

Chromosomal damage and telomere length in peripheral blood lymphocytes of cancer patients

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Received November 29, 2019; Accepted July 24, 2020

DOI: 10.3892/or.2020.7774

Abstract. Accumulation of non-specific structural chromosomal aberrations (CAs) and telomere shortening contribute to genome instability, which constitutes as one of the hallmarks of cancer. CAs arise due to direct DNA damage or telomere shortening. CAs in peripheral blood lymphocytes (PBL), which are considered to be markers of exposure, have been previously reported to serve a role in the pathophysiology and progression of cancer through mechanisms that are

poorly understood. In addition, the prognostic relevance of telomere length (TL) in patients with cancer remains to be elucidated. In the present study, CAs and TL in PBL isolated from patients with newly diagnosed cancer (151 breast, 96 colorectal, 90 lung) and 335 cancer-free control individuals were investigated. These results were then correlated with clinicopathological factors and follow-up data. The accumulation of CAs in PBL was observed with increased susceptibility to breast and lung cancer ($P < 0.0001$), while individuals with longer TL were found to be at a higher risk of breast cancer ($P < 0.0001$). Increased chromatid-type aberrations were also revealed to be associated with lower overall survival of patients with breast and colorectal cancers using a multivariate model. Compared with control individuals, no association was observed between TL and CAs or age in patients with cancer. In conclusion, the present study demonstrates the association between CAs/TL in PBL and the susceptibility, prognosis and survival of patients with breast, colorectal and lung cancer.

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Abbreviations: ACs, aberrant cells; aOR, adjusted odds ratio; BC, breast cancer; CART, classification and regression tree; CAs, chromosomal aberrations; CA_{tot}, total chromosomal aberrations; CI, confidence interval; CIN, chromosomal instability; CRC, colorectal cancer; CSAs, chromosome-type aberrations; CTAs, chromatid-type aberrations; Her2, ErbB2 (Her2/neu) receptors; HR, hazard ratio; LC, lung cancer; OR, odds ratio; OS, overall survival; PBL, peripheral blood lymphocytes; PCR, polymerase chain reaction; PRs, progesterone receptors; RFS, recurrence-free survival; rs, Spearman's ρ ; RTL, relative telomere length; TL, telomere length; TNM, Tumor-Node-Metastasis

Key words: structural chromosomal aberrations, telomere length, peripheral blood lymphocytes, breast cancer, colorectal cancer, lung cancer, cancer susceptibility, patient long-term survival

Introduction

Tumorigenesis is a multistep process that is driven by a number of genetic alterations, such as chromosomal rearrangements, which result in selective advantages during the process of cellular transformation, tumor development and progression (1,2). The majority of cancers also undergoes substantial epigenetic alterations that alter the expression profile of critical genes during the malignant transformation process (3).

Acquired non-specific structural chromosomal aberrations (CAs) describe a group of multiple and diverse intra- or inter-chromosomal rearrangements. Structural CAs can be of either chromatid-type (CTAs) or chromosome-type (CSAs) depending on the stage of cell cycle and mechanism of clastogenic compounds involved. They arise as a consequence of either direct mutagenic effects and/or dysfunctions in

DNA repair during the entire lifetime of an individual (4-6). Measurement of CAs in peripheral blood lymphocytes (PBL) is conventionally used for monitoring environmental and occupational exposure to clastogens (7,8). Several European prospective and retrospective studies have previously demonstrated that increased frequencies of CAs in PBL can be applied as predictive markers for increased cancer susceptibility (9-12).

A number of CAs can occur as the primary events during cancer initiation, following which disease progression drives additional chromosomal changes, which can be used to reflect prognosis and cancer progression (13). Compared with specific chromosomal rearrangements that occur in tumor tissues during carcinogenesis, non-specific structural CAs in PBL can serve as a marker of cancer susceptibility. This hypothesis, which was summarized by Boffetta *et al* (14), was based on the presumption that genetic damage in PBL is reflective of changes in cells undergoing malignant transformation.

The formation of CAs has also been previously associated with the process of telomere shortening (15-17). Telomeres are unique chromatin structures at the ends of linear eukaryotic chromosomes characterized by tandem repeats (TTAGGG)_n and associated proteins (18,19). Telomeres progressively shorten due to limitations in DNA synthesis after successive cycles of mitosis (20). Excessive telomere shortening can lead to proliferation arrest and cellular apoptosis. Additionally, loss of telomeres can result in a telomere crisis, causing genome instability leading to structural CAs (21). Therefore, telomere length (TL) serves a critical role in the maintenance of genome integrity and cancer initiation/progression.

In the present study, the potential association between acquired non-specific structural CAs and TL in the PBL of patients with a sporadic form of the breast (BC), colorectal (CRC) and lung cancer (LC) and control individuals was investigated. In addition, the potential prognostic relevance of CAs and TL was also addressed.

Materials and methods

Study population. The present study was performed by pooling data from cytogenetic studies performed between January 2006 and May 2013. The study population consisted of three groups of newly diagnosed and histologically confirmed individuals with BC (n=151), CRC (n=96) and LC (n=90), along with a group of control individuals (n=335). Patients were recruited at the 3rd Faculty of Medicine, Charles University (Prague, Czech Republic) and the Jessenius Faculty of Medicine, Comenius University and University Hospital (Martin, Slovakia).

Control individuals who were matched for both age and sex, including 145 women for comparison with women with BC, were recruited at the Blood Centre of Faculty Hospital Kralovske Vinohrady (Prague, Czech Republic) and from the Department of Surgery, General University Hospital (Prague, Czech Republic).

The present study adhered to the ethical guidelines of the Helsinki Declaration. All participants were informed about all aspects of the study, agreed with the purpose of the study and procedures to be undertaken, following which they provided informed consent. The present study design was approved by the local Ethics Committees of all participating hospitals.

