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Germ line variation in TP53 regulatory network genes associates with breast cancer survival and treatment outcome

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Abstract

Germ line variation in the TP53 network genes *PRKAG2*, *PPP2R2B*, *CCNG1*, *PIAS1* and *YWHAQ* was previously suggested to have an impact on drug response *in vitro*. Here, we investigated the effect on breast cancer survival of germ line variation in these genes in 925 Finnish breast cancer patients and further analyzed 5 SNPs in *PRKAG2* (rs1029946, rs4726050, rs6464153, rs7789699) and *PPP2R2B* (rs10477313) for 10-year survival in breast cancer patients, interaction with *TP53* R72P and *MDM2*-SNP309, outcome after specific adjuvant therapy, and correlation to tumor characteristics in 4701 invasive cases from four data sets. We found evidence for carriers of

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PRKAG2-rs1029946 and *PRKAG2*-rs4726050 having improved survival in the pooled data (HR 0.53, 95% CI 0.3–0.9; $P = 0.023$ for homozygous carriers of the rare G-allele and HR 0.85, 95% CI 0.7–0.9; $P = 0.049$ for carriers of the rare G allele, respectively). *PRKAG2*-rs4726050 showed a significant interaction with *MDM2*-SNP309, with *PRKAG2*-rs4726050 rare G-allele having a dose-dependent effect for better breast cancer survival confined only to *MDM2* SNP309 rare G-allele carriers (HR 0.45, 95% CI 0.2–0.7; $P = 0.001$). This interaction also emerged as an independent predictor of better survival ($P = 0.047$). *PPP2R2B*-rs10477313 rare A-allele was found to predict better survival (HR 0.82, 95% CI 0.6–0.9; $P = 0.018$), especially after hormonal therapy (HR 0.66, 95% CI 0.5–0.9; $P = 0.048$). These findings warrant further studies and suggest that genetic markers in TP53 network genes such as *PRKAG2* and *PPP2R2B* might affect prognosis and treatment outcome in breast cancer patients.

Keywords

TP53; *MDM2*; *PRKAG2*; *PPP2R2B*; breast cancer; hormonal therapy

Introduction

Several studies have investigated the association of single nucleotide polymorphisms (SNP)s with breast cancer risk or survival (1–4). We have previously investigated the role of TP53 and *MDM2* genetic variants on breast cancer survival (5, 6). The TP53 regulatory network mediates cell cycle arrest or apoptosis in response to stress conditions such as DNA damage, hypoxia and oncogene activation (7). Recent data suggests that germ line variation in genes involved in this stress response network could affect the predisposition to cancer development or patient survival and drug response (8–10). A candidate gene study on 187 genotyped SNPs that reside in 138 genes implicated in mediating the TP53 stress response identified seven SNPs in five genes with significant genotype-drug response association *in vitro* by using data generated by the NCI anticancer drug screen (NCI60 screen) (9). The analysis suggested *MDM2* SNP309 (rs2279744) having an effect on sensitivity to chemotherapeutic agents in cells harboring the wild type *TP53* gene (9). *MDM2* is a negative regulator of TP53 and *MDM2* SNP309 has been shown to increase *MDM2* expression (8, 11). In addition, six other SNPs, residing in five different genes *CCNG1* (Cyclin G1), *PIAS1* (Protein inhibitor of activated Stat1), *PPP2R2B* (PP2A, regulatory subunit B, beta isoform), *PRKAG2* (AMP-activated protein kinase γ 2 subunit) and *YWHAQ* (14-3-3 ϵ), whose products have been shown to regulate TP53 through protein-protein interactions and post-translational modifications, were indicated for genotype-drug response association (9). The alleles of two of these SNPs, those in *YWHAQ* and *CD44*, that associate with weaker growth response to chemotherapeutics were found to associate with poorer overall survival as well as earlier age of diagnosis in patients with soft tissue sarcomas (10). Suggestive evidence was recently reported for an association of *PPP2R2B* (rs319217) and breast cancer risk and recurrence, especially after adjuvant endocrine therapy (12). Additionally, a recent integrated copy number aberration-expression study in primary breast tumors highlighted putative cancer genes, with deletions found in *PPP2R2A* (PP2A, regulatory subunit B, alpha isoform) (13). We recently reported that *PRKAG2*-

rs1860746 within the motif sequence of a *TP53* binding site in the third intron of the gene increases susceptibility to ER-negative breast cancer development (14). (13)

