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Polymorphisms in telomere-associated genes, breast cancer susceptibility and prognosis

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ABSTRACT

Telomeres are essential structures for maintaining chromosomal stability and their length has been reported to correlate with cancer risk and clinical outcome. Single nucleotide polymorphisms (SNPs) in genes encoding telomere-associated proteins could affect telomere length and chromosomal stability by influencing gene expression or protein configuration in the telomeres. Here, we report the results of the first association study on genetic variation in telomere-associated genes and their effect on telomere length, breast cancer (BC) susceptibility and prognosis. We genotyped 14 potentially functional and most informative SNPs in nine telomere-associated genes (*TERT*, *TEP1*, *TERF1*, *TERF2*, *TERF2IP*, *ACD*, *POT1*, *TNKS* and *TNKS2*) in 782 incident BC cases and 1559 matched controls. Relative telomere length (RTL) varied statistically significantly between the genotypes of the SNPs rs446977 (*TEP1*, $p = 0.04$), rs938886 (*TEP1*, $p = 0.04$) and rs6990097 (*TNKS*, $p = 0.04$). However, none of them was associated with BC susceptibility and only rs6990097 correlated with regional lymph node metastasis (odds ratio (OR) 1.38, 95% confidence interval (CI) 1.08–1.77). The strongest association with BC susceptibility was observed for rs3785074 (*TERF2*, OR 0.51, 95% CI 0.31–0.83) and rs10509637 (*TNKS2*, OR 1.33, 95% CI 1.08–1.62). Haplotype and diplotype analysis confirmed the association of the *TNKS2* gene with BC susceptibility. rs3785074 (*TERF2*) was additionally associated with histologic grade (OR 1.44, 95% CI 1.08–1.92) and negative oestrogen receptor status (OR 2.93, 95% CI 1.13–7.58). None of the SNPs showed a significant correlation with survival of the breast cancer patients. With these results, none of the SNPs represents any valuable prognostic marker for BC.

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1. Introduction

Chromosomal instability is a hallmark of human tumours. Mechanisms maintaining chromosome stability include processes involved in the regulation of the cell cycle, mitosis and telomere length.[1] Telomeres are specialised nucleoprotein complexes that cap the ends of eukaryotic chromosomes.[1,2] They are required to stabilise and protect the ends of the linear chromosomes. They are composed of a high number of repeats of a hexanucleotide element, TTAGGG, stretching to about 4–15 kb length in human cells and they are capped with numerous associated proteins. The number of the telomere repeats decreases with each cell division and eventually reaches a critical state. This shortening is thought to lead to a loss of structural integrity of the telomere nucleoprotein, resulting in activation of p53 and Rb tumour suppressor pathway and leading to cellular senescence or apoptosis. Some cell clones, however, manage to escape from the crisis and enter into an immortal growth state by acquisition of telomere maintenance functions, suggesting that dysfunctional telomeres increase chromosome instability and probability of oncogenesis.[1,2] Strikingly, it was found that the *TERT* gene, which encodes the catalytic subunit of the telomerase holoenzyme, is expressed at significant levels in about 90% of human tumours, whereas in normal cells its expression is extremely low.[1,2]

Telomere length in the breast tumour tissue has been shown to correlate strongly with the established prognostic markers as well as the clinical course, with short telomere length predicting an unfavourable clinical outcome.[3] Four recent studies have also evaluated the impact of relative telomere length (RTL) of peripheral blood cells on breast cancer (BC) susceptibility and clinical outcome.[4–7] While two of the studies did not find any association between RTL and BC susceptibility,[4,5] one study suggested an association between short telomeres and BC susceptibility[6] and another study reported significantly longer telomeres in the BC patient group compared with the controls.[7] Furthermore, this study showed that RTL carries prognostic information for patients with advanced disease.[7]

We hypothesised that common genetic variation in the form of single nucleotide polymorphisms (SNPs) in genes encoding telomere-associated proteins could affect chromosomal stability by influencing gene expression or protein configuration in the telomeres. This might lead to telomere dysfunction and in the end affect cancer susceptibility and clinical outcome. The nine genes we focused on, encode proteins which are either directly involved in telomere elongation or are known to be telomere binding proteins necessary for telomere stability and for maintaining telomere structure[1,2,8–11] (Fig. 1). Many of these genes have been shown to be differentially expressed in breast tumour and normal tissues; some of them carrying prognostic information.[12]

