Leukocyte telomere length associates with nasopharyngeal carcinoma risk and survival in Hong Kong Chinese

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Abstract (249 words)

Telomere shortening occurs as an early event in tumorigenesis. The *TERT-CLPTM1L* locus associates with nasopharyngeal carcinoma **(**NPC) risk. It remains unknown if leukocyte telomere length (LTL) associates with NPC risk and survival. The relative LTL (rLTL) was measured by quantitative-PCR in 2996 individuals comprised of 1284 NPC cases and 1712 matched controls. The odds ratio (OR) and 95% confidence intervals (CI) were calculated by logistic regression. The hazard ratio (HR) and 95% CI were calculated by Cox regression for survival analysis with rLTL and other clinical parameters in 1243 NPC with a minimum follow-up period of 25 months. NPC patients had significantly shorter telomere length than controls. Shorter rLTL significantly associated with increased NPC risk, when the individuals were dichotomized into long and short telomeres based on median-split rLTL in the control group (OR=2.317; 95% CI=1.989–2.700, p=4.10x10^{-27}). We observed a significant dose-response association (p_{trend}=3.26x10^{-34}) between rLTL and NPC risk with OR being 3.555 (95% CI=2.853-4.429) for the individuals in the first quartile (shortest) compared with normal individuals in the fourth quartile (longest). A multivariate Cox regression analysis adjusted by age demonstrated an independent effect of rLTL on NPC survival for late stage NPC patients, when the individuals were categorized into suboptimal rLTL versus the medium rLTL based on a threshold set from normal (HR=1.471, 95% CI=1.056–2.048, p=0.022). Shorter blood telomeres may be markers for higher susceptibility for NPC risk. Suboptimal rLTL may be a poor prognostic factor for advanced NPC patients, as it associates independently with poor survival.

Running title: Role of LTL for NPC risk and survival of stage IV patients

Novelty and Impact

The current study provides evidence of an association of individuals with shorter blood telomere length and increased risk of NPC and prognostic role of suboptimal blood telomere length for late stage NPC is the first to strongly implicate the etiologic role of telomere biology in NPC development and disease progression.

Introduction (4761 words)

Nasopharyngeal carcinoma (NPC) is an epithelial squamous cell carcinoma arising in the nasopharynx. NPC worldwide incidence is ethnically and geographically distinct. NPC is endemic disease occurring frequently in southern China, northern Africa and Alaska. The incidence in Hong Kong ranges from 10 - 20 / 100,000 [1]. This incidence remains high for Chinese migrants to southeast Asia or North America, but is lower for second and third generation offspring born in North America compared to those born in Southern China, suggesting NPC etiology is multifactorial, including ethnicity, host genetics, Epstein-Barr virus (EBV) infection, and environmental factors [2]. The peak age for NPC occurs in the upper forties, when oftentimes the patients are the main economic support for the family. Hence, this cancer may seriously impact the livelihood of the family. NPC is usually not diagnosed until advanced disease stage and identifying biomarkers for early detection is important.

Human telomeres consist of ~10 - 15 kb long hexa-nucleotide repeats (TTAGGG)n at the ends of chromosomes. Telomere complexes contain shelterin proteins to protect DNA ends from being recognized as double-stranded breaks (DBSs). It shortens progressively with every cell division due to the lagging DNA strand end replication problem. The telomere shortening from cell division is initially a protection mechanism against tumor progression [3, 4] because cells with critically short telomeres undergo replicative senescence to prevent the accumulation of genomic instability. The growth arrest is known as replicative aging in normal cells and crisis in cancer cells [5]. Telomere biology is crucial in aging and cancer initiation. Telomere length variation plays a causal role in age-related diseases including human cancer [6]. The telomere length from NPC tumor tissues was ~3.2-fold shorter compared to the adjacent tissues suggesting that telomere shortening is involved in disease pathogenesis [7].

A meta-analysis of genome-wide association studies (GWAS) identified *TERT* as one of the seven loci associated with rLTL [6] . Numerous GWAS implicate the *TERT-CLPTM1L* region at 5p15.33 as playing an important and unique cancer genetic predisposition role in human cancers [8-10]. Common germline SNPs in this region associate with both increased and decreased cancer risks. Our previous association study identified the SNP rs401681 in the *TERT-CLPTM1L* region conferring a protective effect for NPC risk in Hong Kong Chinese [11]. Five independent association studies for NPC risk with large sample sizes including GWAS meta-analysis revealed converging evidence to further substantiate that the *TERT-CLPTM1L* locus strongly and consistently is a critical NPC susceptibility locus [12-16]. Although the report of short LTL contributing to higher risk of human papillomavirus (HPV)-associated oropharyngeal carcinoma (OPC) does not prove causality between shorter LTL and HPV infection, as the possibility of shorter LTL resulting from carcinogenesis cannot be ruled out, it supports the inference that short LTL may play a causal role in the development of virally-associated cancers [17]. NPC development is intimately associated with the viral co-factor, EBV. Hence, the association of EBV and TERT loci with NPC prompted us to hypothesize that short blood telomeres associate with NPC risk.

Telomere attrition predicts age-related disease and all-cause mortality [18-22]. There were association reports of either shortest or longest LTL with cancer survival [20, 23-27]. To date, no study has been done to examine rLTL effect on NPC risk and survival. Hence, we aim to address whether blood telomere lengths carry any prognostic information for NPC survival.