Samples and data collection. Blood samples (2x5 ml) were obtained from control individuals and patients with cancer. Fresh blood samples treated with heparin were used immediately for cytogenetic chromosomal analysis after transportation at 4°C whereas blood samples treated with EDTA were frozen at -20°C for subsequent DNA isolation and TL measurement.

Information regarding patient characteristics, including demographics, family history of cancer, smoking habit, occupational history, body mass index and the presence of other diseases, including hypertension, diabetes mellitus, cardiovascular disease, along with their treatment regimen, were collected at the time of diagnosis using a structured questionnaire. Information on disease characteristics, including tumor location, tumor-node-metastasis (TNM) stage, histopathological grade, histological classification and the presence of hormonal receptors and administration of hormonal therapy in cases of BC were all collected following surgical resection. The characteristics of the study population were partially described previously by Vodenkova *et al* (12). Detailed data of the subjects in the present study are provided in the Table S1. The last follow-up date of the patients in the present study was July 2017.

The exclusion criteria for the recruitment of cancer patients in the present study were as follows: i) Personal history of previous malignancies; ii) hereditary forms of cancer; iii) prior use of radiotherapy or chemotherapy, to avoid the presence of additional chromosomal damage related to the treatment; and iv) prior use of X-rays, such as those used for diagnostic purposes. In addition to data from questionnaires and medical reports, the last criterion was also verified by comparing the frequencies of typical post-radiation aberrations including dicentric and ring chromosomes, in the study population. The frequencies of these CAs did not differ between the patients with cancer and corresponding controls (12). For survival analyses, the following additional exclusion criteria were applied for patients: i) Those who had either missing data regarding their living status <5 years; ii) underwent a 2nd surgery for unknown reasons; or iii) had experienced duplicate tumors. Consequently, 4 patients with LC were disqualified from the final analysis. The entire data set comprised 208 patients who were still alive and 125 patients who were deceased as a result of their respective cancers.

Chromosomal analysis. Levels of structural CAs in PBL were evaluated using the standard cytogenetic method as previously described by Vodicka *et al* (11). Briefly, blood cultivation was performed in complete medium (Chromosome medium P; cat. no. EKAMTB-100; EuroClone S.p.A.) for 50 h at 37°C. After 48 h cultivation, cell division was stopped using colchicine (Sigma-Aldrich; Merck KGaA) during the first metaphase of mitosis. After a cytogenetic procedure, microscopic slides were stained conventionally using Giemsa (Sigma-Aldrich; Merck KGaA). Microscopical analysis of 100 metaphases with 46±1 chromosomes was performed blindly by two independent scorers for each sample. The percentages of aberrant cells (ACs), total chromosomal aberrations (CA_{tot}), CTAs (chromatid breaks and exchanges) and CSAs (chromosome breaks, terminal and interstitial deletions, dicentric and ring chromosomes with their difragments and abnormal chromosomes) were calculated. Representative microscopic images of chromosomes with different types of CAs are presented in Fig. S1.

Standardization procedure has been applied for scoring CAs in the former Czechoslovakia and later in both Czech Republic and Slovakia (22). To minimize inter-laboratory and inter-scorer differences, random exchanges of microscope slides were performed between the two laboratories in Prague and Martin as a part of the standard operating procedure.

Measurement of TL. TL was measured as relative telomere length (RTL) using monochrome multiplex real-time qPCR assay as previously described by Cawthon (23) with slight modifications (24-26). All reactions were performed in triplicates in an optical 384-well reaction plate. Real-time PCR experiments were performed on a Viia 7 Real-time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) using two simultaneous programs to acquire the respective cycle threshold values for the telomere sequences and albumin gene. RTL was expressed as the ratio between telomere (T) and albumin (S; single-copy gene), which is also known as the T/S ratio. Examples of the results from RTL measurements are presented in Fig. S2.

Statistical analysis. Statistical analysis of the data was performed using the SAS 9.4 software (SAS Institute Inc.). The results were processed into graphs using Statistica 12 (StatSoft, Inc.). $P < 0.05$ was considered to indicate a statistically significant difference. Multiple pairwise comparisons were corrected using the Bonferroni method ($P = 0.025$). Descriptive statistical analysis was conducted for the measured parameters on the whole data set in addition to individual groups.

Differences between controls and patients were tested using the median two-sample test or χ^2 -test. Association between the examined variables were tested using Spearman's rank correlation coefficient, which is expressed by Spearman's ρ value (r_s) and graphically plotted using linear regression. Subjects were stratified into two groups based on the frequency of CAs and TL using the median values measured for control individuals. The effect on cancer risk was determined by logistic regression and calculated by estimating the odds ratios (ORs) with the 95% confidence intervals (CIs). ORs were adjusted for age and sex (aORs). Only age was used for the adjustment of ORs for patients with BC, since all corresponding controls were female.

Clinical outcomes were evaluated by calculating patients' 5-year overall survival (OS) and recurrence-free survival (RFS). OS was defined as the time from the date of diagnosis to the date of death, which would then be marked as a negative event or the last follow-up (July 2017), which would be marked as censored data. RFS was calculated from the date of diagnosis to the date of local recurrence, distant metastasis discovery or death, which would then be marked as a negative event, or last follow-up (patients without any event) which is defined as censored data. The relative risk of mortality or recurrence was estimated using the hazard ratio (HR) with 95% CIs using Cox regression. Multivariate survival analysis, referred to as a classification and regression tree (CART) (27), was performed using the Cox regression model to identify the most prognostically significant interactions between the factors investigated and the OS and RFS of the patients. Covariates used in the CART were baseline and clinicopathological factors alongside all types of CAs and TL.