In this study we selected putatively functional SNPs and most informative tagging SNPs (tagSNPs) from genes encoding telomere-related proteins in order to evaluate whether genetic variation in these genes affect BC susceptibility or clinical outcome. We genotyped a population-based series of 782 incident BC cases with detailed clinical data and up to 15 years of follow-up together with 1559 age- and gender-matched controls. Additionally, we tested whether the selected SNPs affect telomere length in a subset of 100 BC cases, for which RTL data in peripheral blood cells were available from a previous study.[7]

2. Materials and methods

2.1. Study population

The case–control study was carried out using a female Swedish study population consisting of 782 incident BC cases and 1559 controls. Seven hundred forty nine of the 782 incident BC cases with the age- and gender-matched controls were drawn from the population-based Västerbotten intervention project and the mammary screening project, which contain blood samples collected between January 1990 and January 2001 from an ethnically homogenous population living in a geographically defined region in North Sweden.[13] All cases are prospective cases that were identified from the cohorts by record linkage to the regional cancer registry. The controls were selected from the same cohort as the corresponding case. They were matched with the case by age at baseline (±6 months) and the time of sampling (±2 months). The controls had to be alive at the time of diagnosis of the corresponding case and without any previous cancer diagnosis, except carcinoma in situ of cervix uteri. The remaining 33 prospective BC samples were collected consecutively during the same time period from untreated patients referred to the Department of Oncology for newly diagnosed breast cancer. Their controls, also matched for age and gender, were selected from the Västerbotton intervention cohort. All participants gave informed consent to the use of their samples for research purpose.

The blood samples were stored at −80 °C until the time of sample selection and DNA isolation for genotyping analyses from buffy coats using FlexiGene DNA Kit (Qiagen). The samples were randomly divided on the 96-well plates. Whole genome amplification (WGA) was performed with the GenomiPhi DNA amplification kit (Amersham Biosciences, Piscataway, NJ) according to the method described by Wong and colleagues[14] and using the Φ29 DNA polymerase as described by Paez and colleagues, respectively.[15] The amplification results were controlled by genotyping all samples for two frequent SNPs using TaqMan allelic discrimination assays. The SNPs were genotyped previously using the original genomic DNA. Less than 0.10% of the WGA genotypes could not be determined or did not agree with the data of the genomic DNA. The genotyping for this study was performed blinded by the case–control status of each sample using whole genome amplified DNA.

Clinical data for the unselected BC cases were retrieved from the registry managed by the Northern Sweden Breast Cancer Group (Table 1). Follow-up data were available for every patient and it was performed until 26th April 2007. Information about the date of death was collected from the Swedish population register with a BC-specific follow-up until 31st December 2004. The median follow-up time for BC-specific survival was 4.7 years. The study was approved by the ethical committee of Karolinska Institute Syd and Umeå University.

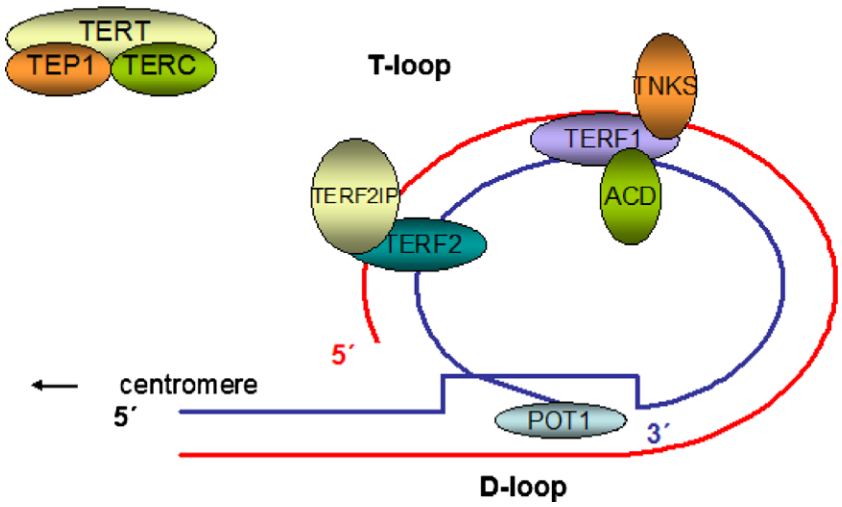

Fig. 1 – Telomere structure and the telomere-associated proteins selected for the present study. The telomere forms a looped structure (T-loop), in which the single-stranded 3′end folds back to interact with the more proximal double-stranded telomere sequence through strand invasion at the D-loop. Sequence specific binding proteins, such as TERF1, TERF2 and POT1, are bound to the telomeric DNA and can recruit additional proteins, such as ACD, TNKS and TERF2IP to the complex to protect the chromosome ends. The telomerase enzyme complex, with the three major components, the RNA component (TERC), the catalytic subunit (TERT) and the telomerase-associated protein (TEP1), is required for the addition of telomere repeats to the ends of the chromosomes. During telomere DNA repeat sequence synthesis, the telomerase complex associates at the telomeric end.

