non keratinizing squamous cell carcinoma (N = 6)	2/6 (33.33)	4/6 (66.66)	
	Other 2 (N = 4)	3/4 (75.0)	1/4 (25.0)	
Stage	Stage I (N = 4)	4/4 (100)	0/4 (0.00)	0.276
	Stage II (N = 8)	4/8 (50.00)	4/8 (50.0)	
	Stage III (N = 19)	10/19 (52.63)	9/19 (47.36)	
	Stage IV (N =18)	8/18 (44.44)	10/18 (55.55)	

Note. N: Number; %: Percentage; Age category 1 was categorized based on the median values; Other 2: Types of NPC were basaloid squamous cell carcinoma in one case and keratinizing type I; *Significant p-value < 0.05; & #Chi-square or Fisher exact test

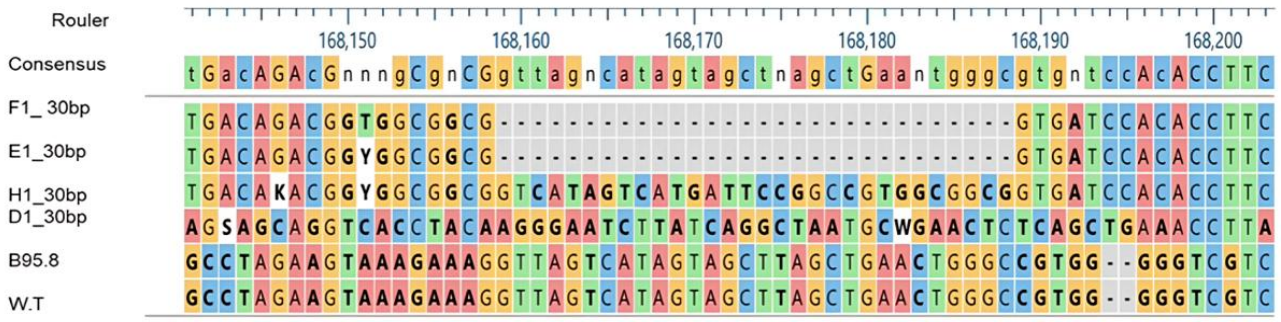

Figure 4. Alignment of B95.8 EBV (V01555) with four samples from NPC patients (dot region shows the 30bp deletion site: [F1; E1] samples with 30bp deletion & [H1; D1] samples without 30bp deletion) (Source: Authors' own elaboration)

Mahdavifar reported that in Saudi Arabia, in 2012, the incidence (male-to-female) was 1.3 to 0.6, 0.7 to 0.5, and 0.3 to 0.1 [19]. Another study in Jordan in 2012 found that the percentage of UDNPC among males was higher than that among females with 1.45:1 ratio [20]. These findings are consistent with our research, which studied 71 confirmed NPC tissue samples from 2003 to 2024, of which 51 (76.1) were males and 16 (23.9%) were females of wide range of ages with a mean age of 53 years old, representing a 3.2 to 1 ratio. Some causes may be related to sex differences and how differently males and females are exposed to environmental risk factors, including smoking and food habits. Variations in X chromosomes have been suggested to play a role in the genetic feature-related advancement of NPC [21].

According to WHO the predominant type of NPC is type III (up to 65% of NPC) [22]. In this study, most NPC samples were diagnosed as type III, which formed the majority of cases 42 of 52, 80.8%), followed by NKC (WHO type II), 6 of 52 (11.5%), and 4 of 52 (7.7%) with keratinizing squamous cell SCC (WHO type I) and other types. A related study in Jordan found that Type III is the most dominant type [20] which agrees with our study. Most patients 19 of 49 (38.8%); 18 of 49 (36.7%) were classified as being in stage III and IV. NPC can spread to other regions as it is still detected at an advanced stage. The most frequent location for metastatic NPCs is the bone, followed by the liver, lungs, distant lymph nodes, and the brain [23]. Most biopsies were from post-nasal space (26 of 65, 40.0%), followed by nasopharyngeal (21 of 65, 32.3%). Other biopsies were collected from different metastatic regions. Metastases to the cervical lymph nodes appear to be the most frequent presenting characteristic, which is consistent with the findings of Matalka. Indeed, the early diagnosis of NPC may improve the outcome of treatment and patient prognosis, which is the primary aim of this study to identify a biomarker to diagnose NPC at an early stage.