In the *TP53* gene, a common polymorphism at a proline-rich region of TP53 (*TP53* R72P, rs1042522) which results in an arginine to proline change, has been shown to exert a differential effect on apoptosis. *TP53*-R72 was shown to induce apoptosis faster and more efficiently than *TP53*-72P in patients with wild type TP53 tumor status (15, 16). 72P on the other hand, possesses an enhanced ability to induce *CDKN1A* (P21/Cip1) gene transcription which in turn might associate with inferior apoptotic potential and tendency to cell senescence (17). We have previously observed that among breast cancer patients with TP53-negative tumors, the *TP53* rare homozygous (CC) genotype carriers had a worse survival compared to the common allele carriers (GG/GC) (6, 18). We also showed some evidence of predisposition to worse breast cancer survival for *TP53* R72P in combination with the *MDM2* SNP309 variant (6). We did not find evidence for an association between breast cancer survival and *MDM2* SNP309 alone or by TP53 protein expression although this had been suggested in some previous studies (19). Neither the *TP53* R72P nor the *MDM2* SNP309, separately or in interaction, is associated with breast cancer risk in the general European population (20, 21) whereas *MDM2* SNP309 has been reported to be associated with earlier (>10 years) age of onset of breast cancer in Li-Fraumeni patients (8, 22).

Altogether there is evidence that common germ line variants in the TP53 regulatory pathway may affect prognosis but there are few studies that have attempted to investigate other genes than *TP53* itself and *MDM2*. Here, we investigated breast cancer survival effect of germ line variation in *PRKAG2*, *PPP2R2B*, *CCNG1*, *PIAS1* and *YWHAQ* in 925 Finnish breast cancer patients and further analyzed 5 SNPs in *PRKAG2* and *PPP2R2B* in a total of 4701 invasive cases from the Breast Cancer Association Consortium (BCAC) for overall survival, interaction with *TP53* R72P and *MDM2* SNP309 as well as outcome after specific adjuvant therapy. Additionally, we studied the effect of *PRKAG2* and *PPP2R2B* expression in breast tumors on breast cancer survival.

Patients and Methods

Four European studies within the BCAC contributed to this study with data on altogether 4701 invasive breast cancer cases from the Helsinki Breast Cancer Study (HEBCS), Amsterdam Breast Cancer Study (ABCS), Hannover Breast Cancer Study (HABCS) and Polish Breast Cancer Study (PBCS) as described also in previous publications from the BCAC (20, 23). All contributing groups included data on conventional prognostic and predictive markers: tumor grade, size, histological type, estrogen receptor (ER) and progesterone receptor (PR) and nodal status; Her2 status was available in three out of four studies, namely ABCS, HEBCS and PBCS. Each study collected the data independently; from pathology reports and/or Tissue Micro Arrays, medical records and from tumor registries as previously described (23). In brief, ABCS obtained ER and PR status from TMAs. The status was considered positive when >10% of cells were stained. HABCS and HEBCS obtained ER and PR status from medical reports. The status was considered positive when >10% of cells were stained. PBCS obtained ER and PR status from TMAs and medical reports combined (Intensity score (0, 1, 2 and 3) X percentage of cells stained (0–

100%)) 10 was considered positive (total score range 0–300). ABCS obtained HER2 status from TMAs with score 3+ being considered HER2 positive. HEBCS obtained HER2 status from TMAs using CISH-result (0–1=neg, 2–3=pos); if CISH result were not available IHC was used (0–1=neg, 3=pos; score 2 was not used). HER2 status was not available for HABCS. PBCS obtained HER2 status from TMAs with Score 3+ in 20% stained tumor cells being considered HER2 positive (23). The studies and tumor characteristics are described in table 1.