Results

Case-control study. Levels of structural CAs, including ACs, CA_{tot}, CTAs and CSAs, were found to be significantly higher in patients with BC and LC compared with those in the corresponding control groups. Following multiple testing corrections, no increased chromosomal damage was observed in patients with CRC compared with that in the control individuals (Table I). Individuals with higher frequencies of chromosomal damage were revealed to be at a higher risk of BC (ACs, aOR=4.81, $P < 0.0001$; CA_{tot}, aOR=4.49, $P < 0.0001$; CTAs, aOR=2.43, $P = 0.0004$; CSAs, aOR=2.31, $P = 0.0008$) and LC (ACs, aOR=3.82, $P < 0.0001$; CA_{tot}, aOR=3.44, $P < 0.0001$; CTAs, aOR=3.30, $P < 0.0001$). Although no association between CAs and CRC risk was found in the present set of patients, both age and sex were revealed to be significantly associated with the risk of CRC and LC (Table II).

Patients with BC exhibited significantly longer TL compared with control individuals ($P < 0.0001$; Table I), where longer TL was associated with an increased risk of BC (aOR=6.49; $P < 0.0001$; Table II). By contrast, patients with CRC and LC demonstrated moderately shorter TL compared with that in control individuals (Table I), which in turn was not associated with the risk of either types of cancer (Table II). A negative correlation was observed between the different types of CA and TL in individuals in the control group (ACs, $r_s = -0.21$, $P = 0.0004$; CA_{tot}, $r_s = -0.22$, $P = 0.0003$; CTAs, $r_s = -0.10$, $P = 0.10$; CSAs, $r_s = -0.22$, $P = 0.0002$; Fig. S3A). In addition, a significant correlation was also recorded between TL and age ($r_s = -0.62$; $P < 0.0001$) and between CSAs and age ($r_s = 0.17$; $P = 0.001$) in control individuals. Associations between all types of CAs tested and TL (Fig. S3B-D) or between all the studied parameters and age (data not shown) in patients with cancer were not detected, except for in patients with LC, who exhibited a significant correlation between ACs/CA_{tot}/CSAs and age (AC, $r_s = 0.23$, $P = 0.03$; CA_{tot}, $r_s = 0.22$, $P = 0.04$; CSAs, $r_s = 0.22$, $P = 0.04$).

Survival analyses. Significant differences were observed in both OS and RFS among patients with BC, CRC and LC ($P < 0.0001$, data not shown). After 5 years, the alive/dead proportion of patients with BC was 134/17 (88.7%), 51/45 (53.1%) for patients with CRC and 23/63 (26.7%) for patients with LC. The RFS of patients was also found to significantly differ by cancer type ($P < 0.0001$, data not shown). For BC, 122 patients survived but 29 patients experienced an event during the follow-up period. Patients with CRC were notably more likely to experience an event and therefore demonstrated reduced RFS, with 36 patients surviving asymptotically, events occurring in 60 patients. In patients with LC, the RFS was almost identical to that in OS, with 20 patients showing asymptomatic survival and 66 afflicted with event occurrence. As a result, each of the individual cancer groups were analyzed separately.

Univariate survival analyses. All data from the univariate analysis of the impact of well-established prognostic factors such as baseline and disease characteristics in addition to the CAs, including ACs, CA_{tot}, CTAs, CSAs, and TL on the OS and RFS of patients were generated using the Cox regression hazard

Table I. The levels of chromosomal damage (ACs, CA_{tot}, CTAs, CSAs) and TL in each cancer patient group and their corresponding controls [data were modified according to Vodenkova *et al* 2015 (12)].

	Female controls (N=145)							Breast cancer patients (N=151)							
	Mean	SD	95% CI	Range	Min	Median	Max	Mean	SD	95% CI	Range	Min	Median	Max	P-value
ACs	1.63	1.20	1.43-1.82	6	0	2	6	2.62	1.57	2.36-2.87	7	0	3	7	<0.0001^a
CA _{tot}	1.72	1.31	1.50-1.93	6	0	2	6	2.73	1.64	2.47-2.99	7	0	3	7	<0.0001^a
CTAs	1.00	0.99	0.84-1.16	4	0	1	4	1.63	1.34	1.41-1.85	6	0	1	6	<0.0001^a
CSAs	0.70	0.90	0.55-0.84	5	0	0	5	1.10	1.03	0.93-1.27	4	0	1	4	0.0005^a
TL	1.54	0.82	1.40-1.69	3.17	0.61	1.23	3.78	1.88	0.79	1.72-2.03	5.06	0.57	1.77	5.63	<0.0001^a
	Controls (N=335)							Colorectal cancer patients (N=96)							
	Mean	SD	95% CI	Range	Min	Median	Max	Mean	SD	95% CI	Range	Min	Median	Max	P-value
ACs	1.76	1.30	1.62-1.90	6	0	2	6	2.13	1.45	1.83-2.42	6	0	2	6	0.03
CA _{tot}	1.89	1.45	1.73-2.04	7	0	2	7	2.26	1.67	1.92-2.60	8	0	2	8	0.06
CTAs	1.07	1.00	0.96-1.18	4	0	1	4	1.46	1.31	1.19-1.72	7	0	1	7	0.02
CSAs	0.81	1.10	0.69-0.93	6	0	0	6	0.80	1.00	0.60-1.00	4	0	1	4	0.78
TL	1.77	0.81	1.67-1.86	3.18	0.60	1.57	3.78	1.54	0.41	1.44-1.64	2.13	0.90	1.48	3.03	0.33
	Controls (N=335)							Lung cancer patients (N=90)							
	Mean	SD	95% CI	Range	Min	Median	Max	Mean	SD	95% CI	Range	Min	Median	Max	P-value
ACs	1.76	1.30	1.62-1.90	6	0	2	6	2.84	1.49	2.53-3.16	6	0	3	6	<0.0001^a
CA _{tot}	1.89	1.45	1.73-2.04	7	0	2	7	2.88	1.53	2.56-3.20	7	0	3	7	<0.0001^a
CTAs	1.07	1.00	0.96-1.18	4	0	1	4	1.83	1.34	1.55-2.11	6	0	2	6	<0.0001^a
CSAs	0.81	1.10	0.69-0.93	6	0	0	6	1.06	0.99	0.85-1.26	4	0	1	4	0.0007^a
TL	1.77	0.81	1.67-1.86	3.18	0.60	1.57	3.78	1.54	0.93	1.22-1.85	5.42	0.77	1.35	6.19	0.06

