



## Research Article

# Association of Leucocyte Telomere Length with Nasopharyngeal Carcinoma Risk and Prognosis

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## Abstract

**Objectives:** Cancer development and/or progression can be imputed to telomere shortening or lengthening. The current research was planned to assess the relation between leucocyte telomere length (LTL) and nasopharyngeal carcinoma (NPC) development, and to evaluate the association between this biomarker and patients' clinical outcomes, mainly response to chemo-radiotherapy, survival and EBV-DNA load.

**Methods:** Leucocyte telomere length was measured using a singleplex quantitative polymerase chain reaction (qPCR) method in 104 NPC patients and 52 healthy controls. The association between LTL and NPC development was assessed using a Khi-deux test. Odds ratios (ORs) and 95% confidence intervals (CI) were calculated by conditional logistic regression to evaluate the relationship LTL and patient's characteristics. The correlation between LTL and 4-years patient's survival outcomes was assessed by Kaplan–Meier and Cox-regression analyses.

**Results:** Data analysis revealed a significant association between LTL and risk of NPC development ( $p < 0.0001$ ). LTL was also significantly associated with gender ( $p = 0.003$ ), cigarette smoking ( $p = 0.02$ ) and pre-EBV-DNA load ( $p = 0.017$ ) in NPC. However, no significant association was obtained between LTL and age, alcohol consumption, TNM classification, disease stage, response to chemo-radiotherapy and survival outcomes of NPC patients ( $p > 0.05$ ).

**Conclusion:** The study finding highlighted the close association between LTL and NPC development and showed a significant association with gender, cigarette smoking and pre-EBV-DNA load, suggesting that LTL could be a promising biomarker for better management of NPC in Morocco. Nevertheless, more studies are needed to highlight the value of LTL as a biomarker for the prediction of the response to treatment and prognosis of NPC.

**Keywords:** Clinical outcomes, leucocyte telomere length, Morocco, nasopharyngeal carcinoma

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Nasopharyngeal carcinoma (NPC) is an epithelial carcinoma with a distinct geographical and racial distribution, the highest prevalence being reported in Southeast

Asia.<sup>[1,2]</sup> Morocco, and according to the Cancer Registry of Casablanca, belongs to an intermediate risk area with an estimated incidence of 1.8 / 100 000 person/year.<sup>[3]</sup> The

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heterogeneous distribution of NPC suggests its multifactorial etiology, including host genetic predisposition, Epstein Barr virus (EBV) infection and numerous environmental and dietary factors.<sup>[4,5]</sup>

NPC diagnosis is often difficult and late, resulting in a high rate of treatment failure and mortality.<sup>[6]</sup> Accordingly, the main challenge faced by clinicians is to identify new diagnosis and prognosis biomarkers that could be used for early cancer diagnosis and patient outcomes improvement.<sup>[5]</sup> Currently, several molecular biomarkers, including mutation status, epigenetic alterations and genetic regulation are being widely evaluated and may be valuable in NPC management.<sup>[7-10]</sup>

Telomeres are ribo-nucleoprotein structures of 10–15 kbp tandem arrays of telomeric DNA repeats (TTAGGG) at the extreme ends of chromosomes.<sup>[5,11]</sup> Their main function is to maintain genomic stability during cell replication and to protect chromosomal material against degradation, recombination and chromosomal end-to-end fusion.<sup>[12,13]</sup> Furthermore, leucocyte telomere length (LTL) is dynamic among individuals and varies according to many factors such as age and tissues type.<sup>[14]</sup> In normal cells, telomerase ensures progressive synthesis of telomeric DNA repeats at the 3'ends of linear chromosomes, thereby reversing DNA loss after replication.<sup>[14]</sup> Once telomere reaches a critically short length, the process of cell senescence is initiated, leading to cell cycle arrest and/or apoptosis.<sup>[14]</sup>

Telomere dysfunctions are largely reported to be associated with the process of age-related diseases, including cancers. In fact, excessive shortening of telomere, which is one of the hallmarks of human cancer cells, was identified as being associated with genomic instability and increased risk of cancer.<sup>[15]</sup>