2.2. *Selection of SNPs*

We focused on a group of nine telomere-associated genes, *TERT*, *TEP1*, *TERF1*, *TERF2*, *TERF2IP*, *ACD*, *POT1*, *TNKS* and *TNKS2*. SNPs located in promoters, 3′UTRs, reported nonsynonymous SNPs and most informative tagging SNPs, were selected in these genes using the data from the International HapMap Project (http://www.hapmap.org/) (Supplementary material). They were sequenced for verification in a set of 32 BC samples as described below.

Promoter polymorphisms within or close to transcription factor binding sites may alter gene expression and contribute to tumourigenesis. To identify putative transcription factor binding sites we used the TESS – Transcription Element Search System tool (http://www.cbil.upenn.edu/tess/). SNPs located in the 3′UTR of the genes may have an effect on microRNA binding sites. MicroRNAs can down-regulate protein translation or even degrade mRNA. To identify putative microRNA binding sites we used the MicroInspector tool (http://mirna.imbb.forth.gr/microinspector/). Non-synonymous SNPs were included in our analyses due to their potential effect on protein function. The PolyPhen tool (http://genetics.bwh.harvard.edu/pph/) was used to predict a possible impact of an amino acid substitution on the structure and function of human proteins. Additionally, an estimation of the impact of the different SNPs according to their linkage disequilibrium (LD) with other SNPs was done using the Haploview software.[16]

2.3. *SNP screening by sequencing*

PCR was performed on 32 Swedish BC samples with 5 ng DNA in a 10 μL reaction volume as described earlier by Vaclavicek and colleagues.[17] Primer sequences and annealing temperatures are available from the corresponding author on request. The PCR product was purified with ExoSapIT (USB Amersham, Uppsala, Sweden) at 37 °C for 40 min followed by 85 °C for 15 min. The Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) was used to carry out the sequencing reaction. The ABI PRISM 3100 Genetic Analyser (Applied Biosystems) was used and the obtained sequences were aligned with DNAStar SeqMan™II software (DNASTAR Inc., Madison, WI)

2.4. *Genotyping*

Genotyping was performed using an allele specific PCR based KASPar SNP Genotyping System purchased from KBiosciences (Hoddesdon, Great Britain). Thermocycling was performed according to the KBiosciences' PCR conditions with a DMSO concentration of either 0% or 5% and a Mg^{2+} concentration range between 1.8 mM and 2.2 mM. Detection was performed using an ABI PRISM 7900 HT Sequence Detection System with SDS 2.2 software (Applied Biosystems, Weiterstadt, Germany). The sample set contained 104 duplicate samples as quality controls for the KASPar assays. For less than 0.1% the genotypes of the duplicate samples did not agree with each other. The genotype completion rate varied between 97.0% (rs2929586) and 98.7% (rs6990097).

2.5. *Haplotype/diplotype analysis*

Haplotypes and diplotypes were inferred using the Haplotype procedure of SAS/Genetics. The frequency of a specific haplotype or diplotype was estimated by summing the carrier probability for all women. The relationship between haplotypes and BC was assessed by logistic regression with the wild type

Table 1 – Characteristics of the Swedish breast cancer samples at diagnosis.

Characteristics	Samples, *N* (%)
Age at diagnosis, mean (range, SD)	58.0 (30.6–86.2, 8.95)
Oestrogen receptor	
Positive	480 (61.3)
Negative	134 (17.1)
Unsure	30 (3.8)
Missing	138 (17.8)
Progesterone receptor	
Positive	345 (44.1)
Negative	212 (27.1)
Unsure	82 (10.5)
Missing	143 (18.4)
Oestrogen/progesterone receptors	
Er+/Pr+	321 (41.0)
Er+/Pr–	88 (11.2)
Er–/Pr+	17 (2.2)
Er–/Pr–	113 (14.4)
Missing	243 (31.1)
Tumour size in mm	
⩽20 mm	501 (64.1)
>20 mm	227 (29.0)
Missing	54 (6.9)
Histologic grade	
1	159 (20.3)
2	357 (45.7)
3	224 (28.6)
Missing	9 (1.2)
Regional lymph node metastasis	
Negative	468 (59.8)
Positive	220 (28.1)
Missing	94 (11.1)
Stage at diagnosis	
0	2 (0.3)
I	405 (51.8)
II	325 (41.6)
III	26 (3.3)
IV	15 (1.9)
Missing	9 (1.2)
Distant metastasis	
Negative	758 (96.9)
Positive	13 (1.7)
Missing	11 (1.4)
Cases	782
Controls	1559

N = number of samples.

haplotype as the reference. Furthermore, the influence of each haplotype was addressed by comparing the distribution of homozygous, heterozygous and non-carriers for each haplotype (by logistic regression).