Materials and Methods

Retrospective NPC case-control study

In total, 1284 NPC patients were recruited, since 2009 to 2016, from five Hong Kong public hospitals including Queen Mary Hospital (QMH), Queen Elizabeth Hospital (QEH), Tuen Mun Hospital (TMH), Pamela Youde Nethersole Eastern Hospital (PYNEH) and Princess Margaret Hospital (PMH). Study protocols were approved by the Hospital Institutional Review Board and written consents were obtained from all patients. In total, this included 2996 participants. The 1712 control population consisted of 1195 healthy controls collected from the Red Cross and 517 cancer-free hospital controls from QMH. Blood samples were recruited from 1284 NPC new cases and collected at diagnosis prior to any therapy. Patients enrolled with routine staging procedures included physical examination and imaging tests. Staging was defined according to the American Joint Committee on Cancer (AJCC) TNM system. Table 1 summarizes the clinical information of the study population of 2996 individuals. There were 973 men (75.8%) and 311 women (24.2%) with median age of 52 (95% CI, 29.1 – 75.1). Figure S1 shows the workflow of the study for NPC risk and survival analysis. Figure S2 shows the details of age and gender matching of case and controls. Controls were selected by frequency matching to the cases on the basis of age (±10 years) and gender. Blood samples were collected in EDTA tubes. Buffy coats of both cases and controls were frozen in -80 °C until further analysis after removal of plasma.

Real-time PCR measurement of rLTL

Peripheral blood leukocyte (PBL) DNA extraction was performed using Illustra™ blood genomicPrep Mini Spin Kit (GE Healthcare, Little Chalfont, UK) following manufacturer's instructions. DNA was accurately quantified by the Qubit dsDNA HS Assay Kits with the Qubit 2.0 Fluorometer. Relative telomere length was assessed by singleplex Q-PCR assays with a reaction volume of 15 μl in 1X KAPA SYBR® FAST mastermix (Kapa Biosystems, ABI

Prism™) and 10 ng of DNA in 384-well plate by the Roche LightCycler 480 System II. The final reagent concentrations in the reaction mix were 1X KAPA SYBR® FAST master mix (Kapa Biosystems, ABI Prism™), 1M betaine and 1 mM DTT. Primer pairs used for telomere gene and albumin gene, are telg (at 180 nM), telc (at 900 nM), albu (at 900 nM) and albd (at 900 nM). Their 5' to 3' sequences are ACA CTA AGG TTT GGG TTT GGG TTT GGG TTT GGG TTA GTG T (telg), TGT TAG GTA TCC CTA TCC CTA TCC CTA TCC CTA TCC CTA ACA (telc), CGG CGG CGG GCG GCG CGG GCT GGG CGG aaa tgc tgc aca gaa tcc ttg (albu) and GCC CGG CCC GCC GCG CCC GTC CCG CCG gaa aag cat ggt cgc ctg tt (albd) [28]. The PCR thermal cycling profile for telomere and albumin amplicons, was: Stage 1: 5 min at 95^{o}C; Stage 2: 2 cycles of 30 s at 95^{o}C, 1 min at 49^{o}C; Stage 3: 50 cycles of 30 s at 95^{o}C, 30 s at 62^{o}C with single acquisition; and Stage 1: 5 min at 95^{o}C; Stage 2: 2 cycles of 15 s at 95^{o}C, 45 s at 49^{o}C; Stage 3: 50 cycles of 15 s at 95^{o}C, 30 s at 62^{o}C with single acquisition, respectively. To avoid any sample position effect on Ct value, each experimental sample was allocated to the same well positions in the telomere and albumin plates. The NPC cell line CNE2 DNA was used as reference sample and three-fold serial dilutions were performed to prepare six concentrations of CNE2, ranging from 0.27 to 66 ng/ul. These standard samples were prepared in duplicates for each plate, which provided data for the generation of standard curves and PCR efficiencies by LightCycler® 480 Software. Each plate contained equal representation of cases and controls that were randomly selected. The laboratory staff were blind to the case and control status.

Measurement of mean rLTL by real-time Q-PCR for quantification of the (T/S) ratio of telomere repeat copy number (T) to single-copy gene copy number (albumin) (S) was calculated by using the $2^{-(\Delta Ct_T - \Delta Ct_S)} = 2^{-\Delta\Delta Ct}$ method by using SYBR Green real-time PCR for quantification of the T/S ratio (TSR) in the patients' blood DNA [28]. Briefly, the PCR amplicon for the T assay

contains a 78 bp hexanucleotide telomeric repeat. The S assay amplifies a segment from the single-copy human albumin gene for normalization of the sample-to-sample variation of input DNA template amount. The normalization of the amount of starting telomeric repeat units relative to single-copy human albumin gene (ΔCt) was calculated by subtracting Ct(S) from Ct(T), as their amounts should be proportional to $2^{-Ct(T)}$ and $2^{-Ct(S)}$, respectively. The relative "telomere copy number" per genome for each sample should be proportional to $2^{-\Delta Ct}$. All experimental samples were assayed in triplicate. Six cell line DNA controls (over 5% of one batch size) were assayed in triplicate in each run with random well position allocation. Their mean and standard deviation of the relative T/S values for the six cell lines from 21 runs in ~3 month-interval were calculated and the inter-assay mean CV for the relative T/S of the six cell lines was 7.7%. To measure intra-assay reproducibility, the coefficients of variation for Ct values for each of the 112 experimental sample DNA triplicates were calculated in a pair of telomere and albumin runs. The mean intra-assay CV for the telomere run was 0.597% and the mean intra-assay CV for albumin run was 0.422%. To examine inter-plate reproducibility, the telomere run of 96 samples was repeated and strong correlation between the PCR efficiency of telomere run ($E_T^{-\Delta CtT}$) of the two runs was displayed ($R^2 = 0.954$).