Long telomeres were rather found to display an increased risk for several types of cancers, which represent the cancer-telomere length paradox. Accordingly, a large discordance was observed between studies assessing the association between LTL and different cancers, including colorectal, endometrial, prostate and lung cancers.<sup>[16-19]</sup>

In NPC, the LTL was reported to be considerably shorter in cancer tissues (4.5kb), compared to tumor adjacent tissues (14.6 kb) and chronic nasopharyngitis (15.8±3.1 kb), indicating that short telomeres may increase NPC risk.<sup>[15]</sup> Of particular interest, another study reported a great link between LTL and development of NPC; patients at advanced disease NPC (stage IV) have shorter LTL, than healthy controls, suggesting that LTL assessment could be a promising biomarker for NPC monitoring.<sup>[5]</sup> Therefore, the present study was planned to assess the association between LTL and the risk of NPC development, as well as the relevance

of this biomarker in predicting treatment response and patients' prognosis.

## Methods

### Study Design

A total of 104 patients with NPC were recruited in Mohammed 6 Center for Treatment of Cancer of Casablanca, between January 2017 and March 2019. Fifty-two controls were randomly selected from healthy individuals. At recruitment, a detailed interview was made with each participant to obtain information on the epidemiological data including age, gender, alcohol consumption, cigarette smoking, childhood habitat and family cancer history. Clinical stage was identified from patients' clinical registers. Patients staging and TNM classification were applied according to the 7th Edition of the International Union against Cancer/American Joint Committee on Cancer system (UICC/AJCC) using imaging approach. Patients underwent radiotherapy treatment with or without chemotherapy induction, according to the hospital practice guidelines. A median cure of 50 Gy in the nasopharyngeal tumor area and 66 Gy in the affected lymph nodes were received by NPC patients. They received a 2 Gy daily fraction five times a week and therapy was completed in five to seven weeks. For patients with advanced disease, neoadjuvant chemotherapy was given using multiple regimens consisting of cisplatin, anthracycline and fluorouracil.

For all NPC patients, EBV DNA quantification before treatment initiation was performed, and the cut-off value used was 4000 UI/mL.<sup>[20]</sup>

### Blood Samples and Genomic DNA Extraction

Peripheral blood samples were collected in Ethylenediaminetetraacetic acid (EDTA) tubes from all patients prior to any therapy, and healthy subjects and stored at -20°C for DNA extraction. Genomic DNA was extracted using a phenol chloroform standard method and accurately quantified on the NanoDrop spectrophotometer (ThermoFisher). DNA samples were used immediately for LTL measurement or stored at -20°C until use.

### Measurements of Leucocyte Telomere Length

Mean leucocyte telomere length was assessed by a validated singleplex q-PCR assays.<sup>[21]</sup> For PCR amplification, primers targeting the telomere gene and the housekeeping *GAPDH* gene are reported in Table 1. Amplification mixtures consisted of a total volume of 10 µl, containing 2X Powerup SYBR Green master mix (ThermoFisher), 500 µM of each primer and 20 ng of genomic DNA. DNA Amplification was performed on StepOne qPCR system (Applied Bio-

**Table 1.** Primers used for qPCR

Gene		Sequence
Tel	F	5'-ACACTAAGGTTTGGGTTTGGGTTTGGGTTTGGGTTAGTGT-3'
	R	5'-TGTTAGGTATCCCTATCCCTATCCCTATCCCTATCCCTAACA-3'
GAPDH	F	5'-GAAGGTGAAGGTCGGAGTC-3'
	R	5'-GAAGATGGTGATGGGATTC-3'

systems, Foster city, CA, USA) under the following conditions: 95°C for 2 minutes followed by 40 cycles of 95°C for 3 seconds and 60°C for 30 seconds. To minimize variation between samples, all samples were assayed in triplicate, and the final analysis used the mean value of the three replicates. The results of LTL from PCR were expressed as the ratio of telomere repeat copy number (T) to single gene copy number (S) (T/S ratio) in experimental samples relative to the standard sample. Five concentrations of the standard human genomic DNA sample were prepared by 10-fold serial dilutions (50 ng, 5 ng, 0.5 ng, 0.05 ng and 0.005ng per well) and used for the standard curve.

