Abstract

Genetic variation at the TERT-CLPTM1L locus at 5p15.33 is associated with susceptibility to several cancers, including epithelial ovarian cancer (EOC). We have carried out fine-mapping of this region in EOC which implicates an association with a single nucleotide polymorphism (SNP) within the TERT promoter. We demonstrate that the minor alleles at rs2736109, and an additional TERT promoter SNP, rs2736108, are associated with decreased breast cancer risk, and that the combination of both SNPs substantially reduces TERT promoter activity.
followed by functional analyses of associated SNPs identified a per-allele model [9]. In this study we carried out fine-mapping, and EOC risk, particularly of the serous histological subtype, using

Results and Discussion

study of EOC [9] (rs11133719, rs7726159, rs2736100 and

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TERT

expression, for their continued proliferation, strongly implicating that the presence of a common haplotype, which is associated with decreased cancer risk, substantially reduces TERT promoter activity. The vast majority of cancers depend on expression of the reverse transcriptase component of telomerase, and cleft lip and palate cancers [8]. This locus harbours a locus at 5p15.33 which has been implicated in susceptibility to melanoma [1,2], glioma [3], lung [4,5], pancreas [6], prostate [2], testicular [7], and bladder cancers [8]. This locus harboursoftERT, encoding the reverse transcriptase component of telomerase, and cleft lip and palate transmembrane protein 1-like (CLPTM1L). GWAS of ovarian and breast cancer have not detected an association with this locus to date, which may be due to poor tagging of this region on the chips used, or the lack of statistical power to detect associations. Using a candidate-gene approach we previously reported evidence that failed OCAC’s quality control criteria [10], 28 SNPs were employed, or the lack of statistical power to detect associations.

Introduction

Genome-wide association studies (GWAS) have identified more than 140 cancer susceptibility loci for 17 different cancers (www.genome.gov/gwastudies), including a locus at 3p15.33 which has been implicated in susceptibility to melanoma [1,2], glioma [3], lung [4,5], pancreas [6], prostate [2], testicular [7], and bladder cancers [8]. This locus harboursoftERT, encoding the reverse transcriptase component of telomerase, and cleft lip and palate transmembrane protein 1-like (CLPTM1L). GWAS of ovarian and breast cancer have not detected an association with this locus to date, which may be due to poor tagging of this region on the chips used, or the lack of statistical power to detect associations. Using a candidate-gene approach we previously reported evidence that failed OCAC’s quality control criteria [10], 28 SNPs were employed, or the lack of statistical power to detect associations.

Using a candidate-gene approach we previously reported evidence of an association between an intronic SNP in TERT (rs7726159) and EOC risk, particularly of the serous histological subtype, using a per-allele model [9]. In this study we carried out fine-mapping, followed by functional analyses of associated SNPs identified within the promoter region of TERT. We directly demonstrate that the presence of a common haplotype, which is associated with decreased cancer risk, substantially reduces TERT promoter activity. The vast majority of cancers depend on expression of telomerase, which requires substantial upregulation of TERT expression, for their continued proliferation, strongly implicating TERT as the predominant gene involved in this association.