Statistical analysis

SPSS version 24 was used for statistical analysis. Logarithmic transformation of TSR (Log TSR) was used for NPC risk and survival analysis so that tests with assumption of normal distribution could be correctly applied. TSR means were compared by independent sample t-test. Individuals were categorized into deciles or quartiles or by median-split to represent long and short rLTL according to the distribution of Log TSR value in the control population. The association between telomere length and the NPC risk was analyzed by logistic regression

adjusted with age and gender. Survival analysis was performed using Kaplan-Meier with the long-rank test and restricted to the 1243 newly diagnosed patients, who were dead and alive with minimum 2-year follow-up. The overall survival was defined as the interval between the date of radiotherapy completion to date of death from any cause. Hazard ratios (HRs) were calculated by univariate and multivariate Cox regression analysis. In the survival analysis, age was used as a continuous variable, and the other clinical parameters including stage and gender, as well as rLTL, were used as a categorical variable. The threshold for suboptimal rLTL was set for short and long telomeres based on the shortest 15th (Log TSR <-0.411) and longest 85th (Log TSR >-0.152) percentiles of Log TSR of our 1712 control cohort (Figure S3). The medium blood telomere length is defined as the Log TSR falling into the intermediate range between 15th and 85th percentile of the control cohort. The 30% cut-off for suboptimal LTL, including both short and long LTL based on the control cohort, is chosen based on the power estimation. With this cut-off, we will achieve 70% of the power for the survival analysis estimated based on the method described previously [29]. In the multivariate analysis, only the variables significantly associated with overall survival in the univariate analysis were considered. The interactions between the variables in the multivariate analysis were examined. Statistical significance of all tests was defined as $p \leq 0.05$, two tailed.

Results and Discussion

Short blood telomeres associate with increased NPC risk

NPC tumorigenesis involves a complex interplay between the environmental, viral, stromal and extracellular matrix, host immune response, and genetic components. Our previous findings aimed to elucidate the genetic etiology of NPC by performing an association study with a candidate gene approach [11]. The association of genetic variations of telomere-related gene at

TERT-CLPTM1L loci for NPC risk prompted us to examine rLTL. Telomere shortening occurs in NPC development evidenced by shorter telomeres detected in primary NPC tissues[7], but the effect of inter-individual variation of LTL for NPC risk remains unknown. We report novel findings from the current case-control association study of NPC risk and blood telomere length with 2996 individuals suggesting that shorter rLTL associates with a substantially higher NPC risk. The log normalized TSR was significantly shorter in 1284 NPC cases (mean Log TSR = -0.343, 95% CI = -0.629 – -0.057) than in 1712 age and sex matched controls (mean Log TSR = -0.283, 95% CI = -0.559 – -0.007) by t-test, $p = 1.245 \times 10^{-29}$ (Table 1 and Figure S3). When the individuals were dichotomized into long and short rLTL based on the median normalized rLTL in the control group, NPC patients with short rLTL had a significantly increased NPC risk (OR=2.317; 95% CI=1.989 – 2.700, $p=4.10 \times 10^{-27}$, Table 2), after adjustment for age and gender. When the individuals were categorized into four groups according to quartile rLTL in the control group, a significant dose-response association between normalized rLTL and NPC risk was observed with OR in the shortest group (first quartile), second, and third quartiles being 3.555, 1.929 and 1.360, respectively, ($p = 3.26 \times 10^{-34}$) (Table 2). When the individuals were categorized into ten groups according to decile rLTL in the control group, NPC patients with the shortest rLTL significantly associated with NPC risk compared to controls in the 10^{th} decile with longest telomere length (OR in the shortest group = 4.388, 95% CI = 3.082 – 6.246, $p = 2.23 \times 10^{-16}$) (Table 2). In the distribution of telomere lengths among the epidemiological and clinical variables (stage, gender and age) in the current cohort of controls and cases, it was observed that males had significantly shorter telomere lengths in the controls and age was negatively correlated with telomere length in the cases (Table S1). This result is consistent with previous studies in which the shorter telomere length is associated with older age and a gender effect is present [30].

Further stratification analysis by utilizing the mean age of 52 showed that the estimated ORs were much higher in the older NPC patients (age > 52) (OR in the shortest group = 6.427, 95% CI = 4.533 – 9.131, $p = 1.59 \times 10^{-25}$) versus that of younger NPC patients (age ≤ 52) (OR in the shortest group = 2.108, 95% CI = 1.579 – 2.815, $p = 4.27 \times 10^{-7}$) (Table 3). The results suggest that there is a stronger risk effect in the older individuals with short rLTL.

Although telomere lengths are often shorter in tumor cells than their normal counterparts, recent studies have demonstrated that both short and long blood telomeres associated with cancer risk in a cancer type-specific manner [31-34]. Short rLTL associated with specific cancer types including the oropharyngeal, bladder, lung, pancreatic, renal, gastrointestinal and head and neck cancers [17, 22, 34-37]. Individuals with the shortest decile rLTL may have approximately four-fold higher risk of NPC compared to individuals with the longest decile rLTL (Table 2). It had been shown that telomere length measurement in easily accessible blood could serve as a surrogate parameter of telomere length in other tissues [38]. Our observation of a higher risk of NPC in patients with shorter telomeres is consistent with the etiological hypothesis that assumes telomere shortening in the peripheral blood is an indication of older biologic age and the shortening in leukocytes reflects the shorter tissue telomeres. Short rLTL associating with NPC risk can be explained by the critical role telomeres and telomerase plays in bypass senescence (M1) and crisis (M2) and the immortalization of cells with critically short telomere length associated with the loss of *p16*/*Rb*/*p53,* thus, resulting in genomic instability that fuels carcinogenesis [5]. Recent cancer risk studies in the Chinese population reported a U-shape association between short and long telomere length and esophageal squamous cell carcinoma and gastric cancer risk; such a U-shape phenomenon was not observed for NPC risk in Hong Kong Chinese [32, 33]. However, our current novel findings suggest patients with short and long telomeres