The table summarizes the results obtained in the case-control study calculated by Median Two-Sample Test. The levels of ACs, CA_{tot}, CTAs, and CSAs were evaluated using the standard cytogenetic method. TL was measured as relative telomere length, and its measurement was conducted using the monochrome multiplex polymerase chain reaction. Statistically significant values are in bold. ^aResults that passed the Bonferroni test for multiple pairwise comparisons. ACs, aberrant cells; CA_{tot}, total chromosomal aberrations; CI, confidence interval; CSAs, chromosome-type aberrations; CTAs, chromatid-type aberrations; N, number of samples; SD, standard deviation; TL, telomere length.

Table II. Association of CA frequencies (ACs, CAtot, CTAs, CSAs) and TL with the risk of particular types of studied cancers [data were modified according to Vodenkova *et al* 2015 (12)].

	Breast cancer (N=151)			Colorectal cancer (N=96)			Lung cancer (N=90)		
	aOR	95% CI	P-value	aOR	95% CI	P-value	aOR	95% CI	P-value
ACs	4.81	2.84-8.12	<0.0001^a	1.19	0.71-1.98	0.52	3.82	2.27-6.42	<0.0001^a
Age (years)	0.99	0.97-1.01	0.27	1.04	1.02-1.06	<0.0001	1.06	1.04-1.08	<0.0001
Sex (0 for females)	-	-	-	1.85	1.10-3.10	0.02	3.18	1.72-5.90	0.0002
CAtot	4.49	2.69-7.50	<0.0001^a	1.04	0.63-1.73	0.87	3.44	2.05-5.77	<0.0001^a
Age (years)	0.99	0.97-1.01	0.18	1.04	1.02-1.06	<0.0001	1.06	1.04-1.08	<0.0001
Sex (0 for females)	-	-	-	1.87	1.11-3.13	0.02	3.32	1.79-6.15	0.0001
CTAs	2.43	1.49-3.97	0.0004^a	1.56	0.96-2.53	0.07	3.30	1.97-5.52	<0.0001^a
Age (years)	0.99	0.98-1.01	0.49	1.04	1.02-1.06	<0.0001	1.06	1.04-1.08	<0.0001
Sex (0 for females)	-	-	-	1.84	1.10-3.09	0.02	3.40	1.84-6.29	<0.0001
CSAs	2.31	1.42-3.77	0.0008^a	0.92	0.57-1.47	0.73	1.75	1.03-2.96	0.04
Age (years)	0.99	0.97-1.00	0.13	1.04	1.02-1.06	<0.0001	1.06	1.04-1.08	<0.0001
Sex (0 for females)	-	-	-	1.87	1.12-3.13	0.02	3.70	2.02-6.76	<0.0001
TL	6.49	3.00-14.04	<0.0001^a	0.76	0.39-1.48	0.42	0.89	0.36-2.21	0.80
Age (years)	1.02	1.00-1.05	0.09	1.03	1.00-1.05	0.04	1.05	1.02-1.08	0.003
Sex (0 for females)	-	-	-	1.66	0.91-3.01	0.10	4.94	1.97-12.39	0.0007

The table summarizes the results obtained in the case-control study. aORs with 95% CIs for all studied types of CAs and TL for individual cancer types are expressed by Logistic regression analysis. The levels of ACs, CAtot, CTAs, and CSAs were evaluated using the standard cytogenetic method. TL was measured as relative telomere length, and its measurement was conducted using the monochrome multiplex polymerase chain reaction. Statistically significant values are in bold. ^aResults that passed the Bonferroni test for multiple pairwise comparisons. ACs, aberrant cells; aOR, adjusted odds ratio; CAtot, total chromosomal aberrations; CI, confidence interval; CSAs, chromosome-type aberrations; CTAs, chromatid-type aberrations; N, number of samples; TL, telomere length.

Table III. Continued.

	Breast cancer patients (N=151)						Colorectal cancer patients (N=96)						Lung cancer patients (N=86)						
	OS			RFS			OS			RFS			OS			RFS			
	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	HR	95% CI	P-value	
ACs	1.99	0.75-5.24	0.16	1.96	0.88-4.39	0.10	1.06	0.57-2.01	0.85	1.13	0.63-2.01	0.68	0.89	0.51-1.56	0.69	0.99	0.58-1.69	0.97	
Under (Ref) vs. Over median value																			
CAtot	1.82	0.69-4.79	0.23	1.78	0.80-3.96	0.16	1.14	0.61-2.13	0.67	1.20	0.68-2.12	0.53	0.89	0.51-1.56	0.69	0.99	0.58-1.69	0.97	
Under (Ref) vs. Over median value																			
CTAs	1.13	0.44-2.94	0.80	0.83	0.37-1.84	0.64	1.33	0.74-2.39	0.35	1.33	0.77-2.28	0.30	1.35	0.79-2.30	0.27	1.34	0.80-2.25	0.27	
Under (Ref) vs. Over median value																			
CSAs	0.47	0.14-1.65	0.24	0.85	0.35-2.02	0.71	1.51	0.76-2.98	0.24	1.64	0.88-3.06	0.12	0.91	0.50-1.65	0.75	0.95	0.53-1.69	0.86	
Under (Ref) vs. Over median value																			
TL	1.02	0.34-3.05	0.97	0.72	0.29-1.77	0.47	1.20	0.59-2.41	0.62	1.21	0.63-2.34	0.57	0.88	0.42-1.86	0.73	0.88	0.42-1.86	0.73	
Under (Ref) vs. Over median value																			