Results and Discussion

To further clarify the association previously reported with TERT, we employed a fine-mapping strategy in nine case-control studies from the Ovarian Cancer Association Consortium (OCAC) (Table S1). SNPs in a region spanning ±250 kb across the TERT, CLPTM1L, SLC6A18 and SLC6A19 genes that were correlated (0.2 < r² < 0.99) with rs7726159, rs11133719, rs2735940 and rs2736100 [9] were selected from the 1000 Genomes low coverage pilot release of April 2009. The SNPs implicated by our previous study of EOC [9] (rs11133719, rs7726159, rs2735940 and rs2736100) were also included in the panel. We genotyped 36 SNPs by iPLEX (Sequenom Inc., in 2,130 invasive EOC cases and 3,975 controls, all of Caucasian ancestry. After excluding monomorphic loci (n = 6) and two SNPs that failed OCAC’s quality control criteria [10], 28 SNPs were analysed for association with risk of EOC. We used single marker and stepwise logistic regression models, adjusted for study and age (at interview for controls and at diagnosis for cases) with a threshold of P < 0.05 for addition (forward stepwise) or removal (backward stepwise) of SNPs (Table S2). We found that rs2736109 showed the strongest association with serous EOC (adjusted OR per-allele 0.86 (0.77–0.96), P = 0.005) (Table S3), but not with invasive EOC risk overall (adjusted OR per-allele 0.96 (0.89–1.05), P = 0.33) (data not shown). Likelihood ratio tests comparing logistic regression models with and without a genotype-by-study interaction term revealed no significant study heterogeneity (P = 0.4), rs2736109 is in a region of low linkage disequilibrium that encompasses the 5′ end of the TERT gene and the TERT promoter (Figure 1). This region also contains the SNPs, rs2736108 and rs2853669, which have pairwise correlations (r²) with each other, and with rs2736109 of greater than 0.6. It has previously been reported that rs2853669 is associated with breast cancer risk [11]. Since other loci confer susceptibility to both EOC and breast cancer [12,13] we investigated associations of TERT SNPs with breast cancer risk in various data sets. First, we genotyped rs2736109 by iPLEX in 4,277 invasive breast cancer cases and 7,000 controls from Australian, German and Thai studies from the Breast Cancer Association Consortium (BCAC) (Table S4). In the combined analysis of the Australian and German studies, there was no association with invasive breast cancer risk overall (adjusted OR per-allele 0.95 (0.90–1.01), P = 0.10), but there was an association in cases ≥50 years at diagnosis that approached significance (adjusted OR per-allele 0.94 (0.88–1.00), P = 0.049). We also found a stronger association for ER-negative tumours (adjusted OR per-allele 0.88 (0.80–0.96), P = 0.022; for ER-positive tumours adjusted OR per-allele 0.98 (0.92–1.05), P = 0.562) (Tables S5, S6, and S7). We found the strongest evidence of association among ER-negative cases over the age of 50 (n = 636) (adjusted OR per-allele 0.84 (0.75–0.93), P = 0.005). We did not find any
association with breast cancer risk in a Thai study (n = 327 cases) genotyped for rs2736109.

Next, we analysed genotypes imputed using MaCH for rs2736109 (r^2 = 0.45) from a breast cancer GWAS of 3,931 cases and 3,622 controls from the United Kingdom, from which neither age nor ER status were available [14]. We observed a significant association between rs2736109 and overall breast cancer risk in this population (OR per-allele 0.91 (0.83–0.99), P = 0.037), and from a weighted meta-analysis of imputed and genotyped data from all studies (OR per-allele 0.94 (0.89–0.98), P = 0.011).

In an additional replication sample set (SEARCH) we genotyped a correlated SNP, rs2736108 (r^2 = 0.96, 1000 Genomes Project, Dec 2009, r^2 = 0.63 based on 345 Australian controls) in 6,788 cases and 6,426 controls because rs2736109 was not amenable to genotyping by TaqMan (the genotyping platform used by this group). We observed a significant association with breast cancer risk overall (adjusted OR per-allele 0.92 (0.87–0.97), P = 0.003), which was restricted to the subset of cases diagnosed at age 50 or older (adjusted OR per-allele 0.91 (0.85–0.97), P = 0.002) (Table S6). Estimates according to ER status showed a significant association between the rs2736108 genotypes and ER-positive tumours (adjusted OR per-allele 0.93 (0.88–0.99), P = 0.031); there was no significant association in ER-negative tumours but the estimated OR was similar (OR per-allele 0.95 (0.85–1.06)) and the sample size much smaller. Comparison of models for both rs2736109 and rs2736108 with and without genotype by age group interaction terms showed no evidence of a statistical interaction on the multiplicative scale (PInteraction ≥ 0.25).