Breast cancer tissue microarray sections were immunohistochemically stained for TP53 expression using mouse monoclonal anti-human TP53-antibody (DO-7, DAKO) as previously described (6, 24) In ABCS, HABCS, HEBCS and PBCS samples were considered positive when >10%, >10%, >20% and >10%, respectively, of the cancer cells were stained. Missing TP53 data could be attributed to missing tumor blocks, loss of cores in the sectioning or staining process or cores not containing enough tumor material (6, 24), or because just a subset of tumors was stained (HABCS). Methods and results are reported according to reporting recommendations for tumor marker prognostic study (REMARK) (25). All studies were done with informed consent from the patients where applicable and permissions from the corresponding Ethics Committee.

SNP selection and genotyping

We initially evaluated the SNPs in the regions of the five genes identified by Vazquez et al. (9), namely *PRKAG2*, *PPP2R2B*, *CCNG1*, *PIAS1* and *YWHAQ*, for survival in breast cancer patients in the HEBCS patients included in BCAC and for whom samples were previously genotyped for a genome-wide case-control breast cancer risk study (26, 27). Of 925 HEBCS invasive breast cancers, 760 cases from the unselected and familial breast cancer series (described in (28–30)) were genotyped with Illumina 550k SNP array and an additional 165 ER-negative cases were genotyped with the Quad610.v1 platform (26, 27). Using Cox regression models for main survival effect as well as stratification by TP53 expression, ER status, adjuvant chemotherapy and adjuvant hormonal therapy we identified five SNPs tagging different haplotypes in *PRKAG2* and *PPP2R2B*, i.e., *PRKAG2*-01 (rs1029946), *PRKAG2*-02 (rs4726050), *PRKAG2*-03 (rs6464153), *PRKAG2*-04 (rs7789699) and *PPP2R2B* (rs10477313), which were significantly associated with overall survival in all patients (*PRKAG2*-02 (rs4726050), *PRKAG2*-04 (rs7789699), *PPP2R2B* (rs10477313)) or in the subgroups defined by tumor TP53 status (*PRKAG2*-01 (rs1029946), *PRKAG2*-03 (rs6464153)), tumor estrogen receptor status (*PRKAG2*-04 (rs7789699), *PPP2R2B* (rs10477313)) or adjuvant hormonal treatment outcome (*PPP2R2B* (rs10477313)) (Supplementary Table 1). None of the SNPs in the studied genes showed survival effect after adjuvant chemotherapy treatment.

PRKAG2-01 (rs1029946) and *PRKAG2*-02 (rs4726050) show low linkage disequilibrium ($r^2=0.22$). *PRKAG2*-02 (rs4726050) tags *PRKAG2* (rs2727567, $r^2=0.89$) which was originally suggested by Vazquez et al. (9), whereas *PPP2R2B* (rs10477313) is independent ($r^2=0.12$) of *PPP2R2B* rs319217 suggested by the same study. (12). For other SNPs suggested by Vazquez et al. (9) (*YWHAQ*(rs6734469), *PPP2R2B*(rs319217 and rs319227), *CCNG1*(rs2069347)), or SNPs that are tagging these SNPs with a $r^2 > 0.80$, no significant

association with survival was observed. *PIAS1* (rs1027154) was not represented or tagged on the SNP genotyping array.