### Follow-up and Clinical Endpoints

All included patients were followed until February 2021. Overall survival (OS) consored as the interval from random assignment to death. Locoregional recurrence-free survival (LRRFS) was identified as the period between the time of randomisation and the first sign of loco-regional recurrence death from any cause or last follow-up. Distant metastasis-free survival (DMFS) was measured from the time of diagnosis until the first distant metastatic lesion, death from any cause, or last follow-up. Progression-free survival (PFS) corresponded to the time from diagnosis to disease progression, death or last follow-up.

### Statistical Analysis

Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS) version 22.0 for Windows. Descriptive statistics were calculated and presented as numbers and percentages. NPC patients were categorized into long and short LTL by median-split, according to the distribution of T/S ratio (TSR) value; and, the upper limit of the overall 95% confidence interval (CI, 1.283–1.327) for the 104 NPC samples was defined as a cut-off value. A *Khi-deux* ( $X^2$ ) test was used to assess the association between LTL and NPC development. Conditional logistic regression was used to evaluate the relationship between LTL and age, gender, cigarette smoking, alcohol consumption, overall cancer stage and TNM classification by calculating the odds ratios (ORs) and 95% CI. The OS, LRRFS, DMFS and PFS among the different patients' groups were assessed by the Kaplan-Meier method and analyzed by the log-rank test.

Univariate analyses were carried out using the Cox proportional hazards regression models. Results were expressed as hazard ratios (HR) with their 95% CI. Differences were considered significant when p values were less than 0.05.

## Results

### Patient's Characteristics

The distributions of the socio-economic and clinico-pathologic characteristics of the 104 NPC patients included in this study are summarized in Table 2. There were 63 men (60.6%) and 41 females (39.4%) with a median age of 45 years ranging from 12 to 80 years old. Patients were divided into two age groups: young patients  $\leq 30$  years (24%) and elder patients  $> 30$  years (76%). 27% of the studied patients were smokers (28/104) and 14.4% were alcohol consumers (15/104). The distribution of patients according to their childhood habitat demonstrates that 47.1% were from rural areas (49/104) and 52.9% were from urban areas (55/104). According to the 7th AJCC edition, patients were mainly diagnosed at advanced stages: 27.9% at stage III (29/104) and 59.6% at stage IV (62/104). Reported results of positron emission tomography/computed tomography (PET/CT) and head and neck magnetic resonance imaging (MRI) have shown that most patients were diagnosed at classes T3 (34.6%) and T4 (41.3%) and with lymph node status N1 (27.9%) and N2 (48.1%). Of note, distant metastasis at diagnosis (M1) was detected in 28.8% of patients.

### Association between LTL and Risk of NPC Development

In the present study, 1.33 was used as the cut-off point to distinguish between short ( $\leq 1.33$ ) and long ( $> 1.33$ ) telomeres. The distribution of NPC cases and controls according to LTL showed that 43.3% of cases (45/104) have long telomeres whereas all healthy controls have short telomeres. Statistical analysis showed a significant association between LTL and NPC development ( $p < 0.0001$ ).

### Correlation between LTL and Socio-Demographic and Clinico-Pathological Characteristics

The correlation between LTL and socio-demographic characteristics and clinico-pathological features of recruited patients is reported in Table 2. Our data showed