The SNPs of interest (rs2736108, rs2736109 and rs2853669) lie within the upstream promoter region of TERT. To determine the functional significance of these sites, we generated combinations of these variants in a luciferase reporter construct containing 3.9 kb of the TERT promoter [15]. Relative promoter activity was determined in an EOC cell line (27/87), a breast adenocarcinoma cell line (MDA-MB-468), and in post-selection normal breast epithelial cells (Bre16) (Figure 2). Introduction of a mutation into the human estrogen-responsive element in the TERT promoter (TERT-ERE) [16] was used as a positive control and confirmed diminished reporter activity. In all three cell types, luciferase activity was substantially reduced for the construct carrying both minor (A) alleles at rs2736108 and rs2736109, but remained unaltered for those with the individual minor allele at either SNP. We observed no change in expression for the minor allele at rs2853669.

Our analysis of Australian controls estimated a frequency of 32% for the A-A haplotype at rs2736108 and rs2736109, suggesting that this relatively common promoter haplotype may lower the risk of ovarian and breast cancer through decreasing TERT expression. This finding provides no support for the hypothesis that decreased telomerase activity predisposes to genomic instability and consequent oncogenic progression but instead our data imply the opposite, namely, that decreased TERT expression confers decreased cancer risk.

Figure 1. TERT-CLPTM1L locus SNPs genotyped in epithelial ovarian cancer cases and controls. Panel A depicts the pattern of linkage disequilibrium using data from the HapMap phase II+III CEU population (2010), where white represents r^2 = 0, and black r^2 = 1. The plot in B represents −log_10(p) against the chromosomal position for genotyped SNPs in the ovarian cancer fine mapping panel. The purple diamond represents the SNP (rs2736109) with the strongest observed association. The point colours represent the strength of LD according to 1000 Genomes Data. doi:10.1371/journal.pone.0024987.g001
We also examined tumour expression and germline variants at the **TERT-CLPTM1L** locus using data from The Cancer Genome Atlas (TCGA) ovarian serous cystadenocarcinoma set. mRNA expression profiling was available for 574 tumour samples using Affymetrix U133A platform. This assay has one probe for **TERT**, but none for **CLPTM1L**. **TERT** was expressed at low levels, as expected, but this does not necessarily reflect low levels of telomerase protein expression or activity [17]. 508 normal DNA samples were genotyped using Illumina 1 M array, and the SNP rs2736108 was typed on this array. We also imputed the TCGA samples with reference to the 1000 Genome data (June 2010 release); rs2853669 was successfully imputed, but rs2736109 failed quality control. There was no evidence suggesting that the genotyped SNP, rs2736108, or the imputed SNP, rs2853669, are associated with expression of **TERT**. However one SNP, rs2735845, in partial linkage disequilibrium with the typed SNP, rs2736108, or the imputed SNP, rs2853669, are associated with expression of **TERT**. However one SNP, rs2735845, in partial linkage disequilibrium with the typed SNP, rs2736108 ($r^2 = 0.306$), was associated with significantly altered transcript abundance of **TERT** ($P = 4 \times 10^{-4}$ and $P = 0.0029$ after correction for multiple testing). Gene copy number aberration was found in **TERT**; it was amplified in 20% of tumour samples. This SNP showed robust association after adjusting for the copy number variation ($P = 1.8 \times 10^{-3}$ and $P = 0.015$ after correction for multiple testing), and it explained 1.85% of variance of **TERT** transcription in ovarian cancer tumours.

A recently published meta-analysis identified a significantly decreased risk of breast cancer with a **TERT** SNP, rs2853669 (OR $0.76$ ($0.64$–$0.91$), $P = 0.002$) [11]. This SNP lies in the same linkage disequilibrium block as rs2736108 and rs2736109 ($r^2 > 0.6$ with rs2736108); therefore, this meta-analysis provides additional support for an association between **TERT** promoter SNPs and breast cancer risk. However, our functional analyses, which were carried out in normal breast epithelial cells and a breast adenocarcinoma cell line did not indicate any change to **TERT** expression with this individual SNP.