The survival effect of the five selected SNPs in *PRKAG2* (*PRKAG2*-01 (rs1029946), *PRKAG2*-02 (rs4726050), *PRKAG2*-03 (rs6464153), *PRKAG2*-04 (rs7789699)) and in *PPP2R2B* (rs10477313) as well as their interaction with *TP53* R72P and *MDM2* SNP309 was further studied in independent and pooled datasets of 1442 Dutch (ABCS), 794 German (HABCS) and 1540 Polish (PBCS) breast cancer patients. The genotyping was performed by each group separately. The *TP53* R72P (rs1042522) and *MDM2* SNP 309 (rs2279744) were genotyped in ABCS, HABCS and HEBCS as previously described (6); PBCS genotyped *MDM2* SNP309 using the same Taqman assay as the ABCS study (6) and *TP53* R72P as described previously (31). The genotyping for the *PRKAG2* and *PPP2R2B* SNPs was carried out using Applied Biosystems TaqMan SNP genotyping assays; primer and probe sequences are available from the authors upon request. There was a higher frequency of rare homozygous genotypes and deviation from Hardy-Weinberg equilibrium for *PPP2R2B* (rs10477313) in the ABCS, however, the results for this SNP were in keeping with the remaining studies. Genotyping for *PRKAG2*-02 (rs4726050) in ABCS and *PRKAG2*-04 (rs7789699) in PBCS failed to produce conclusive calls therefore these genotyping results were not included in the analysis.

RNA expression profile of *PRKAG2* and *PPP2R2B*

We studied the *PRKAG2* and *PPP2R2B* gene expression for survival using microarray data on 187 primary breast tumors that were processed and hybridized to Illumina HumanHT-12 v3 Expression BeadChips and the raw data were quality checked and normalized as previously described (32). The RNA expression of *PRKAG2* and *PPP2R2B* are shown in supplementary table 2.

Statistical analyses

Statistical analyses on the HEBCS discovery data set were conducted in Anduril (data integration framework) (33) and for other analyses using SPSS v15.0.1 (SPSS, inc.). The significance limit was set at 0.05 (two-sided test), and not adjusted for multiple comparisons. P-values for evaluation of proportional differences in germ line variants by tumor characteristics were calculated using Pearson's chi-squared tests or Fisher's exact test (for $n < 5$). Log-rank tests were used to assess the statistical significance of differences between Kaplan-Meier curves for survival analyses among all patients. Univariate (non-adjusted) and multivariate (adjusted) Cox's regression analyses were used to estimate survival hazard ratios overall and in various subgroups (ER, TP53 and adjuvant hormonal treatment groups). Since TP53 status was available for only 4% of patients in HABCS, this data set was not included in the particular survival analysis by TP53. Multivariate Cox's proportional hazards models included the common prognostic factors: grade, tumor size, nodal status, primary metastasis, estrogen receptor and progesterone receptor status, as categorical variables, as well as age of diagnosis (as a continuous variable). All pooled analyses were adjusted for study; inclusion of study as a stratifier instead of a categorical co-variate did not change the result. The RNA expression of *PRKAG2* and *PPP2R2B* was

assumed as categorical variable (median split) in Log-rank test and as a continuous variable in the univariate Cox's regression model.

In order to run models including all patients, missing value categories were included for each separate variable with missing information. However, sensitivity analysis including only the patients with available information for all the variables was additionally performed and did not change the conclusions of the analyses. The proportional hazard assumption was verified visually evaluating log-minus-log curves. To determine interaction effects between SNPs and tumor characteristics or treatment, interaction terms were included in the Cox regression models, including the main effects (2df each) as well. In the analyses with adjuvant treatment stratifications, we excluded patients who had distant metastasis at the time of diagnosis ($M=1$) as they had been extensively treated for metastatic disease. Follow-up was defined as the time from diagnosis to the end of follow-up or date of death due to breast cancer (for breast cancer survival) or any reason (for overall survival), and right censored at 10-years. 96% of the cases were incident breast cancers (entering the study before or in less than 6 months from the date of diagnosis).