The table summarizes the results obtained by univariate assessment of the impact of well-established prognostic factors as well as ACs, CAtot, CTAs, CSAs, and TL on overall (OS) and recurrence-free (RFS) survival expressed by Cox regression hazard model. Statistically significant values are in bold. *The most suitable cut-offs for age (in years): OS and RFS (BC, CRC, LC) = 72,73,57, respectively. * Not calculated because all patients in stage I survived. ACs, aberrant cells; CAtot, total chromosomal aberrations; CI, confidence interval; CSAs, chromosome-type aberrations; CTAs, chromatid-type aberrations; ERs^{+/+}, α-estrogenic receptors positive/negative; Her2^{+/+}, ErbB2 (Her2/neu) receptors positive/negative; HR, hazard ratio; md, missing data; N, number of samples; OS, overall survival; PRs^{+/+}, progesterone receptors positive/negative; RFS, recurrence-free survival; TL, telomere length; TNM, tumor-node-metastasis.

model and are presented in Table III. TNM stage, histopathological grade, histological classification and tumor location were found to modulate OS and RFS differently. Advancing age was found to be associated with lower OS and RFS in patients with BC and CRC. In patients with BC, the absence of both hormonal receptors and hormonal therapy was associated with lower OS and RFS. CAs and TL were not revealed to significantly associate with OS and RFS in patients with any cancer.

Multivariate survival analyses. To assess the prognostic utility of all the variables, the interactive effects of all studied types of CAs and TL together in association with baseline and disease characteristics were explored using a CART analysis. Covariates used in the CART were baseline and clinicopathological factors, specifically age, sex, family history of cancer, smoking status, TNM stage, histopathological grade, histological classification, tumor location, type of subsequent therapy received and the presence of hormonal receptors in breast tumors, in addition to all types of CAs and TL. The first split at the top of the tree denote the prognostic factor with the highest statistical significance. TNM stage was found to be the first parameter to split from the tree for predicting both OS (Fig. 1) and RFS (Fig. 2) in patients with BC and CRC, whereas the laterality of tumor was found to be the point of first split in patients with LC.

In patients with BC, additional factors apart from TNM stage were also revealed to serve key prognostic roles in predicting both OS and RFS. Among the variables found to be key determinants of the OS tree structure in patients with BC were progesterone receptors (PRs), histopathological grade, age and CTAs (Fig. 1A). Among TNM stages 0 + I + II + III, the next split revealed an interaction between PR positivity (positive vs. negative, 94.9 vs. 85.4%) and histopathological grade (grades 1+2 vs. grades 3+4, 98.5 vs. 86.7%). Subsequently, patients with grade 1+2 BC would display better OS if they were aged >48 years (>48 years vs. <48 years, 100 vs. 90%). By contrast, patients with grade 3+4 BC would exhibit superior OS provided their levels of CTAs were <2 (0-2 vs. 3-7, 95 vs. 70%). In terms of RFS, patients with BC were subsequently divided into two categories after the initial split – TNM stage 0 + I + II and TNM III + IV (Fig. 2A). In the TNM 0 + I + II group, the subsequent split was again found to be PR positivity/negativity (positive vs. negative, 94.3 vs. 65.1%), which interacted with human epidermal growth factor receptor 2 (Her2/neu or ErbB2) positivity/negativity. The prognosis of patients with BC negative for PRs was improved by the presence of Her2 receptors (positive vs. negative, 87.5 vs. 50%). Similarly, this was the case in patients with BC positive for PRs, where the next most statistically significant factor for predicted RFS was the presence of Her2 receptors (positive vs. negative, 100 vs. 88.8%). Following stratification for PR positivity, RFS of patients with BC negative for Her2 was then found to be influenced by age (<64 vs. >64 years, 90.8 vs. 83.3%). In addition, patients with BC aged <64 years demonstrated CSA frequency as the terminal node of the CART analysis for RFS. Patients with BC without observable CSAs displayed worse prognosis (0 vs. 1-4, 72.7 vs. 100%).

The only significant split found after the TNM stage was CTA frequency in patients with CRC (Fig. 1B). Patients with CRC in TNM stage III would have dramatically increased OS if they exhibit CTA levels <1 (0-1 vs. 2-7, 84.6 vs. 31%). The only signifi-

cant prognostic factor connected with RFS appeared to be the TNM stage in patients with CRC in the present study (Fig. 2B).

The OS of patients with LC was found to be associated with tumor laterality and histological classification (Fig. 1C), where patients with left-sided LC of the pulmonary type were more likely to survive after 5 years compared with patients with LC of the bronchogenic type (pulmonary vs. bronchogenic, 24.5 vs. 0%). Similarly to OS, patients with left-sided LC of pulmonary histology also exhibited superior RFS (pulmonary vs. bronchogenic, 24.5 vs. 0%; Fig. 2C). Additionally, patients with right-sided LC demonstrated an age-dependent association with prognosis.

Discussion

Both accumulations of chromosomal damage and telomere shortening contribute to genome and chromosomal instability (CIN) (28-30). The primary aim of the present study was to investigate the relationship between the quantity of particular types of chromosomal damage, including aberrant cells (ACs), total chromosomal aberrations (CA_{tot}), chromatid-type aberrations (CTAs) and chromosome-type aberrations (CSAs), and telomere length (TL) in the peripheral blood lymphocytes (PBL) of patients with newly diagnosed cancer and corresponding control individuals. The results were analyzed alongside the baseline, clinicopathological and follow-up data. The impact of increased CAs and shorter TL on the risk of the most common cancers breast cancer (BC), colorectal cancer (CRC) and (lung cancer) LC was also investigated. To the best of our knowledge, no previous study has addressed the association between the frequencies of CAs and TL in the PBL of both patients with cancer and control individuals. The present study also examined the possible prognostic utility of these aforementioned biomarkers in assessing the 5-year OS and RFS of patients with BC, CRC and LC.