We computed a gene-based test [18] of association at **TERT** and **CLPTM1L** which yielded evidence in GWAS data available from dbGAP (http://www.ncbi.nlm.nih.gov/gap) for association with risk of lung, prostate and pancreatic cancer, but not breast cancer overall. Combining data for all cancers in a cross-cancer meta-analysis, revealed a genome-wide significant gene-based $P = 4.1 \times 10^{-7}$ for **CLPTM1L** ($P = 0.008$ after correction for 19,000 genes). A similar result was obtained for **TERT** (all cancer $P = 7.7 \times 10^{-5}$). This gene-based test includes all SNPs within 50 kb of the start/stop site of each gene. Most of the associated SNPs lie in the interval between **TERT** and **CLPTM1L** but with slightly more evidence for SNPs near to **CLPTM1L**, leading to a slightly higher gene-based $P$ value for **CLPTM1L**. These results are based on marker data from Illumina GWAS arrays and hence the exact location of the maximum association test statistic is dependent to some degree upon the arbitrary set of SNPs that are on the arrays. Clearly, additional analysis of the entire **TERT-**
CLPM1L locus is warranted in breast, and other, cancers to validate the associations we have identified and fine map putative causal variants if our findings are confirmed.

The failure to date to identify an association between the TERT-CLPM1L locus and risk of EOC or breast cancer by GWAS may be explained by the pattern of linkage disequilibrium of the relevant SNPs: rs2736100, the tagSNP most commonly identified by GWAS of other cancers is poorly correlated with rs2736108 and rs2736109 ($r^2 = 0.141$ and $0.105$, respectively), and neither of these SNPs are on the Illumina 300 K, 610 K or 650 K arrays used for most cancer GWAS.

In summary, we have demonstrated a direct association between functional SNPs in the TERT promoter, which confer decreased risk of ovarian and breast cancer, and reduced TERT promoter activity. Decreased levels of TERT result in progressive telomere shortening and the onset of cellular senescence, which ultimately acts to suppress tumorigenesis. The association of hypomorphic sequence variants in the TERT promoter with decreased risk of cancer implicates downregulation of telomerase and telomere shortening as an intrinsic tumour suppressive mechanism. It is also possible that TERT variants associated with elevated cancer risk may alter the stringency with which TERT is regulated, potentially facilitating TERT activation and consequently providing a tumorigenic advantage. Potential non-canonical roles of TERT in cell signalling pathways may also underlie cancer risk [19]. Our results add functional insight into the increasingly important role of TERT as a cancer risk factor and demonstrate the need for further mechanistic analysis of this multi-cancer susceptibility locus.

Materials and Methods

Ethics Statement

Approval for this study was obtained from The Queensland Institute of Medical Research Human Research Ethics Committee. All studies were approved by the review boards and ethics committees of their respective institutions, and all participants provided written informed consent.

Genotyping

iPLEX genotyping was carried out using MALDI-TOF spectroscopy utilising Sequenom’s MassARRAY platform and iPLEX GOLD chemistry. 10 ng of genomic DNA was used as template, to which a PCR mix containing Qiagen Hot-StarTaq was added. Shrimp alkaline phosphatase and primer extension steps were carried out using Sequenom’s protocol and reagents. Primers were obtained from Integrated DNA Technologies (Ohio USA). Assays were designed with MassARRAY Assay Design version 3.1 (Sequenom). Raw genotype data were visualised and processed with MassARRAY Typer software version 3.4. TaqMan genotyping (SEARCH) was carried out as previously described [20]. Strict quality control criteria were adhered to as part of the Ovarian Cancer Association Consortium’s guidelines [10].