**Table 2.** The relationship between LTL and clinico-pathological parameters

Parameter	N	LTL (N=104)				OR (CI 95%)	p
		≤1.33		>1.33			
		N	%	N	%		
Gender							
Male	63	43	68.25	20	31.75		0.003
Female	41	16	39.02	25	60.98		
Age							
≤30	25	13	52.00	12	48.00	1.00	
>30	79	46	58.23	33	41.77	0.777 (0.315 - 1.917)	0.584
Cigarette Smoking							
Yes	28	22	78.57	6	21.43	1.00	
No	76	37	48.68	39	51.32	0.3 (0.109 - 0.827)	0.02
Alcohol consumption							
Yes	15	9	60.00	6	40.00	1.00	0.782
No	89	50	56.18	39	43.82	0.855 (0.280 - 2.606)	
Disease stage							
I-II	13	7	53.85	6	46.15	1.00	
III	29	16	55.17	13	44.83	0.948 (0.255 - 3.525)	0.936
IV	62	36	58.06	26	41.94	0.843 (0.253 - 2.802)	0.780
Tumor classification							
T1	8	5	62.50	3	37.50	1.00	0.474
T2	17	8	47.06	9	52.94	1.875 (0.336 - 10.463)	
T3	36	21	58.33	15	41.67	1.190 (0.246 - 5.764)	0.828
T4	43	25	58.14	18	41.86	1.200 (0.254 - 5.678)	0.818
Lymph node status							
N0	8	6	75.00	2	25.00	1.00	
N1	29	20	68.97	9	31.03	1.350 (0.227 - 8.031)	0.742
N2	50	26	52.00	24	48.00	2.769 (0.509 - 15.064)	0.239
N3	17	7	41.18	10	58.82	4.286 (0.661 - 27.785)	0.127
Metastasis status							
M0	74	41	55.41	33	44.59	1.00	
M1	30	18	60.00	12	40.00	0.828 (0.350 - 1.962)	0.669

a significant association between LTL and sex ( $p=0.003$ ) and cigarette smoking ( $p=0.02$ ). In fact, telomeres were most often shorter in men with NPC (68.25%), while most women with NPC have long telomeres (60.98%). Of interest, short telomeres were more frequent in cigarette smokers. Our data further revealed that LTL was not associated with alcohol consumption, overall cancer stage and TNM classification ( $p>0.05$ ).

### The Correlation between LTL and Treatment Response

The correlation between telomere length and treatment outcomes of NPC patients was also investigated and results are reported in Table 3. Data analysis clearly showed

the absence of any significant association between LTL and radio-resistance with an OR=0.662 (CI 95%=0.145-3,018),  $p=0.594$  and chemo-resistance with an OR=0.967 (CI 95%=0.435-2.149),  $p=0.934$ .

### Association between LTL and NPC Patients' Survival Endpoints

In the following study, the estimated survival rates were for a period of 4 years. Our results showed that LTL doesn't correlate with OS ( $p=0.921$ ), LRRFS ( $p=0.628$ ), DMFS ( $p=0.901$ ) and PFS ( $p=0.745$ ) (Table 4). The estimated 4-years rate did not differ between patients with short and long telomeres with 66% vs. 67.9% for OS, 70.6% vs. 69.8% for LRRFS, 50.7% vs. 53.6% for DMFS and 47.8% vs. 39.6% for PFS, respectively.

**Table 3.** Correlation between LTL and response to chemoradiotherapy

Parameter	N	LTL (N=104)				OR (CI 95%)	p
		≤1.33		>1.33			
		N	%	N	%		
Chemo-resistance							
No	53	29	54.72	24	45.28	1.00	0.934
Yes	45	25	55.56	20	44.44	0.967 (0.435-2.149)	
Radio-resistance							
No	61	32	52.46	29	47.54	1.00	0.594
Yes	8	5	62.50	3	37.50	0.662 (0.145-3,018)	

### Association between LTL and EBV-DNA Load

In this study, we have also assessed the correlation between EBV DNA load before treatment initiation (pre-EBV DNA) and LTL in patients with NPC. Our data clearly demonstrated a significant association between LTL and pre-EBV-DNA load with  $p=0.017$  (OR=2.725 and CI 95%= 1.197-6.204). Patients with pre-EBV-DNA load  $\leq 4000$  UI/ml had shorter telomeres than those with EBV-DNA load  $>4000$  UI/ml (Table 5).