The present study successively concludes the series of long-term cytogenetic studies that were launched in 2006, which initiated with the collection of samples from patients with cancer and control individuals. Initially, in the first retrospective case-control study, the frequencies of chromosomal damage were first measured in 300 newly diagnosed patients with cancer matched with 300 control individuals (11). In that previous study, the group of patients was mainly comprised of those with breast, colorectal, prostate, uterus + ovary, head + neck and bladder + kidney cancers. Comparing the entire group of patients with cancer with control individuals, significant differences in the CA frequencies were found. However, following the classification of each individual patient by tumor type, only patients with certain types of solid tumors differed compared with controls in terms of CA frequencies. Therefore, the present study extended the number of patients by focusing on the cancer types with the highest rates of prevalence, namely BC, CRC and LC. In a previous study, increased levels of chromosomal damage markers were reported in patients with BC, CRC and LC compared with control individuals (12). In the present study, a complex regression analysis was performed to determine the predictive power of each type of CAs. Although statistically significant aORs were found for particular types of CAs in patients with cancer compared with those in control individuals, only higher frequencies of ACs and CA_{tot} were found to influence the risk of BC and LC whereas

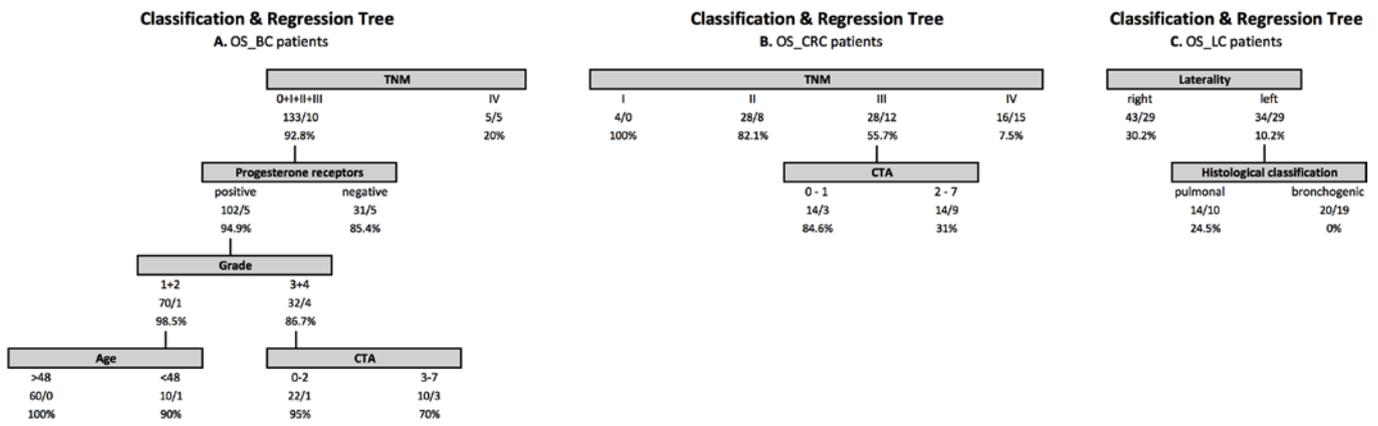


Figure 1. OS classification and regression trees. Classification and regression trees represent the results of multivariate survival analyses of patients with (A) BC, (B) CRC and (C) LC, generated using the Cox regression hazard model. Numbers under each node represent the total number of cases in a particular subcategory/number of events. Percentages below indicate the proportion of patients who survived after 5 years. BC, breast cancer; CRC, colorectal cancer; CTAs, chromatid-type aberrations; LC, lung cancer patients; OS, overall survival; TNM, tumor-node-metastasis.

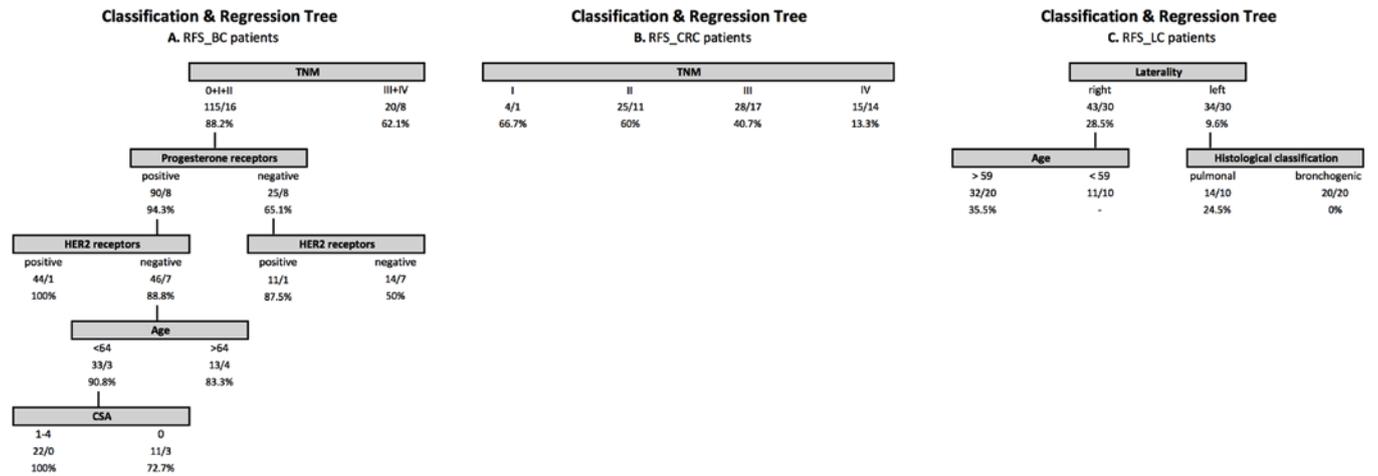


Figure 2. RFS classification and regression trees. Classification and regression trees represent the results of multivariate survival analysis of patients with (A) BC, (B) CRC and (C) LC, generated using the Cox regression hazard model. Numbers under each node represent the total number of cases in a particular subcategory/number of events. Percentages below indicate the proportion of patients who survived after 5 years. BC, breast cancer; CRC, colorectal cancer; CSAs, chromosome-type aberrations; LC, lung cancer; RFS, recurrence-free survival; TNM, tumor-node-metastasis.