2.6. Relative telomere length (RTL) measurement

RTL of peripheral blood cells, using DNA extracted from buffy coat samples (a mixture of granulocytes and lymphocytes), had been measured previously in a BC case population using real-time PCR as described.[7] Samples differing in average telomere length by as little as 11.4% should be distinguishable by this method at the 95% confidence level.[18] One hundred samples of the study population with data of RTL overlapped with the study population genotyped in the present study and could therefore be used for comparison of the telomere length and the genotypes. The allele and genotype distributions in this subset of samples did not differ statistically significant from the ones in the present study.

2.7. Statistical analysis

The observed genotype frequencies in the controls were tested for Hardy-Weinberg equilibrium (HWE) and the difference between the observed and expected frequencies was tested for significance using the χ^2 test. Statistical significance for the differences in the genotype frequencies between the BC cases and the controls was determined by the Wald χ^2 test of heterogeneity with two degrees of freedom. LD between the SNPs was evaluated using the Haploview software.[16] Statistical significance for a different genotype distribution in cases and controls was determined by global χ^2 test. When the overall genotype effect of a SNP on BC susceptibility or on a clinical factor was statistically significant at the level of 0.05, the best model to represent the relationship between the genotypes and BC susceptibility or a clinical factor, respectively, was selected based on likelihood ratio tests (LRTs). Odds ratios (ORs) and 95% confidence intervals (95% CIs) for association between genotypes and BC susceptibility and tumour characteristics were calculated by logistic regression (PROC LOGISTIC, SAS Version 9.1; SAS Institute, Cary, NC). For a polymorphism with a variant allele frequency between 10% and 50%, the study had greater than 90% power to detect an OR of 1.50 at a significance level of 0.05 (PS-software for power and sample size calculation, http://biostat.mc.vanderbilt.edu/twiki/bin/view/Main/PowerSampleSize). The relative risk of death by BC was estimated as hazard ratio (HR) using Cox regression (PROC PHREG, SAS Version 9.1, SAS Institute). Censoring events were death by another cause than BC, moving out of the study and 31st December 2004. Kruskal–Wallis test was used to evaluate the relationship between the genotypes and RTL (Statistical Package for the Social Sciences, SPSS, Version 14).

3. Results

3.1. Association with breast cancer susceptibility

Altogether, we genotyped 14 SNPs in nine telomere-associated genes. For the *TERT* promoter SNP rs2853669, the genotype distribution in controls deviated from HWE ($p = 0.001$), and the SNP was excluded from the BC susceptibility analysis. All the other genotype distributions in controls followed HWE. For two SNPs the global χ^2 test indicated a significant association with BC susceptibility (Table 2). According to likelihood ratio tests, the best model to represent the relationship between the genotypes and BC susceptibility was recessive for *TERF2* and was dominant for *TNKS2*.

The homozygous carriers of the minor allele of the SNP rs3785074, located in intron 3 of the *TERF2* gene, had a

Table 2 – Association of the genotypes in the telomere-associated genes with breast cancer susceptibility.