Results

Genotype distribution of the studied SNPs among the breast cancer patients

For all SNPs, except *TP53* R72P, some heterogeneity in genotype frequencies was observed that appeared to be attributable to HEBCS, HABCS and PBCS, due to normal allele frequency variation in different populations. The frequency variation was mostly pronounced in PBCS for *PRKAG2*-01 (rs1029946) and HEBCS for *PRKAG2*-02 (rs4726050) (Table 2). Significant association was seen between genotypes for *PRKAG2*-01 (rs1029946) and *PRKAG2*-02 (rs4726050) ($p \ll 0.001$), i.e. the rare alleles of both SNPs tended to segregate together.

SNPs in *PRKAG2* and *PPP2R2B* associate with clinical and pathological features of the tumors in the pooled data

The association of the studied SNPs with histopathological characteristics of the tumors is presented in supplementary Table 3. Homozygous carriers of *PRKAG2*-01 (rs1029946) rare G-allele developed more TP53-immuno-negative tumors ($p = 0.005$). Homozygous carriers of *PRKAG2*-02 (rs4726050) rare G-allele were diagnosed with fewer high grade tumors ($p = 0.005$) whereas *PRKAG2*-04 (rs7789699) carriers had significantly more high grade tumors ($p < 0.001$) with a dose-dependent effect of the rare allele associating with higher grade (Chi square for trend: $p = 0.002$). Tumors from homozygous carriers of *PPP2R2B* (rs10477313) rare A-allele were more often progesterone receptor negative ($p = 0.002$).

PRKAG2-01 (rs1029946) and *PRKAG2*-02 (rs4726050) associate with breast cancer survival in the pooled data

The Hazard ratio estimates of univariate analysis by the SNPs among all patients and in applicable subgroups (as derived from discovery analysis) are shown in supplementary table 4. Among all cases, patients carrying the *PRKAG2*-01 (rs1029946) homozygous rare G-allele had better 10-year overall survival compared to AG and AA carriers (Figure 1a, Table

3). The Hazard ratio estimates of univariate analysis by *PRKAG2*-01 (rs1029946) within studies were consistent and are shown in supplementary table 5. In a multivariate Cox's regression analysis, *PRKAG2*-01 (rs1029946) remained an independent variable when adjusted for established prognostic factors as well as study and age of diagnosis (HR_{adjusted} 0.57, 95% CI 0.3–0.9; $p = 0.044$) (Table 3); due to the *PRKAG2*-01 (rs1029946) association with TP53 tumor status that we found in this study the TP53 status was also included in the model as a categorical covariate. In the complete case analysis (including only the patients who had complete information for all the prognostic markers), the HR for overall survival remained the same but was no longer significant (HR_{adjusted} 0.54, 95% CI 0.2–1.0; $p = 0.137$ for GG vs. AA/AG). Patients carrying the rare G-allele of *PRKAG2*-02(rs4726050) showed borderline significance for better overall survival compared to homozygous carriers of the common A-allele ($p = 0.049$), however, the effect was not significant in the multivariate model (Figure 1b, Table 3). The Hazard ratio estimate in univariate analysis by *PRKAG2*-02(rs4726050) showed some inconsistency between the studies (Supplementary table 6).

We did not find evidence of an association with survival for *PRKAG2*-03(rs6464153) and *PRKAG2*-04(rs7789699) in the main analyses. In the pooled data, in contrast to our findings in the HEBCS study (Supplementary Table 1), none of the studied SNPs had significant effect on patient survival in the respective subgroups of TP53 positive or ER positive tumors (Supplementary table 4); the observed effect of *PRKAG2*-04 (rs7789699) on patient survival ($p = 0.044$) in the ER positive subgroup (Supplementary table 4) was driven by HEBCS data only, with no effect in the other studies.