and CTAs were found to influence the risk of LC from a clinical standpoint. Specifically, patients with elevated ACs and CA_{tot} frequencies were at a higher risk of BC by 48 and 45%, respectively and 38 and 34% for LC, respectively. In addition, higher quantities of CTAs were found to increase the risk of LC by 33%. These observations confirmed the findings from previous studies, which were summarized by Bonassi *et al* (10). A cohort study conducted in Central Europe previously suggested that CSAs rather than CTAs are more accurate predictors of cancer risk (14). However, a pooled analysis involving >1,000 control individuals revealed an equally strong predictive power of both CTAs and CSAs for cancer (9).

Bonassi *et al* (10), did not find any of the covariates tested, specifically sex, smoking habit, age, time from the test and occupational exposure, to be potential modifiers of the effect of CA levels on the overall cancer risk in the whole cohort. In previous studies (11,12), an effect of age and smoking on cancer risk was found, where these factors were employed to adjust the effect of CAs on BC, CRC and LC risk. The difference in the role of potential effect modifiers could be due to the

difference in studied populations. Bonassi *et al* (10) conducted a study on cancer-free subjects who underwent CA screening for biomonitoring purposes, while our research studied groups consisting of newly diagnosed cancer patients. Cancer incidence is frequently dependent on age which, along with TNM stage, are well-established prognostic factors. However, data on CAs in the present study could be biased by the differences in stage/grade of the tumor and other clinicopathological characteristics at the time of diagnosis.

Measurement of CA frequencies in patients with cancer and healthy individuals was supplemented by TL determination in the present study. Telomeres are nucleoprotein structures that protect chromosomal ends from exonucleolytic degradation and the inappropriate activation of DNA repair pathways, which frequently results in the end-to-end fusion of non-homologous chromosomes (31). Li *et al* (15) and Xu *et al* (16), provided evidence that CAs may arise as a consequence of telomere dysfunction and the mechanisms associated with telomere shortening instead of direct DNA damage *per se*. Additionally, sub-telomeric regions appear to be hotspots for the formation

of symmetrical exchanges between homologous chromatids. Cryptic aberrations in these regions have been previously shown to be associated with a number of human congenital abnormalities (4).

Comparing the TL between individuals in cancer and control groups, patients with BC were found to exhibit significantly longer TL compared with control individuals. In addition, patients with BC exhibited the longest TL of all groups investigated in the present study, including individuals in the CRC, LC and control groups. This observation may be explained by the fact that females typically harbor longer telomeres compared with males (32), where the set of patients with BC in the present study are comprised of only women. A number of hypotheses have been postulated to clarify this association, one of which is the presence of estrogen (33). An estrogen-responsive element has been previously demonstrated in telomerase reverse transcriptase (*hTERT*), a catalytic subunit of the enzyme telomerase (34). Telomerase is responsible for extending the telomeric DNA at the very tips of linear chromosomes, thereby preventing the loss of genetic material in proliferating cells (18). Estrogen is also known to stimulate telomerase activity in breast cancer cells (35) where a functional estrogen response element has been identified in the proximal promoter of the *hTERT* gene. Therefore, direct estrogen effects have been considered to be a part of the telomerase induction mechanism (36). Supporting this, overexpression of estrogen has been reported to be one of the typical features of breast tumors and risk factors for BC.

The present set of patients with BC with longer telomeres were found with a 65% increased risk of BC. However, previous prospective and retrospective studies both showed that the association between TL in PBL and the risk of BC remains controversial. Several studies found an elevated risk among women with longer telomeres (37,38) whereas other studies showed short telomere lengths to be associated with increased risk of BC (39-41). In addition, a number of previous studies also found no significant association between these two parameters (42-44).

Regarding the relationship between CAs and TL, Hemminki *et al* (17) previously demonstrated that shorter TL correlates with elevated levels of CAs in control individuals. In the present study, the same trend of significant negative correlation between ACs/CA_{tot}/CSAs and TL in the entire group of controls was observed independently. It has been widely reported that telomeres progressively shorten with increased age. Initial TL can vary among individuals, where the rate of telomere shortening reflects replicative exhaustion during aging (45). In the absence of a telomere maintenance mechanism, cells eventually undergo replicative senescence (20). In the present group of control individuals, dependence of TL on age was found, where telomeres gradually shortened with advancing age. Age was also revealed to be associated with the accumulation of CSAs in control individuals. The association between this finding and the lower DNA repair rate in older subjects remains to be elucidated.

In the present study, it should be noted that apart from the correlation found between TL and age in control individuals, all the remaining statistically significant results should not be overinterpreted since the Spearman's ρ values were small. It means that even though the relationships analyzed were statisti-

cally significant, their biological relevance may remain limited. In a previous study, Bernadotte *et al* (45) proposed that instead of measuring the average TL, it would be reasonable to consider the quantity of shortened telomeres as an indicator of cellular and tissue proliferative potential. This is because cells do not exhaust their proliferative potentials in a simultaneous manner. Notably, patients with cancer did not exhibit any associations between TL and any type of CAs. Since there is no available literature that previously described this phenomenon, it can only be hypothesized that in complex and multifactorial diseases such as cancer, as yet unknown interactive pathways may exist that contribute to DNA damage and formation of CIN. Therefore, there is a need to conduct additional studies to elucidate this relationship between CAs and TL in the PBL of patients with cancer. We also did not record any clear correlation between TL and age in cancer patients. This was in contrast with the results obtained from the majority of previous studies covering this topic, which were summarized by Xu *et al* (46) and found that TL negatively correlated with age in patients with cancer.