Gene	Genotype	Cases, N (%)	Controls, N (%)	Global p value	Best model	Comparison	OR (95% CI)	p Value
ACD rs6979	CC	214 (27.9)	386 (25.5)	0.40				
	CT	368 (47.9)	758 (50)					
	TT	186 (24.2)	372 (24.5)					
POT1 rs12538333	GG	297 (38.8)	594 (38.9)	0.72				
	GA	346 (45.2)	712 (46.6)					
	AA	122 (15.9)	221 (14.5)					
TEP1 rs4246977	AA	337 (43.9)	616 (40.4)	0.21				
	AG	346 (45.1)	722 (47.3)					
	GG	84 (11)	187 (12.3)					
rs1760890	TT	599 (77.4)	1200 (78.2)	0.84				
	TG	163 (21.1)	316 (20.6)					
	GG	12 (1.6)	19 (1.2)					
rs1760897	TT	445 (58.3)	864 (56.8)	0.54				
	TC	278 (36.4)	563 (37)					
	CC	40 (5.2)	94 (6.2)					
rs1760904	TT	277 (36.3)	565 (37.2)	0.77				
	TC	374 (49)	742 (48.8)					
	CC	112 (14.7)	212 (14)					
rs938886	CC	427 (55.5)	874 (57)	0.88				
	CG	289 (37.5)	555 (36.2)					
	GG	54 (7)	104 (6.8)					
TERF1 rs2929586	AA	368 (48.4)	729 (48.2)	0.93				
	AG	326 (42.8)	641 (42.4)					
	GG	67 (8.8)	142 (9.4)					
TERF2 rs3785074	TT	457 (60.3)	921 (60.4)	0.02	Recessive	CC versus (TT + TC)	0.51 (0.31–0.83)	0.007
	TC	279 (36.8)	521 (34.2)					
	CC	22 (2.9)	82 (5.4)					
TERF2IP rs2233807	CC	531 (69.5)	1041 (68.5)	0.71				
	CT	218 (28.5)	446 (29.3)					
	TT	15 (2)	33 (2.2)					
TERT[a] rs2853669	TT	409 (53.4)	818 (53.9)					
	TC	310 (40.5)	558 (36.7)					
	CC	47 (6.1)	143 (9.4)					
TNKS rs6990097	TT	396 (51.2)	791 (51.4)	0.89				
	TC	315 (40.8)	616 (40.1)					
	CC	62 (8)	131 (8.5)					
TNKS2 rs2066276	TT	248 (32.3)	502 (33)	0.10				
	TC	394 (51.4)	725 (47.6)					
	CC	125 (16.3)	295 (19.4)					
rs10509637	AA	564 (73.2)	1190 (78.2)	0.005	Dominant	(AG + GG) versus AA	1.33 (1.08–1.62)	0.006
	AG	197 (25.6)	302 (19.9)					
	GG	10 (1.3)	29 (1.9)					

N = number of samples; OR = odds ratio; CI = confidence interval.

a HWE deviation in controls $p = 0.001$, therefore, the SNP was not analysed for breast cancer susceptibility.

decreased risk for BC (OR 0.51, 95% CI 0.31–0.83). Carriers of the minor allele G of the SNP rs10509637, located in intron 8 of the *TNKS2* gene, had an increased risk for BC (OR 1.33, 95% CI 1.08–1.62).

3.2. *Haplotype/diplotype analysis*

A haplotype and diplotype analysis for the genes *TEP1*, where 5 SNPs were genotyped, and *TNKS2*, where 2 SNPs were genotyped, was carried out. The SNPs that were selected for haplotype analysis were not in LD, the highest r^2 value between these SNPs was 0.09. For *TEP1*, the haplotype analysis showed a significant inverse correlation between the haplotypes GTTTC (OR 0.77, 95% CI 0.63–0.94) and ATCCC (OR 0.68, 95% CI 0.49–0.94) and BC susceptibility. In the diplotype analysis, only a dominant model was considered due to small numbers of homozygote individuals and the associations did not remain statistically significant (Table 3). For *TNKS2*, the analysis indicated an increased OR for the TG haplotype (OR 1.21, 95% CI 1.0–1.46) and a statistically significantly increased risk of

BC for carriers of at least one TG haplotype (OR 1.46, 95% CI 1.12–1.90).

3.3. Association with breast tumour characteristics at the time of diagnosis and with survival

When examining the genotype distribution by tumour characteristics at the time of diagnosis, only a few associations were observed (Supplementary Table 1). For the SNP rs3785074, located in the intron 3 of the *TERF2* gene, a significant correlation with high histologic grade was observed (global *p* value 0.03). The correlation was explained best by the additive model (per allele OR 1.44, 95% CI 1.08–1.92). Additionally, for this SNP, there was a significant association with negative oestrogen receptor status indicated by a global *p* value of 0.04 and the recessive model (CC versus (TT + TC); OR 2.93, 95% CI 1.13–7.58).

For the SNP rs6990097, located in the promoter of the *TNKS* gene, the global *p* value of 0.05 indicated a possible association with regional lymph node metastasis. The additive model explained best this association: the C allele carriers were more likely to have a lymph node metastasis than homozygotes for the T allele (per allele OR 1.38, 95% CI 1.08–1.77).

For the SNP rs2066276, located in the promoter of the *TNKS2* gene, the global *p* value of 0.002 indicated a possible association with histologic grade. The best model to describe this association was based on three genotypes. However, while the heterozygous genotype showed a correlation with low histologic grade 1 + 2 (TC versus TT: OR 0.63, 95% CI 0.44–0.90), the variant homozygous genotype CC associated rather with high histologic grade 3 (CC versus TT: OR 1.27, 95% CI 0.81–2.01).

None of the SNPs showed a significant correlation with survival (data not shown).