Numerous studies have demonstrated a robust association between EBV infection and many forms of carcinoma, including type III NPC. It was shown that NKNPC was highly associated with EBV infection [24]. In this study, although the most dominant type of NPC was type III, the study included other types in low percentages, all of which yielded positive results for EBV, which means that there is an association between types I, II, and III of NPC with EBV. Among the most important features of EBV NPC is the presence of an EBV episomal genome and the requirement of a very high number of viral traits for harmful modification. Viral inert characteristics have been identified to provide a variety of malignant growth features, which, in turn, have been demonstrated to lead to the genesis of NPC. EBNA1 is necessary for replication and mitotic isolation of EBV episomes and for the protection of EBV genomes in

actively infected cells. Furthermore, EBNA1 maintains cell viability following DNA damage by triggering genomic instability and certain cellular properties via transcriptional enactment [25].

The relationship between EBV DNA load and patient outcomes is being redefined with emerging artificial intelligence (AI)-based modelling [26]. It was validated that advanced NPC patients with high plasma EBV DNA levels had substantially inferior progression-free survival, and risk-stratification models integrating EBV DNA copy number as the key variable demonstrated superior prognostic accuracy compared to traditional clinical metrics [26]. These AI-enhanced tools not only better predict patient outcomes but hold promise for guiding individualized treatment decisions [26].

The recent studies affirm the central significance of EBV in regulating molecular pathways such as autophagy and reinforce the value of EBV DNA and associated autophagy markers as key biomolecular prognostic tools in NPC [26, 27]. Future research into specific gene targets within these pathways offers exciting prospects for both improved risk stratification and the design of next-generation targeted therapies for EBV-associated NPC.

The prediction of EBV presence among 71 confirmed Jordanian NPC patients was performed successfully in this study. By detecting the EBNA1 gene in 81.7% (58/71) in combination with the LMP1 (with or without deletion) in 97.18% (69/71) using PCR. The presence of EBV in all 71 (100%) NPC samples was verified. It is important to detect the EBNA1 gene in patient samples in combination with targeted LMP1, as this protein is expressed in both the latent and lytic stages of infection [28, 29]. In a study using brush samples from NPC patients in Vietnam, EBNA-1 and LMP1 were detected in 46.32% and 45.26% of patients, respectively, the results were compared with a non-cancerous samples, and the difference was found to be statistically significant, confirmed the association between these two genes of EBV and NPC [30]. The lower percentage in the Vietnam study when compared to our investigation could be explained by two factors. First, the sample types could have affected the sensitivity for detecting these genes, as well as the geographic distribution of NPC and the prevalence of EBV. Another study performed by Yap in a nasopharyngeal biopsy set indicated that the EBNA1-LMP1 combination was the most appropriate pairwise combination of genes for the diagnosis of NPC in nasopharyngeal biopsy. Of the 35 NPC specimens, 34 were positive for EBNA1, 31 were positive for EBNA2, and 32 were positive for LMP1 [31]. This combination was used in the present study. These results are in line with the findings in [31], indicating a high incidence among Jordanian patients with NPC, suggesting that it can be used for the prediction of EBV among Jordanian patients with NPC, with little variation from a previous study on increased expression of the LMP1 gene. This implies that LMP1 protein plays a major role in the etiology of EBV-associated cancer and is the most prominent viral oncogenic protein studied.

Several recent studies highlight that LMP1 remains a principal oncogenic driver in NPC by engaging host cell survival and transformation pathways, notably through its complex interaction with autophagy and signal transduction cascades [9, 26, 27, 32]. Importantly, new diagnostic assays for circulating EBV DNA and secreted LMP1 protein are being validated and are poised to improve disease monitoring and patient outcomes [9, 26, 27, 32]. Furthermore, AI-based prognostic models incorporating EBV DNA levels now provide a robust approach for individualized risk stratification in advanced NPC [9, 26].