***PRKAG2*-02 (rs4726050) interaction with *MDM2* SNP309 (rs2279744) affects patient survival in the pooled data**

For possible interaction with genetic variation in *TP53* or *MDM2*, we stratified the analyses by the *TP53* R72P or *MDM2* SNP309 genotype. Of all the five SNPs, the *PRKAG2*-02 (rs4726050) rare G-allele was found to have a dose-dependent effect for better breast cancer survival confined only to *MDM2* SNP309 heterozygous and/or rare homozygous carriers ($HR_{\text{non-adjusted}}$ 0.45, 95% CI 0.2–0.7; $P = 0.001$ for *PRKAG2*-02 GG in *MDM2* SNP309 TG/GG compared to common homozygous) (Figure 2). The interaction term between *MDM2* SNP309 TG/GG and *PRKAG2*-02 (rs4726050) GG remained significant in multivariate Cox' regression model (HR_{adjusted} 0.59, 95% CI 0.3–0.9; $P = 0.047$). None of the remaining SNPs showed interaction with *TP53* R72P or *MDM2* SNP309.

***PPP2R2B* (rs10477313) associates with patient survival after adjuvant hormonal therapy in the pooled data**

In the pooled data, patients carrying *PPP2R2B* (rs10477313) rare A-allele (GA/AA) showed improved survival compared to homozygous carriers of the common G-allele ($p = 0.018$). *PPP2R2B* (rs10477313) also emerged as an independent prognostic factor in the multivariate Cox's regression analysis (HR_{adjusted} 0.83, 95% CI 0.7–0.9; $p = 0.034$) (Table 3). We further investigated the association between the *PPP2R2B* (rs10477313) and survival stratified by adjuvant hormonal therapy as suggested by the initial analysis in HEBCS study (Supplementary table 1). Among patients who had received adjuvant hormonal therapy, the

PPP2R2B (rs10477313) rare A-allele (GA/AA) associated with better overall survival compared to GG genotype carriers ($HR_{\text{non-adjusted}}=0.66$, 95% CI 0.5–0.9; $P = 0.048$ for GA/AA vs. GG) whereas no such effect was observed in patients who had not received hormonal therapy ($HR_{\text{non-adjusted}} 0.87$, 95% CI 0.7–1.1; $P = 0.200$) (Figure 3, Table 3). While nearly all of the 1278 cases treated with hormonal therapy had hormone receptor positive tumors (98%), no significant survival effect was seen among all ER positive patients as such (1597 cases, Supplementary table 4), nor among patients with ER positive tumor who did not receive hormonal therapy ($n=218$, $HR_{\text{non-adjusted}}=0.97$, 95% CI 0.7–1.2; $P = 0.813$ for GA/AA vs. GG). These data suggest that the observed survival effect is likely due to improved response to hormonal treatment. The Hazard ratio estimates of univariate analysis by *PPP2R2B* (rs10477313) within studies are shown in supplementary table 7. Furthermore, in the multivariate model, the SNP remained as an independent predictor of survival after hormonal therapy ($HR_{\text{adjusted}} 0.48$, 95% CI 0.2–0.8; $p = 0.014$) (Table 3). Interaction analysis in all patients indicated that the interaction term between adjuvant hormonal therapy and *PPP2R2B* (rs10477313) GA/AA genotype was significant in multivariate Cox' regression model ($p = 0.039$). We did not observe any significant effect of *MDM* SNP309 or *TP53* R72P on survival after adjuvant hormonal or chemotherapy treatment.

RNA expression of *PRKAG2* and *PPP2R2B* associates with patient survival

In the HEBCS data set studied for gene expression, we did not find any significant correlation between the studied *PRKAG2* and *PPP2R2B* SNPs and RNA expression of these genes. In the gene expression analysis *per se*, however, higher *PRKAG2* expression correlated with poor breast cancer outcome ($HR_{\text{univariate}} 8.6$, 95% CI 1.3 to 56.1; $P = 0.024$) (Supplementary figure 1). The *PPP2R2B* expression also associated with a significant effect on breast cancer survival: patients with higher expression of *PPP2R2B* had better prognosis ($HR_{\text{univariate}} 0.03$, 95% CI 0.002 to 0.565; $P = 0.019$) (Supplementary figure 2). There was insufficient statistical power for survival analysis by *PPP2R2B* gene expression in the hormone treated subgroup of patients with ER or PR positive tumors