### Discussion

The relation between LTL and cancer development was previously documented and discussed, and results are very controversial. Two epidemiological studies have shown a significant association between longer telomeres and a higher risk of developing colorectal and endometrial can-

cers.<sup>[16,17]</sup> However, another Chinese study did not reveal any association between LTL and the risk of lung cancer.<sup>[19]</sup> In NPC, our results have clearly showed that patients with NPC have a longer LTL than the control group, and that LTL was significantly associated with risk of the development of this cancer ( $p<0.0001$ ). In contrast, the single case-control study assessing LTL as a biomarker of NPC risk showed that in peripheral blood leucocytes of patients with NPC, the LTL was shorter compared to controls, which may lead to genomic instability and involves an association between the size of the telomeric extremities and the risk of developing several cancers.<sup>[5]</sup>

In the present study, the prevalence of NPC is higher in males as compared to females, which is in agreement with widely previous reports highlighting the predominance of male gender among NPC patients. Indeed, most pub-

**Table 4.** Association between LTL and clinical survival endpoints.

	LTL		HR (CI 95%)	p
	≤1.33 (%)	>1.33 (%)		
OS	66.0	67.9	0.964 (0.468-1.968)	0.921
LRFS	70.6	69.8	0.801 (0.326-1.967)	0.628
DMFS	50.7	53.6	0.963 (0.530-1.750)	0.901
PFS	47.8	39.6	1.096 (0.631-1.902)	0.745

**Table 5.** Association between LTL and EBV-DNA load

Parameter	N	LTL (N=104)				OR (CI 95%)	p
		≤1.33		>1.33			
		N	%	N	%		
Pre-EBV DNA							
≤4000 UI/ml	44	31	70.45	13	29.55	1.00	0.017
>4000 UI/ml	60	28	46.67	32	53.33	2.725 (1.197-6.204)	

lished papers showed that male-to-female ratio is about 2 and can reach 3 in some countries.<sup>[22]</sup> Of particular interest, the present study highlighted that males have significantly short telomeres compared to females NPC patients ( $p=0.003$ ). This observation is well-aligned with data from Risques et al. reporting a significantly shorter telomeres in males than in females with Barrett's esophagus ( $p=0.027$ ).<sup>[23]</sup> In contrast, no significant interaction was found between LTL and gender neither in patients with bladder Cancer ( $p=0.674$ )<sup>[24]</sup> or in patients with head and neck cancer (HNC) ( $p=0.291$ ).<sup>[25]</sup>

Regarding age, our data didn't reveal any significant interaction while comparing LTL between young patients and patients elder than 30 years. In the same line, other investigators reported the absence of differences in LTL according to the age of patients with HNC ( $p=0.278$ ).<sup>[25]</sup> Nevertheless, recent studies revealed a relationship between shorter telomere length and older age in patients with NPC and breast cancer.<sup>[5,26]</sup>

In the present study, cigarette smoking was found to be associated with LTL. Indeed, smokers have shorter telomeres than non-smokers ( $p=0.02$ ). These findings are in agreement with a large meta-analysis, based on 84 studies, reporting that smokers had shorter telomeres than non-smokers.<sup>[27]</sup> However, in patients with bladder cancer, no significant correlation was observed between LTL and smoking status ( $p=0.875$ ).<sup>[24]</sup> According to most scientific studies, smoking is a well-known health risk factor, and exposure to the toxic chemicals in cigarettes can induce both oxidative stress and permanently damaged telomere DNA.<sup>[27]</sup>

Our data further revealed no significant association between LTL and alcohol consumption status in NPC. These results are inconsistent with those of Alves-Paiva et al. on 97 patients with HNC that have reported a strong association between LTL and the high percentage of non-alcohol drinking patients in the fourth quartile (Q4) ( $p=0.032$ ).<sup>[25]</sup>

In agreement with previously known facts, we didn't find any significant association between LTL and, TNM classification and disease stage in NPC. Consistent with our results, a case-control study conducted in Germany observed no significant correlation between LTL and metastatic disease ( $p=0.612$ ) and overall stage ( $p=0.178$ ) in patients with bladder cancer.<sup>[24]</sup> These results were further confirmed by data reported by Antwi et al. among patients with localized, locally advanced, and metastatic pancreatic cancer.<sup>[12]</sup>