associated with a poorer survival rate in late stage IV NPC patients (Figure 1Ab). The association of short rLTL with higher NPC risk may be attributed to rLTL is suggested to be a marker of immune competence [24, 26, 39]. Short LTL associated with higher risk of infections and infection-related deaths possibly due to age-related impaired adaptive immune response [39]. NPC is a lymphoepithelioma with frequent lymphoid cell infiltration in the nasopharynx [1], suggesting NPC tumorigenesis is highly related to immune surveillance. For a subset of NPC patients (N=858) with EBV infection status available in the current study, there were 784/858 (91.4%) EBV-positive NPC patients detected by the EBV serology or EBV plasma tests. There is no statistically significant difference in the telomere length between EBV-positive and EBV-negative NPC patients (Log TSR_{EBV+} = -0.361 Vs Log TSR_{EBV-} = -0.349, p = 0.561, t-test). It remains unknown whether the substantially higher NPC risk for patients with shorter rLTL may be due to the role EBV plays in NPC tumorigenesis. EBV infection induces acute infectious mononucleosis (IM), particularly common among teenagers and young adults. The affected IM patients develop massive expansion of CD8+ T-cells to control the spread of EBV. Substantial telomere shortening occurs in those EBV-specific CD8+ memory T-cells. The telomere length depends on the telomere maintenance capability in the memory CD8+ T-cell pool upon persistent EBV re-stimulation over time [40]. It is unknown if EBV infection in NPC patients results in similar telomere attrition and clonal expansion of EBV-specific CD8+ T-lymphocytes. During aging, the decline of the adaptive immune system may be attributed partially to T cell replicative senescence, which is characterized by short telomere length, loss of the CD28 co-stimulatory molecules and higher pro-inflammatory cytokines [41]. Shorter LTL associated with higher risk of HPV-associated oropharyngeal carcinoma [17]. However, it remains unknown for any such viral-associated correlations of short telomere length and EBV status in NPC risk.

Future studies are required to more comprehensively examine the relationship between rLTL and the EBV infection status in order to provide evidence to address this issue.

Gender, stage, and age are independent prognostic factors for NPC patient survival

Of the 1243 NPC patients, there were 25.7 % (320/1243) deaths. The median follow-up of 923 alive NPC patients was 52 months and ranged from 25 to 87 months. The prognostic factors of NPC survival are summarized for HRs according to different clinical parameters in Table 4 with univariate Cox regression analysis. Age, as a continuous variable, male gender, stage IV, were all significantly associated with poor survival. Since short or long blood telomere lengths had been reported for association with cancer deaths [18, 20, 23, 25, 26], we hypothesize that both short and long blood telomere lengths may be associated with poor survival in NPC patients. Patients with the shortest blood telomeres (Log TSR < -0.411) and longest blood telomeres (Log TSR >-0.152) were associated significantly with survival compared to patients with medium blood telomeres (Log TSR between -0.411 and -0.152) by univariate analysis with Cox regression (HR, 95% CI = 1.323, 1.059 – 1.652, p = 0.014) (Table 4). Three clinical variables, including age, gender, and stage and the rLTL groups were tested for their interactions that remain significant in the multivariable Cox analysis. The results show that age was potentially interacting with stage and gender, with a p = 0.025 and p = 0.063, respectively. Thus, we included a three-way interaction in the analysis (Table S2). The multivariate Cox regression analysis showed that age, gender, stage, and their interaction (age*gender*stage) illustrated in Table S2, but not the rLTL, are significant prognostic factors for NPC patient survival (Table 4). A strong linear correlation between age and rLTL (p = 1.56 x 10^{-19}) was found in the NPC cases, which explained only a trend of association result for rLTL and survival in the multivariate analysis.

Blood telomere length is an independent prognostic marker of stage IV NPC patients

The overall survival rate of NPC patients was 74.6% (916/1228). However, when the NPC patients were stratified according to TNM stage, a significantly lower survival rate of 57.4% (198/345) was observed in late stage IV NPC, compared with earlier stages I/II and III, with 87.3% (165/189) and 79.7% (553/694), respectively (Figure 1Aa). Given the interaction observed between age, gender and stage, we stratified the patients into six subgroups according to their status of early (I+II+III) or late (IV) TNM stage and short or medium or long rLTL based on the observation of interaction between age, gender and stage. Kaplan-Meier analyses showed that the patients with long rLTL at early TNM stage (I/II/III) had the best survival, whereas those with short and long rLTL in late TNM stage IV had the worst survival, with HR being 3.48 and 5.27, respectively (Figure 1Ba). Since males had about 2-fold higher risk of death, the cohort was further stratified by gender; the Kaplan-Meier analyses showed similar effects of worst survival with higher HR for both male and female patients with short and long rLTL at late TNM stage IV (Figures 1Bb and c).

The survival analysis was performed in the earlier stage (I/II and III) and late stage (IV) groups, respectively (Table S3). The independent prognostic value of gender was only observed in the early-stage group, while age remains a strong independent prognostic factor in both early and late-stage groups. Interestingly, in the late stage IV patients having poorer survival, the association between rLTL and overall survival becomes significant and is independent from other clinical parameters. Kaplan-Meier survival analysis further indicated late stage IV NPC patients with suboptimal blood telomere length are associated with a poorer survival rate, 47.2% (58/123), compared with those with medium blood telomere length, 63.1% (140/222), Log-rank test, $p = 0.003$ (Figure 1Ab). When the late stage NPC patients were categorized into short, medium, and long rLTL, Kaplan-Meier survival analysis consistently indicated patients with

short and long blood telomeres had poorer survival rates of 49.0% (47/96), and 40.7% (11/27), respectively, compared to those with medium blood telomere length, 63.1% (140/222), Log-rank test, $p = 0.014$ (Figure 1Ac). We constructed a multivariate Cox regression model for prognosis in the late-stage IV patients using the stepwise forward likelihood ratio approach. Only age and the blood telomere length were included in the final model with the estimated HR (95% CI) for suboptimal versus medium blood telomeres being 1.471 (1.056 – 2.048, $p = 0.022$) (Table 5).