Although CAs in PBL are proven markers of exposure, the role of this parameter in the pathophysiology and progression of cancer remain poorly understood (47). This uncertainty also applies for the prognostic value of TL in PBL for patients with cancer. The hypothesis that variations in CA frequencies and TL are determinants of prognosis is plausible to explain the heterogeneity in the clinical outcomes of patients with cancer. This therefore warrants further attention. In the present study, the prognostic power of CAs and TL was determined in all groups of patients with cancer. Association was not observed between any types of CAs and overall survival (OS) or recurrence-free survival (RFS) using univariate survival analysis. However, outputs obtained using the CART analysis revealed the involvement of CTAs in predicting the survival/mortality of patients, such that elevated CTAs was found to be a terminal node for lower OS for patients with BC at TNM stages 0 + I + II + III and for patients with CRC at TNM stage III. For CRC, an increase in CTAs >1 was found to worsen OS by ~55%. Of note, patients with BC without any CSAs exhibited inferior RFS. Based on these observations, different types of CAs may serve different roles in both cancer risk and patient prognosis. Numerous studies have previously explored the relationship between chromosomal rearrangements/CIN in PBL and the prognosis of patients with leukemia and lymphoma, the collective findings of which are summarized by Tanaka *et al* (48). However, no available accounts currently exist that explored such a relationship in solid tumors. Therefore, there remains a necessity in performing further extensive studies regarding this topic.

In terms of TL, no association could be found between telomere shortening and OS or RFS in the groups of patients with cancer, using either univariate or multivariate survival models. Several studies investigated the possible effects of TL on cancer prognosis, both in PBL and tumor tissues. A previous meta-analysis (46) of 29 studies revealed the association between shortened TL in blood and inferior OS and RFS in solid cancers, which is even stronger in hematological malignancies. The biological relevance of results on TL obtained from blood, and that obtained from target tissues remains unclear (49) PBL is indeed an available tissue that is suitable for repeated monitoring, which has a specific gatekeeping role in cancer, namely immunocompetency.

Kroupa *et al* (50) recently demonstrated that patients with CRC containing smaller TL ratios between the tumor tissue and the adjacent cancer-free mucosa were associated with superior OS. By comparing the outputs obtained from both univariate and multivariate analyses among the individual groups of patients with cancer, survival in patients with LC was not affected by altering any of the genetic markers studied. Even the well-established parameter TNM stage was not found to influence survival. This was most likely due to the majority of patients with LC being diagnosed at advanced stages, mainly at TNM III + IV.

In summary, results from the present study suggest that increased frequency of ACs, CA_{tot}, and CTAs in PBL may be considered as a marker of BC and LC risk. It was also observed that individuals with longer TL in PBL were at a higher risk of BC. Accumulation of CTAs in PBL was associated with worsened OS in patients with BC and CRC after stratification according to disease characteristics using a multivariate survival model. Neither the OS nor the RFS of the patients was found to be influenced by TL alterations. Notably, unlike those observed with control individuals, patients with cancer did not exhibit any associations between TL and CA frequencies or TL and age. The present study is associated with a number of limitations, particularly concerning the small sample sizes of individuals included, which served as one of the possible reasons for the reduced statistical power. In addition, outputs acquired using CART analysis should be treated with caution, since only individuals with full sets of data in all of the implied variables were included, which could have introduced selection bias. Nevertheless, the present study was performed based on validated and well-established methods with clearly defined clinical data.

In conclusion, increased frequency of chromosomal damage and TL in surrogate tissues, namely lymphocytes, which mediate antitumor immune responses, may to be applied to reflect cancer risk and progression. This underlines its potential applications in clinical oncology, which was confirmed by the rapidly rising number of similar studies. Therefore, additional studies with larger cohorts and consistent data on CAs and TL are warranted.

Acknowledgements

Not applicable.

Funding

The present study was funded by The Grant Agency of the Charles University (grant no. GAUK 800216 to SV), The Grant Agency of the Czech Republic (grant no. GACR 18-09709S to PV), Internal Grant Agency of the Ministry of Health of the Czech Republic (grant no. AZV 15-27580A to PV), Research program of Charles University, (grant no. PROGRES Q 28 Oncology to ZP, PV and LV), the project Biomedical Center Martin Slovakia co-financed from EU sources (grant no. ITMS 26220220187 to LM), The National Sustainability Program I (NPU I) provided by the Ministry of Education Youth and Sports of the Czech Republic (grant no. LO1503) and Charles University Research Centre program (grant no. UNCE/MED/006 'University Center of Clinical and Experimental Liver Surgery' to PV and LV).

Availability of data and materials

The datasets generated and/or analyzed during the present study are not publicly available to protect the personal identity of patients but are available from the corresponding author on reasonable request.

Authors' contributions

SV, KH, RaK and PV conceived the study. LM, MA and MS provided the hospital-based samples. ReK, LM and LV performed data collection of the patients. SV performed data curation. SV, ZP and LM performed chromosomal analysis. MK and SR performed the measurement of telomere length. SV performed data analyses and interpreted the results with assistance from MK, KH, RaK and PV. SV wrote and edited the manuscript. ZP, LM, LV, KH, RaK and PV revised the manuscript. ZP, RaK and PV supervised the project. LV administered the project. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The present study adhered to the ethical guidelines of the Helsinki Declaration. All participants were informed about all aspects of the study, agreed with the purpose of the study and procedures to be undertaken, following which they provided informed consent. The present study design was approved by the local Ethics Committees of all participating hospitals.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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