Worldwide, NPC patients are most likely treated by radiotherapy with/without chemotherapy.<sup>[28]</sup> This is mainly due to the deep localization of the nasopharynx and the difficulty to use surgical approaches.<sup>[29]</sup> In the review of

the effects of telomere lengths on chemo-radioresistance, we found no evidence that telomere length was associated with resistance to radiotherapy. It's widely accepted that irradiation could induce more damage in individuals with short telomeres than those with long telomeres, and accordingly, it was assumed that there is a link between mechanisms causing radio-sensitivity and those responsible of telomere damage.<sup>[28]</sup> In this field, signals generated by genes involved in DSB (double strand breaks) repair, LTL regulation, cell cycle checkpoint control and apoptosis can be converted to telomeres. Consequently, telomeres may act as molecular "sensors" that express the cell's ability to react to genotoxic stress. For instance, if telomere maintenance is dysfunctional, it is likely that cells with telomere abnormalities will not be able to respond effectively to genotoxic stresses, including ionizing radiation.<sup>[30]</sup> In the present study, no significant association was found between LTL and radiotherapeutic ( $p=0.594$ ) and chemotherapeutic ( $p=0.934$ ) responses. This could be explained by the fact that the radiation dose is not sufficient to induce DNA damage and/or the follow-up time is not sufficient to adequately appreciate the impact of LTL on responses to radiation.

To determine if survival outcomes was associated with changes in telomere length, we compared the correlation between LTL and OS, LRRFS, DMFS and PFS. Our results proved that telomere length doesn't correlate with survival outcomes of NPC patients. Lu et al. reported also that LTL was not associated with breast cancer clinical features or patient survival.<sup>[31]</sup> In contrast, it has been observed that patients with short telomeres were at risk of poor survival (HR=2.05, CI 95%=1.33–3.16 and  $p=0.001$ ) compared to the patients with long telomeres in the primary cutaneous melanoma.<sup>[32]</sup>

Scientific evidence has clearly shown that EBV infection is closely associated with NPC carcinogenesis.<sup>[33]</sup> In our previous study, we have clearly demonstrated that pre-EBV DNA load was significantly associated with disease progression and patients' survivals (OS) ( $p<0.05$ ).<sup>[20]</sup> Furthermore, in the present study, patients with low pre-EBV-DNA load ( $\leq 4000$  UI/ml) had shorter telomeres compared with those with high EBV-DNA load ( $>4000$  UI/ml) (OR=2.725 and CI 95%=1.197-6.204,  $p=0.017$ ). It remains unclear whether the significantly higher risk of NPC in patients with longer LTL may be due to the role of EBV in NPC tumorigenesis. In this context, it has been documented that EBV infection can induce different key phenotypic properties of malignant transformation, including the escape from replicative cycle. Indeed, EBV latent proteins such as LMP1 have the ability to regulate telomerase activity in NPC cell lines.<sup>[34]</sup> A high number of chromosomes with abnormal telomeres

(usually loss or duplication of telomere signaling and telomere fusions) was found as early as one week after EBV infection. Notably, relatively low telomerase activity levels were detected at day 14, whereas telomere signaling was significantly stronger in EBV-infected cells. EBV infection thus promotes an increase in LTL in the absence of a parallel increase in telomerase activity.<sup>[34]</sup>

The present study is very informative and has much strength mainly related to the study design, the technical approach used for assessment of LTL and the availability of clinico-pathological parameters that served to appreciate the association between telomere length and NPC development. However, the main limitations of the study are the relatively small sample size and the short-term period of patients' follow-up that could not thoroughly in favor of using LTL as a prognostic biomarker.

## Conclusion

The study finding highlighted the close association between LTL and NPC development and showed a significant association with gender, cigarette smoking and EBV load, suggesting that LTL could be a promising biomarker for better management of NPC in Morocco.

## Disclosures

**Ethics Committee Approval:** The study protocol was agreed by the Ethics Committee of Ibn Rochd Hospital of Casablanca, Morocco and informed consents were provided from all participants..

**Peer-review:** Externally peer-reviewed.

**Conflict of Interest:** None declared.

**Authorship Contributions:** Concept – M.K., I.C., M.M.; Design – M.K., I.C., M.E.M.; Supervision – M.K., M.E.M., I.C., K.B.; Materials – M.K., N.T., N.B., A.B., A.B.; Data collection &/or processing – I.E.A., A.G., S.M.B.; Analysis and/or interpretation – I.E.A., H.C., M.E.A., A.G.; Literature – I.E.A.; Writing – I.E.A., A.G.; Critical review – M.K., M.E.M., K.B., I.C.

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