In general, short rLTL associates with cancer mortality [18, 20, 22], as critically short rLTL results in replicative senescence and genome instability due to chromosome ends being fused and recognized as double-strand break DNA damage. However, the prognostic value of rLTL has cancer type-specific predictive effects on survival. Short rLTL predicts poor survival in colorectal, bladder and gastric cancers [24-26]. Long rLTL predicts poor survival in gliomas, prostate, and renal cell carcinomas [23, 27, 42]. However, this current NPC study suggests for the first time that late stage IV NPC patients with short or long, i.e. suboptimal, blood telomere length associates with poor survival compared to those carrying medium rLTL. The prognostic effect of blood telomere length is dependent on age, since no significant association, but only a trend, was observed after the addition of age as the covariate in the multivariate COX regression analysis in all NPC patients. However, when the sample cohort was stratified by early stage I, II and III versus late stage IV, since the stage IV NPC patients show substantially poorer survival, suboptimal blood telomere length was independently associated with poor survival for advanced NPC patients (stage IV). Although the sample size in our study of survival was relatively large (N=1243) and the medium follow-up time was 52 months, an additional validation cohort is necessary. The independent prognostic biomarker role of blood telomere length was suggested to be due to altered immune functions in colorectal cancer patients [26]. A subgroup of prostate

ancer patients with longer LTL had a poorer clinical outcome and this is possibly linked to the uppressed immune system, so that blood cells of those patients undergo fewer cell divisions and ave less severe telomere attrition [27]. Although the association of both long and short TL with oor prognosis is unexpected, but it is biologically plausible. Emerging evidence suggested the isequilibrium of telomere maintenance may act as a double-edged sword in carcinogenesis. lomere attrition is an initial anti-tumor protection mechanism limiting the cell division cycles hat every cell can undergo. As a result, almost all precancerous lesions possess shortening elomeres and, hence, results in chromosomal instability, when cells survive the "crisis" ypassing senescence or apoptosis [19]. In contrast, long telomeres may promote cancer evelopment, as it allows for more cell divisions and an accumulated higher number of bnormalities [43]. Another possible hypothesis is the "immune-hypothesis", where the patients ith long telomeres may have greater suppression of the immune response; the leukocytes in heir less active immune system undergo fewer cell divisions and there is less telomere attrition, hus, resulting in poorer immune surveillance. Two studies in kidney cancer and hepatocellular arcinoma showed a positive correlation between blood telomere length and the level of mmunosuppressive regulatory T cells (Tregs), in line with the immune-hypothesis [44, 45]. Further n-depth studies are warranted to characterize the immune-related differences between patients ith long and short telomeres. Another possibility that long rLTL associates with poor survival as attributed to the greater challenge for DNA repair and replication for long telomeres, which re more prone to suffer from oxidative damage and secondary structure formation. A recent eview suggested the defects in the Fanconi Anemia pathway led to dysregulation of telomere engtth homeostasis [46]. Telomere attrition predicts age-related disease and all-cause mortality [18-1]. Our current data provide new information of short rLTLs not only increasing NPC risk, but

that telomere length homeostasis may be crucial for survival prognosis in advanced stage IV NPC patients. The imbalance of telomere length equilibrium and not only telomere attrition predicts poor survival in NPC patients with aggressive stage IV cancer (Figure 1Ab and 1Ac). Our preliminary observation suggests a critical role of telomere maintenance in the hematological system for NPC risk and survival. The etiologic and prognostic role of mean LTL may be related to the pro-inflammatory processes generated by immune cell senescence [21]. The imbalance of blood telomere length equilibrium may be representing the age-related decline of the immune system and, hence, affects health. Independent studies are required to validate the novel findings for association of increased NPC risk with short blood telomere length and the prognostic effect for poor survival of late stage IV NPC patients with suboptimal blood telomere length versus those with medium blood telomere observed in the current study.

The current study design is a retrospective case-control association study, which has the inherent limitation of possible reverse causation, as we could not know if the telomere shortening is the cause or the consequence of NPC, since the blood samples were collected after NPC diagnosis [47]. Our data may fit an alternative hypothesis that other unknown factor(s) causal for NPC development may shorten the telomere length. To indirectly address this concern, we estimated the ORs after stratified analysis of early stage and late stage patients. The estimated ORs are slightly higher in the late-stage patients compared to early-stage patients, although it does not reach statistical significance (Table S4). A recent review pointed out the relationship between leukocyte telomere length and cancer risk is a dynamic problem with fluctuations over time, dependent not only on cancer-type, but also histology [48]. Our result may not be generalizable to other endemic regions of NPC with different races, which affect telomere length, as this is the first study reporting the association of rLTL with NPC risk and prognosis in

the Hong Kong Chinese population. Future studies in other endemic regions are warranted to validate our observations in the Hong Kong NPC cohort in different ethnic groups. Future large cross-sectional and prospective study design with multiple blood collections scheduled with long follow-up time are warranted to more accurately assess the dynamic changes of LTL with cancer risk longitudinally. The primary concern of a retrospective case control association study is potential selection bias. We only included newly diagnosed NPC patients with collected blood samples before treatment and age and gender matched with controls to alleviate selection bias. Our studies may have potential confounding factors including genetic factors, as well as non-genetic factors such as psychological, stress, aging, life-style habits like exercise, smoking, and nutrition, and other environmental exposures like oxidative damage, that affect telomere length. Further epidemiological studies are required to more systematically address the impact and interactions between rLTL and other genetic, psychological, sociological and environmental factors with NPC risk.

The blood telomere length measurement is simple and non-invasive. Future studies may explore the potential usefulness of combining EBV plasma test and rLTL assay together to increase precision to predict survival. We expect the accumulated knowledge in blood telomere length will provide invaluable information for the identification of high-risk NPC individuals. Ultimately this may result in earlier cancer detection and also better identification of the subgroup of patients having aggressive tumors with poor prognosis. Such knowledge will translate into improved overall survival.