Many variants of LMP1 have been investigated in endemic and non-endemic areas, like (30bp deletion, 69bp deletion, and XhoI loss). Our study focused on the association between a 30bp deletion and NPC in Jordan. A 39 out of 71 (54.92%) tissue samples with confirmed EBV displayed a 30bp deletion mutation. However, there is no evidence that this variant can be used as a biomarker for NPC diagnosis and prognosis. Additional studies conducted in endemic regions, particularly in China, confirmed that the LMP1 30bp deletion is a prognostic marker for NPC and revealed that the percentage of deletion mutations in NPC tissue was seven times greater than that in healthy tissue [33]. The prevalence of 30bp deletion of LMP1 in non-endemic regions has been studied in tissue biopsy from Serbians, where the percent was 28.6%, as well as from Europe and North Africa, where the percentage was 55-75% [34]. In comparison to our study, the prevalence of the 30bp deletion was higher than the proportion in Serbians and agrees with that in Europe and South Africa. In addition, Trinh et al. report that the Americas and North Africa had the lowest prevalence of this mutation in NPC patients (64% and 59%, respectively), while Asia has the greatest rate (79%) [35]. EBV strains identified from NPC Tunisian and Moroccan patients and healthy blood donors from Qatar were shown to have the 30bp deletion in Arab nations [36]. Genetic variation plays an important role in these differences, suggesting that the 30bp deletion may be used as a biomarker for the early diagnosis and prognosis of NPC in Jordan. Our results showed three variants of the LMP1: mutant type with 30bp deletion at 40.85%, wild type without deletion at 42.25%, and heterogeneous type at 14.08%. Another study in Malay found these heterogeneities of LMP1 among NPC patients in 5/29 (17.2%) of blood sample [17]. The heterogeneity variant may have been caused by several EBV infections by different strains or may have been produced from a single EBV strain during the clonal proliferation of EBV-infected cells over time [37].

The relationship between LMP1 30bp deletion and histological type is important not only for epidemiology or NPC diagnosis, but also for the prognosis and monitoring of patients [38]. One study conducted among Taiwanese patients found no association between clinical and histological features and the presence of a 30bp deletion [39], whereas, the opposite was found among Malaysian patients [17].

In this study, statistical analysis was performed between the presence of a 30bp LMP1 deletion and the histological type, age, sex, and stage to further understand the effect of 30bp in the pathogenesis of NPC using Fisher's exact test. A significant difference was found with higher prevalence of mutation among elderly people (56 years old and above) and in tissue sources and the 30bp deletion with high prevalence among all metastatic regions specifically cervical lymph node region that suggest that the oncogene features for the existent of 30bp deletion mutation among NPC. No significant association was found with the other studied features.

However, further research is necessary, even though a high percentage of this 30bp deletion suggested that it has transformation capacity during NPC carcinogenesis. It may not affect the clinical situation or pathological symptoms of established NPC after exposure to carcinogens.

Limitations and Future Research Directions

Certain limitations of our study directly impact on the findings, particularly regarding the disease prognosis. It was challenging to obtain treatment and follow-up data for each patient, which is important for understanding how a 30bp deletion in LMP1 may contribute to disease progression; thus, further investigations should be conducted. This study used conventional PCR, which is less sensitive and less specific than the other types of PCR. The use of real-time PCR, which can detect viral DNA even at small concentrations, or Multiplex PCR, which can combine multiple LMPs, can increase the sensitivity and specificity of the test.

CONCLUSION

In conclusion, 54.92% of Jordanian NPC tissue samples had an LMP1 30bp deletion discovered in their NPC tissues. This was within the range reported in other studies. Four percent of the mutations were found to be heterogeneous. Histological type, illness stage, and a 30bp deletion of LMP1 were not significantly associated. Further investigation is necessary to determine the impact of this LMP1 mutation on the progression and outcome of NPC type III NPC in Jordan.

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