3.4. Association with relative telomere length

For the analysis of association between the genotypes and RTL, data on RTL were available for 100 BC samples that were overlapping between our present study and the study by Svenson and colleagues.[7] According to Kruskal–Wallis test, the distribution of RTL varied statistically significantly between the genotypes of the SNPs rs4246977 (*TEP1*, $p = 0.04$), rs938886 (*TEP1*, $p = 0.04$) and rs6990097 (*TNKS*, $p = 0.04$). For the SNP rs4246977, the rare GG genotype seemed to associate with shorter RTL compared to the other genotypes (Fig. 2A). For the SNPs rs938886 and rs6990097, the rare genotypes, GG and CC, respectively, seemed to associate with longer telomeres compared to the other genotypes (Fig. 2B and C, respectively).

4. Discussion

Telomerase activity and the role of telomeres in cancer has been a well-discussed topic during the last years.[1,2] As several genes are involved in the mechanism of telomere elongation and stabilisation, we decided to investigate genetic variation in nine major telomere-associated genes (*ACD*, *POT1*, *TEP1*, *TERF1*, *TERF2*, *TERF2IP*, *TERT*, *TNKS*, *TNKS2*). We detected several common SNPs in the promoter, the coding region and the 3′UTR of the investigated genes. Furthermore, in some genes, tagSNPs were selected in order to cover a multitude of the existing SNPs in the gene. We focused on the effects of 14 putatively functional and informative SNPs on BC susceptibility, traditional prognostic markers and survival. Although many of the SNPs had a potential to affect gene expression or protein function, only few impacts on BC susceptibility or clinical outcome in our Swedish study population were observed.

The homozygous carriers of the minor allele C of the *TERF2* SNP rs3785074 had a decreased risk for BC, but they were more likely to have tumours with high histologic grade and negative oestrogen receptor status. However, as no association with BC-specific survival was observed, the SNP rs3785074 does not represent a valuable prognostic marker. The International HapMap Project contains information of six SNPs in the *TERF2* gene, which are located within one haploblock. Our selected SNP rs3785074 captures four of the

Table 3 – Association of the haplotypes and the diplotypes in the telomere-associated genes *TEP1* and *TNKS2* with breast cancer risk.

Haplotype	Cases, N (%)	Controls, N (%)	OR (95% CI)	*p* Value	Diplotype	Cases, N	Controls, N	OR (dominant model[a]) 95% CI	*p* Value
Gene: TEP1: Promoter, rs4246977; rs1760890; rs1760897; rs1760904; rs938886 3′UTR[b]									
A-T-T-T-C	352 (22.5)	640 (20.6)	1			40	72		
G-T-T-T-C	244 (15.6)	577 (18.6)	0.77 (0.63–0.94)	0.009	G-T-T-T-C/any	228	529	0.78 (0.51–1.18)	0.23
G-T-T-**C**-C	113 (7.2)	219 (7)	0.94 (0.72–1.22)	0.63	G-T-T-C-C/any	109	214	0.92 (0.58–1.44)	0.71
A-T-T-**C**-C	126 (8.1)	212 (6.8)	1.08 (0.84–1.4)	0.55	A-T-T-C-C/any	117	206	1.02 (0.65–1.6)	0.92
A-T-T-T-**G**	111 (7.1)	212 (6.8)	0.95 (0.73–1.24)	0.71	A-T-T-T-G/any	103	202	0.92 (0.58–1.44)	0.71
A-T-**C**-T-C	77 (4.9)	163 (5.2)	0.86 (0.64–1.16)	0.32	A-T-C-T-C/any	74	161	0.83 (0.51–1.33)	0.43
A-T-**C**-**C**-C	60 (3.8)	160 (5.1)	0.68 (0.49–0.94)	0.02	A-T-C-C-C/any	59	152	0.7 (0.43–1.14)	0.15
Gene: TNKS2: Promoter; rs2066276; rs10509637; 3′UTR[c]									
T-A	681 (43.9)	1385 (45)	1		T-A/T-A	138	326		
C-A	650 (41.9)	1329 (43.2)	0.99 (0.87–1.13)	0.94	CA/any	523	1031	1.2 (0.96–1.5)	0.12
T-**G**	217 (14)	365 (11.9)	1.21 (1–1.46)	0.05	TG/any	207	335	1.46 (1.12–1.9)	0.005

N = number of samples; OR = odds ratio; CI = confidence interval.

[a] Due to a small number of homozygotes, ORs and 95% CIs are presented only for the dominant model.

[b] The order of SNPs; minor alleles in haplotypes are typed in bold.

[c] The order of SNPs; minor alleles in haplotypes are typed in bold.