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Table 1: Clinical information of study population for 2996 individuals for rLTL analysis

	Case N = 1284 (%)	Control N = 1712 (%)	Total N = 2996 (%)	*p*[a]
Age				
Mean ± SD	52.11 ± 11.743	51.72 ± 11.659	51.89 ± 11.695	NS
Median	52	52	52	
Range	12 – 89	16 - 91	12 – 91	
Sex				NS
Female	311 (24.2)	415 (24.2)	726 (24.2)	
Male	973 (75.8)	1297 (75.8)	2270 (75.8)	
Normalized TSR				
Mean ± SD	0.480 ± 0.174	0.548 ± 0.173	0.519 ± 0.177	**4.192×10^{-26}**
Median	0.458	0.534	0.502	
95% CI	0.139 - 0.821	0.209 - 0.887	0.172 - 0.886	
Log TSR				
Mean ± SD	-0.343 ± 0.146	-0.283 ± 0.141	-0.309 ± 0.146	**1.245×10^{-29}**
Median	-0.339	-0.273	-0.299	
95% CI	-0.629 - -0.057	-0.559 - -0.007	-0.595 - -0.023	

[a] independent sample t-test between cases and controls

Table 2: rLTL association with NPC risk by logistic regression analysis (N = 2996)

Variable	Case N = 1288 (%)	Control N = 1712 (%)	OR (95% CI)	p^a
By median				
0 (Short)	897 (69.9)	856 (50.0)	**2.317 (1.989 – 2.700)**	**4.10×10^{-27}**
1 (Long)	387 (30.1)	856 (50.0)	1 (reference)	
By quartile			***p* for trend 3.26×10^{-34}**	
0 (Shortest)	581 (45.2)	428 (25.0)	**3.555 (2.853 – 4.429)**	**1.19×10^{-29}**
1	316 (24.6)	428 (25.0)	**1.929 (1.530 – 2.431)**	**2.66×10^{-8}**
2	223 (17.4)	428 (25.0)	**1.360 (1.067 – 1.732)**	**1.30×10^{-2}**
3 (Longest)	164 (12.8)	428 (25.0)	1 (reference)	
By decile			***p* for trend 1.51×10^{-29}**	
0 (Shortest)	261 (20.3)	171 (10.0)	**4.388 (3.082 – 6.246)**	**2.23×10^{-16}**
1	209 (16.3)	171 (10.0)	**3.497 (2.447 – 4.998)**	**6.41×10^{-12}**
2	180 (14.0)	171 (10.0)	**3.009 (2.097 – 4.318)**	**2.27×10^{-9}**
3	128 (10.0)	172 (10.0)	**2.125 (1.464 – 3.085)**	**7.40×10^{-5}**
4	119(9.3)	171 (10.0)	**1.984 (1.363 – 2.889)**	**3.53×10^{-4}**
5	101(7.9)	171 (10.0)	**1.683 (1.147 – 2.470)**	**8.00×10^{-3}**
6	80(6.2)	172 (10.0)	1.327 (0.893 – 1.971)	0.16
7	73 (5.7)	171 (10.0)	1.219 (0.815 – 1.823)	0.33
8	73 (5.7)	171 (10.0)	1.216 (0.813 – 1.817)	0.34
9 (Longest)	60 (4.7)	171 (10.0)	1 (reference)	

[a] *p* for logistic regression adjusted for age and sex

Table 3: Age stratification analysis of rLTL association with NPC risk

Variable	Case N = 1288 (%)	Control N = 1712 (%)	OR (95% CI)	p^a
Age <= 52				
By quartile	Total N = 686	Total N = 905	***p* for trend** 6.01×10^{-7}	
0 (Shortest)	249 (36.3)	234 (25.9)	**2.108 (1.579 – 2.815)**	**4.27×10^{-7}**
1	183 (26.7)	213 (23.5)	**1.716 (1.270 – 2.319)**	**4.40×10^{-4}**
2	142 (20.7)	234 (25.9)	1.216 (0.894 – 1.656)	0.213
3 (Longest)	112 (16.3)	224 (24.8)	1 (reference)	
Age > 52				
By quartile	Total N = 602	Total N = 807	***p* for trend** 2.14×10^{-32}	
0 (Shortest)	334 (55.5)	194 (24.0)	**6.427 (4.533 – 9.113)**	**1.59×10^{-25}**
1	133 (22.1)	215 (26.6)	**2.324 (1.605 – 3.366)**	**8.00×10^{-6}**
2	81 (13.5)	194 (24.0)	**1.575 (1.059 - 2.342)**	**0.025**
3 (Longest)	54 (9.0)	204 (25.3)	1 (reference)	

[a] *p* for logistic regression adjusted for age and sex

Table 4: Univariate and multivariate Cox regression survival analysis of age, gender, TNM stage, and blood telomere length in all NPC patients

Clinical parameters	New Cases	Hazard ratio (95% CI)	*p*
Univariate analysis	**N = 1243**		
Age	-	**1.039 (1.030 - 1.049)**	**3.037×10^{-16}**
Gender			
Female	301	Reference	
Male	940	**2.072 (1.516 – 2.833)**	**5.000×10^{-6}**
Total	1245		
Stage			
I + II + III	883	Reference	
IV	345	**2.790 (2.233 - 3.486)**	**1.717×10^{-19}**
Total	1232		
Log TSR			
Medium (-0.411 - -0.152)	793	Reference	
Short (<-0.411) and Long (>-0.152)	448	**1.323 (1.059 – 1.652)**	**0.014**
Total	1241		
Survival			
Yes	923		
No	320		
Total	1243		
Multivariate analysis	**N = 1232[a]**		
Age			
	-	**1.041 (1.031 - 1.051)**	**1.476×10^{-15}**
Gender			
Female	300	Reference	
Male	928	**2.265 (1.515 – 3.386)**	**6.800×10^{-5}**
Stage			
I + II + III	883	Reference	
IV	345	**4.745 (2.848 – 7.906)**	**2.266×10^{-9}**
Log TSR			
Medium (-0.411 - -0.152)	786	Reference	
Short (<-0.411) and Long (>-0.152)	442	1.224 (0.974 - 1.538)	0.083

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Interaction			
Age*Gender*Stage	-	**0.988 (0.979 – 0.997)**	**0.013**

The median follow-up of 923 alive NPC patients was 52 months and ranged from 25 to 87 months. Forty-one NPC patients with follow-up time of less than 25 months were excluded from survival analysis.