Statistical methods

We used single marker and stepwise logistic regression models to screen 28 SNPs in non-Hispanic white ovarian cancer cases (n = 2,130) and controls (n = 3,975) from nine OCAC studies (Supplementary Table 1). Genotype data for all the previously reported TERT SNPs [9] has been excluded from the current analysis. Stepwise models were adjusted for study and age (at interview for controls and at diagnosis for cases), with a threshold of $P ≤ 0.05$ for addition (forward stepwise) or removal (backward stepwise) of SNPs. All single marker risk estimates were obtained from unconditional logistic regression models adjusted for age (where available) and additionally for study where data was pooled across multiple studies. Assuming a log additive model of inheritance, the per-allele odds ratios (ORs) and their 95% confidence intervals (CIs) associated for selected SNPs were estimated by fitting the number of rare alleles carried as a continuous covariate. Ovarian cancer risk associated with SNP genotypes were obtained for all invasive cases as well as a subset of serous cases. Breast cancer risk estimates were obtained for invasive cases and by estrogen receptor (ER) status. Separate comparisons were made for cases diagnosed before 50 vs. $≥ 50$ years of age to explore effect modification by advancing age of diagnosis. Summary estimates from pooled analyses using genotyped and imputed SNP data were obtained from weighted meta-analysis of study-specific parameter estimates ($β$ coefficients and Standard Error). The minor allele frequency (MAF) for each SNP was estimated from the control population for each study. Study heterogeneity and risk differences associated with age groups (<50 vs. $≥ 50$) were assessed using the likelihood ratio test to compare logistic regression models with and without a multiplicative interaction term. All tests for association were two-tailed, statistical significance was assessed at $P ≤ 0.05$, and were performed in STATA SE v.11 (StataCorp, USA), and SAS v. 9.1. Tests for study heterogeneity and age group interaction tests were implemented in the R project for Statistical Computing (http://www.r-project.org/).

VEGAS applied to dbGAP data

To evaluate evidence for association at TERT and CLPM1L with various cancers, we applied the gene-based test implemented in VEGAS (all SNPs in gene test) [18] to data from dbGAP. In brief, we selected cancer cases and controls from dbGAP that were genotyped on ~550,000 SNPs (Illumina 610 quad or Illumina HumanHap550 arrays). Studies were CGEMS breast (1145 cases, 1142 controls), CGEMS pancreatic cancer (2326 cases, 2351 controls), GENEVA lung cancer (EAGLE and PLCO combined 2748 cases, 2840 controls) and CGEMS prostate (1145 cases, 1054 controls). For full details see dbGAP website (http://www.ncbi.nlm.nih.gov/gap). In each case, following standard quality control, the genomic control lambda was as would be expected if cases and controls were well matched; breast, pancreatic, lung, prostate lambda values 1.01, 1.01, 1.03, 1.03, respectively. The VEGAS gene based $P$ values from each of the four dbGAP studies were combined in a meta-analysis using Fisher’s method for combining $P$ values.

Luciferase assays

Variants were introduced into pGL3-hTERT-3915 [15] by site-directed mutagenesis (Agilent Technologies). The A-A vector was generated by introducing the variants sequentially. Cells were transfected using siPORT NeoFX Transfection Agent (Ambion), according to the manufacturer’s instructions, and harvested after 48 h. Cells were washed with phosphate buffered saline (PBS) and lysed with 200 µL/well lysis buffer (25 mM tris pH 7.8, 2 mM EDTA, 10% glycerol, 1% Triton X-100, 0.2 mM DTT). Luciferase activity was assayed in triplicate for each transfection using 20 µL hlsate and 50 µL reconstituted luciferase assay reagent (Promega). Luminescence was measured immediately for 4 s in each well with a Wallac Victor1 1420 multilabel counter. The experiment was repeated three times and the results averaged. Data were analysed by one-way ANOVA with post hoc Dunnett’s tests in GraphPad Prism version 5.03 (GraphPad Software).
Supporting Information

Table S1  Participating EOC case-control studies. (DOC)
Table S2  Per allele OR for all SNPs in EOC. (DOC)
Table S3  The association of rs2736109 with EOC, by study. (DOC)
Table S4  Participating invasive breast case-control studies. (DOC)
Table S5  The association of rs2736109/rs2736108 with risk of invasive breast cancer, by study. (DOC)
Table S6  Breast cancer risk by age. (DOC)
Table S7  Breast cancer risk by ER status. (DOC)

References


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Author Contributions

Conceived and designed the experiments: GC-T RRR GC-T LAK KKHA TM LFAGM. Wrote the paper: JB HAP GC-T.

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References