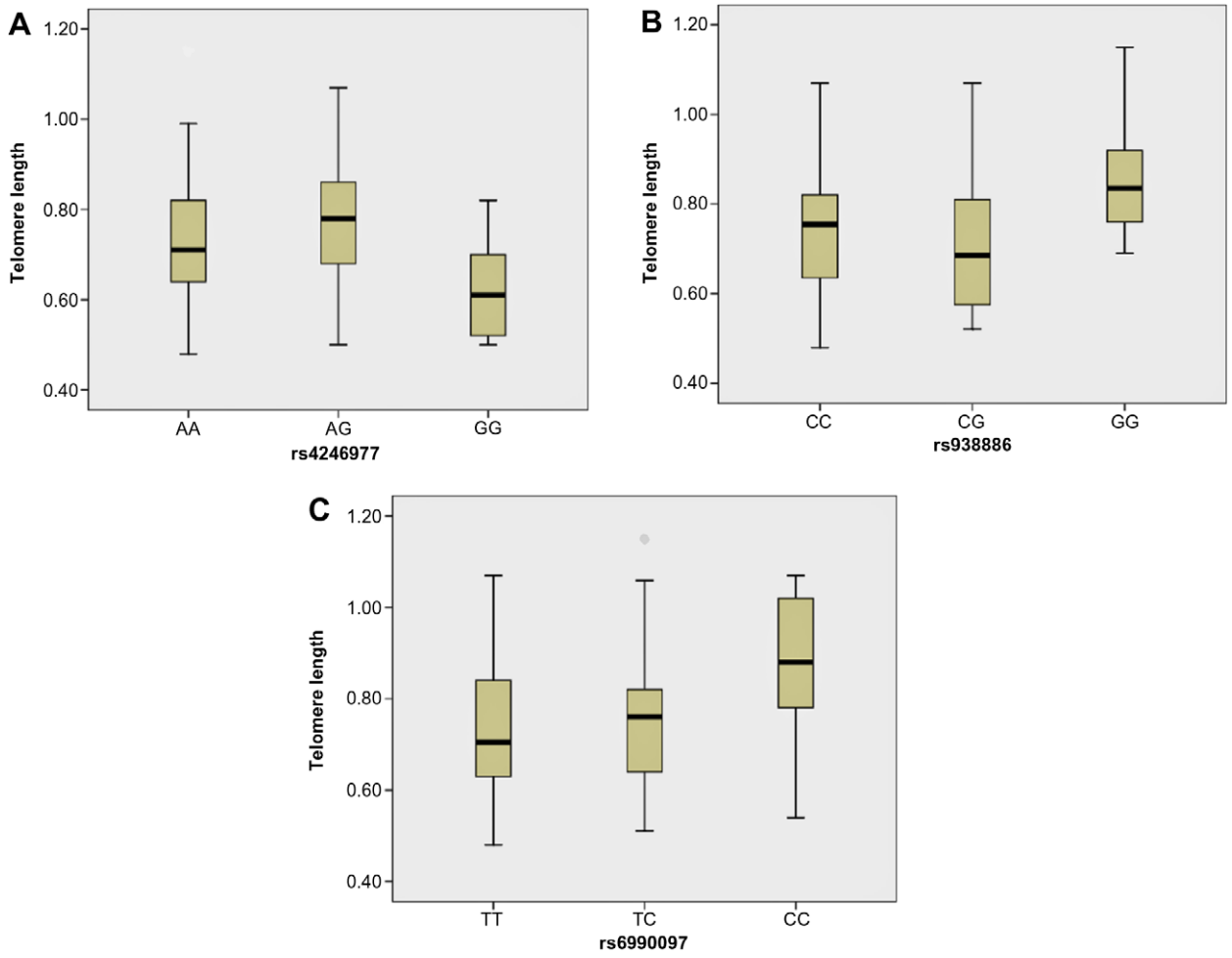

Fig. 2 – Association of relative telomere length (RTL) with the genotypes of the SNPs rs4246977 (*TEP1*), rs938886 (*TEP1*) and rs6990097 (*TNKS*). The analysis was based on 100 breast cancer samples for which RTL was available from a previous study by Svenson and colleagues. Kruskal–Wallis test was used to evaluate the statistical significance of the relationship between the genotypes and RTL.