[a]320 NPC patients died

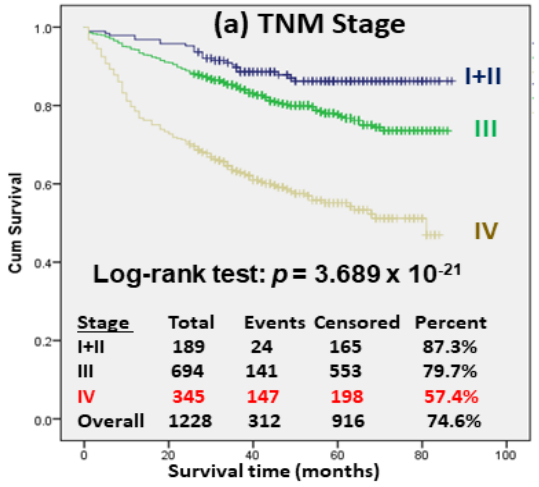
Table 5: Multivariate Cox regression model for prognosis in the late-stage IV patients.

Variables	Cases N = 345[a]	Hazard ratio (95% CI)	*p*
Age			
	345	**1.025 (1.011 - 1.038)**	**3.37×10^{-4}**
Log TSR			
Medium (-0.411 - -0.152)	222	Reference	
Short (<-0.411) and Long (>-0.152)	123	**1.471 (1.056 – 2.048)**	**0.022**

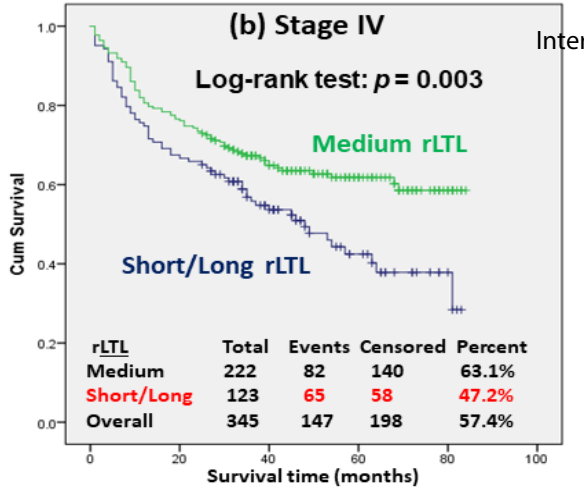
[a]147 patients died.

Figure Legend

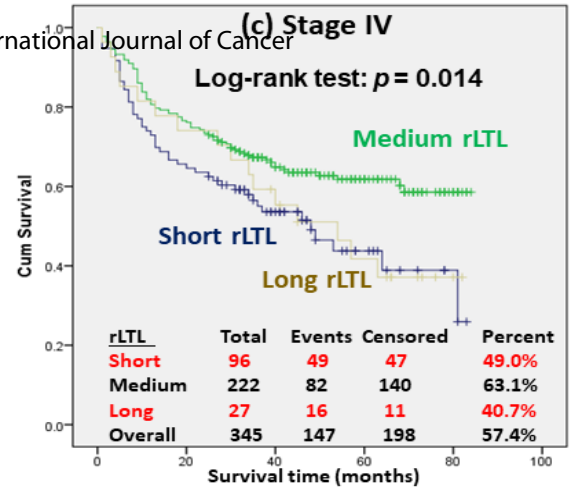
Figure 1(A): Kaplan-Meier survival analysis of NPC patients comparing (a) TNM Stage in all NPC patients (N= 1228) (I+II, III, IV) and sub-stratified as stage IV NPC patients (N=345) with (b) medium (Log TSR between -0.411 and -0.152) versus short (Log TSR <-0.411) / long (Log TSR >-0.152) blood telomere length and (c) short versus medium versus long blood telomere length by the Log-rank test. **(B):** Kaplan-Meier survival analysis of NPC patients subgrouped by the rLTL (short/medium/long) and stages (I/II/III versus IV) by the Log-rank test (a) all NPC; (b) male NPC patients; and (c) female NPC patients. Hazard ratios and 95% CIs were calculated by multivariate Cox proportional hazards regression model, adjusted for age and sex in (a), adjusted for age in (b) & (c).



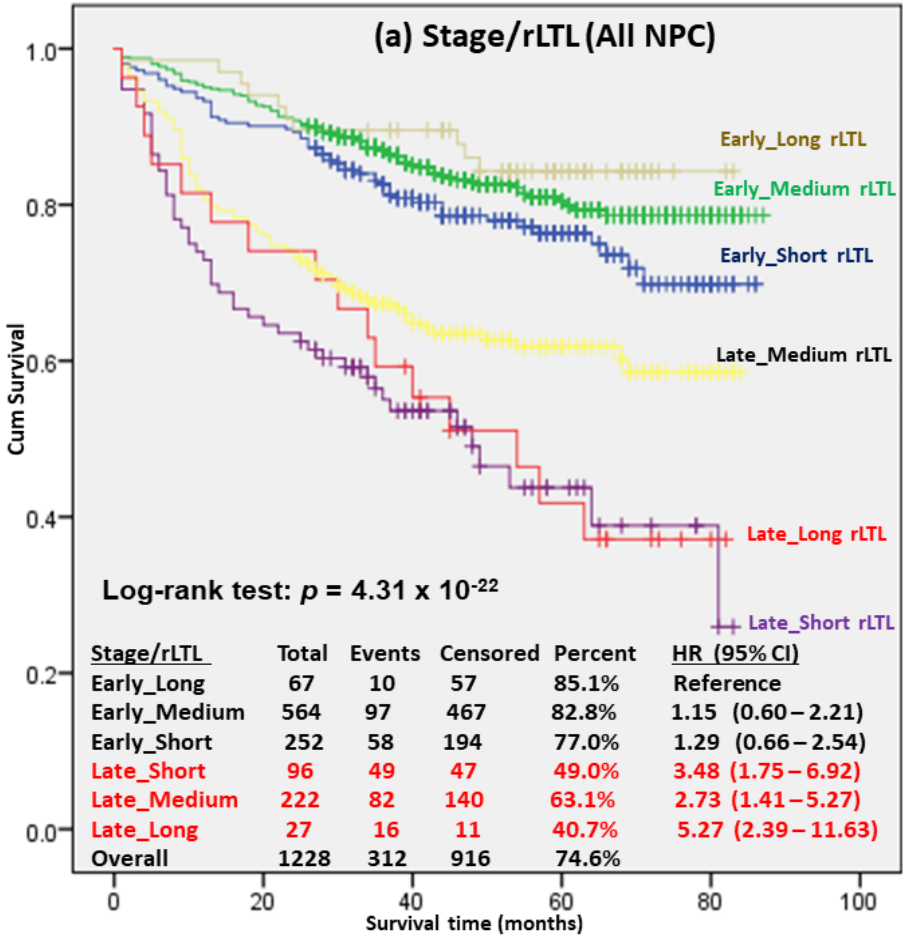
Stage	Total	Events	Censored	Percent
I+II	189	24	165	87.3%
III	694	141	553	79.7%
IV	345	147	198	57.4%
Overall	1228	312	916	74.6%



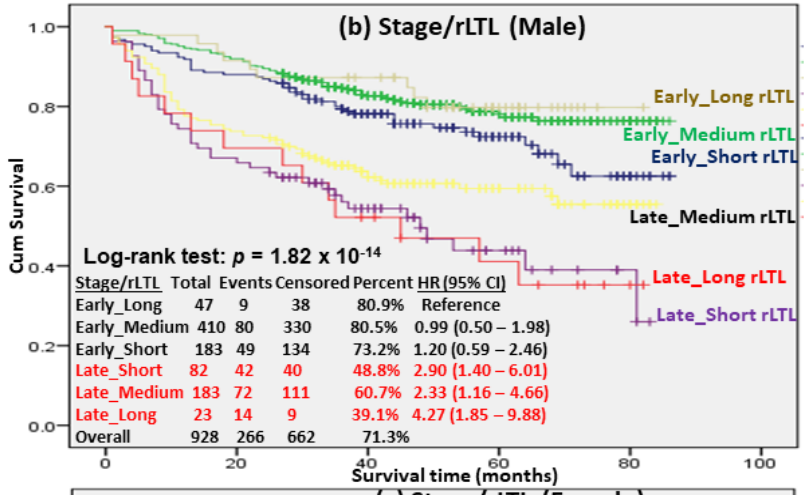
rLTL	Total	Events	Censored	Percent
Medium	222	82	140	63.1%
Short/Long	123	65	58	47.2%
Overall	345	147	198	57.4%



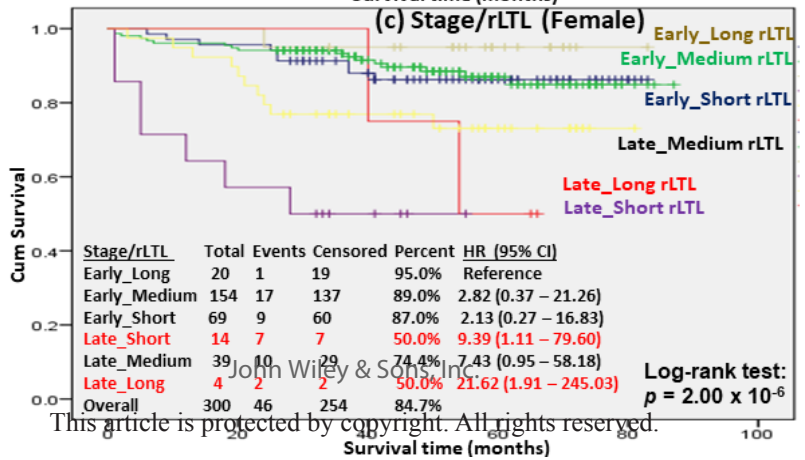
rLTL	Total	Events	Censored	Percent
Short	96	49	47	49.0%
Medium	222	82	140	63.1%
Long	27	16	11	40.7%
Overall	345	147	198	57.4%



Stage/rLTL	Total	Events	Censored	Percent	HR (95% CI)
Early_Long	67	10	57	85.1%	Reference
Early_Medium	564	97	467	82.8%	1.15 (0.60 – 2.21)
Early_Short	252	58	194	77.0%	1.29 (0.66 – 2.54)
Late_Short	96	49	47	49.0%	3.48 (1.75 – 6.92)
Late_Medium	222	82	140	63.1%	2.73 (1.41 – 5.27)
Late_Long	27	16	11	40.7%	5.27 (2.39 – 11.63)
Overall	1228	312	916	74.6%	



Stage/rLTL	Total	Events	Censored	Percent	HR (95% CI)
Early_Long	47	9	38	80.9%	Reference
Early_Medium	410	80	330	80.5%	0.99 (0.50 – 1.98)
Early_Short	183	49	134	73.2%	1.20 (0.59 – 2.46)
Late_Short	82	42	40	48.8%	2.90 (1.40 – 6.01)
Late_Medium	183	72	111	60.7%	2.33 (1.16 – 4.66)
Late_Long	23	14	9	39.1%	4.27 (1.85 – 9.88)
Overall	928	266	662	71.3%	



Stage/rLTL	Total	Events	Censored	Percent	HR (95% CI)
Early_Long	20	1	19	95.0%	Reference
Early_Medium	154	17	137	89.0%	2.82 (0.37 – 21.26)
Early_Short	69	9	60	87.0%	2.13 (0.27 – 16.83)
Late_Short	14	7	7	50.0%	9.39 (1.11 – 79.60)
Late_Medium	39	10	29	74.4%	7.43 (0.95 – 58.18)
Late_Long	4	2	2	50.0%	21.62 (1.91 – 245.03)
Overall	300	46	254	84.7%	