SNPs with $r^2 = 1.0$. All these SNPs are located in the introns and a possible functional importance of these regions is unknown. Further studies are necessary, especially because *TERF2* is located on chromosome 16q22.1, which is one of the most frequent targets of loss of heterozygosity in BC.[19] Additionally, a decreased expression of the *TERF2* gene with increasing severity of BC progression has been reported.[12]

The other SNP associated with BC susceptibility was rs10509637 in the *TNKS2* gene, with carriers of the minor allele G having an increased risk for BC. This association was confirmed in the haplotype and diplotype analysis, which showed an increased risk for the carriers of the TG haplotype. This haplotype contained the major allele T of the promoter SNP rs2066276 and the minor allele G of the SNP rs10509637, which is located in intron 8. The promoter SNP, rs2066276, tags eight intronic SNPs; rs10509637 tags six further intronic SNPs ($D' = 1$, $r^2 \geqslant 0.8$) and one SNP in the 3′UTR, rs2258946 ($D' = 1$, $r^2 = 0.86$). A previous study has shown a decreased expression of the *TNKS2* gene with increasing severity of BC progression,[12] suggesting that transcription might be affected. However, the effect is unlikely caused by the variants genotyped or captured in our study, because according to the MicroInspector tool, the 3'UTR SNP is not located within any miRNA binding site and no data about the functional importance of the introns are available.

In silico analyses predicted a high biological plausibility for the *TEP1* gene to be involved in BC development with several promoter SNPs located within putative transcription factor binding sites (TESS) and several nsSNPs having a possible/probably damaging effect on the protein structure (Polyphen). Additionally, our RTL analysis suggested an association of the SNPs rs4246977 and rs938886 with telomere length. Nevertheless, we observed no effect of the investigated SNPs (rs4246977, rs1760890, rs1760897, rs1760904 and rs938886) on BC susceptibility or clinical outcome. In a previous study, Savage and colleagues used a tagSNP approach and genotyped five SNPs across the *TEP1* gene, none of them was associated with BC.[20] Two of the SNPs (rs1760897 and rs1760904) were also genotyped by us and one (rs1713449) was in LD ($D' = 1.0$, $r^2 = 1.0$) with rs938886 analysed by us. Thus, our results on putatively functional SNPs and the results of Savage and colleagues on tagSNPs complement each other and exclude with high probability an effect of genetic variation in the *TEP1* gene on BC susceptibility.

The promoter SNP rs6990097 in the *TNKS* gene was associated with lymph node metastasis: the C allele carriers were more likely to have lymph node metastases than homozygotes for the common T allele. The CC genotype also correlated with increased telomere length and long telomeres in peripheral blood cells have been associated with a worse outcome of the disease.[7] rs6990097 is in high LD with two other promoter SNPs and one SNP in the 3′UTR, suggesting that the observed effect might be related to transcriptional regulation. In fact, TNKS expression has been shown to be up-regulated in BC.[12,21] However, the SNP did not correlate with any other clinical marker or survival, making it an unlikely candidate for a prognostic marker. The SNPs investigated in the genes *TERF1*, *TERF2IP*, *ACD* and *POT1* were not associated with BC susceptibility, clinical factors or RTL.

The strengths of our study include the analysis of SNPs in several genes encoding the main telomere-associated proteins, the reasonable large sample size (782 cases, 1559 controls) with prospectively collected blood samples, the long follow-up time (up to 15 years) and the detailed clinical data. We focused on SNPs with the highest probability of an influence on gene transcription or protein product, and on the most informative tagSNPs in order to cover a multitude of genetic variation within a gene. With the present sample size of the Swedish study population we had a power of >90% to detect an OR of 1.5 for the risk of BC. As the SNPs were selected based on their putative function, we would expect to have a higher OR than in the recent genome-wide association studies.[22–24] However, as our SNP selection focused on the most informative common SNPs in these genes, we cannot exclude an effect of other SNPs present in the gene. The long and variable follow-up time brought also some limitations to our study. Many of the cases diagnosed in the early 1990s did not have complete clinical data, making some subgroup analyses small and decreasing the power to detect associations with genotypes. This was especially true for analyses of the relationship between the genotypes and hormone receptor status as well as between the genotypes and telomere length. As BC has a relatively good survival, more than 75% of the patients were alive at the end of the present follow-up period, the use of recurrence as an end-point of survival would have been more robust than the use of death by BC. However, such data were not available.

In conclusion, identifying genetic variation within telomere-associated genes could provide valuable clinical tools for cancer prediction and prognosis. In this first association study combining the data of genotypes of SNPs in the main telomere-associated genes, telomere length, BC risk, traditional prognostic markers and survival, no consensus in association between these markers was observed. However, as telomere-associated genes play a significant role in maintaining telomere length and telomere stability.[1,2] and with that may influence cancer susceptibility and prognosis,[3,7,12,25,26] their impact on different cancer types needs further investigation.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.08.012.

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