Discussion

We have here investigated germ line variants in the TP53 network genes *PRKAG2* and *PPP2R2B* for survival among breast cancer patients as well as their interaction with *TP53* R72P and *MDM2* SNP309 variants. SNPs in *PRKAG2* and *PPP2R2B* were previously suggested to have significant genotype-drug response association *in vitro* (9). We initially identified four SNPs tagging different haplotypes in *PRKAG2* and one SNP in *PPP2R2B* affecting patient survival by ER, somatic TP53 status on primary breast tumors, or survival after hormonal treatment in HEBCS. The effect of these variants was further tested in 4701 invasive breast cancer cases from the BCAC.

For two SNPs in the *PRKAG2* gene we found suggestive evidence for improved survival in the pooled analysis: the *PRKAG2*-01 (rs1029946) rare homozygous GG genotype as well as the *PRKAG2*-02(rs4726050) rare G-allele (AG/GG) were found to predict better survival in breast cancer patients, with rare allele of *PRKAG2*-01 (rs1029946) having an independent

effect in the multivariate analysis. *PRKAG2* gene encodes the gamma-2 regulatory subunit of AMP-activated protein kinase (AMPK) which is a conserved cellular energy sensor in eukaryotes as well as a modulator of TP53 (34). In nearly every tissue type, AMPK is regulated by LKB1 (35, 36), a tumor suppressor gene whose germ line mutations predispose to the hereditary Peutz-Jeghers cancer syndrome with also 70% breast cancer risk by the age of 70 years (37). Being a highly sensitive cellular energy sensor, AMPK is directly activated by AMP or ADP upon minor increased cellular AMP/ATP ratio. Once activated, AMPK inhibits biosynthetic pathways and activates catabolic pathways in order to conserve and generate more ATP (38). In addition, when nutrients are scarce, AMPK acts as a metabolic checkpoint inhibiting cellular growth (39). AMPK negatively regulates mTORC1 by activating its inhibitor Tuberine (TSC2) or by direct inhibitory phosphorylation of the mTORC1 subunit Raptor which results in regulation of cell growth and metabolism (39). Furthermore, activated AMPK triggers the TP53 pathway under energy shortage conditions, that is, in low glucose state AMPK directly phosphorylates TP53 to induce G1/S cell-cycle arrest whereas under extensive glucose starvation, persistent activation of AMPK promotes accelerated TP53-dependent cellular senescence (40). In contrary to the metabolic tumor suppressor role of AMPK, there is evidence suggesting that under hypoxia and energy shortage, AMPK might give tumor cells a survival advantage by maintaining their energy homeostasis and protecting them from apoptosis (41–43). Although the underlying mechanisms remain poorly defined, it has been demonstrated that AMPK promotes cell survival by both up-regulating NF- κ B-dependent expression of Bcl-2 and Survivin (two important anti-apoptotic proteins) and maintaining intracellular ATP levels in endothelial cells (34, 42, 43). Interestingly, our analysis by *PRKAG2* RNA expression in breast cancer tumors showed significant correlation of *PRKAG2* expression with patient survival indicating a role for this gene in breast cancer progression and patient outcome. However, the actual functional mechanism of how the studied variants affect *PRKAG2*/AMPK2 protein and patient survival is still to be explored. Both *PRKAG2*-01 (rs1029946) and *PRKAG2*-02 (rs4726050) are intronic variants with no predicted effect on the protein sequence; *PRKAG2*-02 (rs4726050) is tagging the *PRKAG2* (rs2727567) variant previously suggested for *in vitro* drug response by Vazquez et al. (9). Whether the studied SNPs might have some regulatory effect on the gene or whether another variant in linkage disequilibrium with these may be the causal allele remains to be determined.

Furthermore, we observed a significant ($P = 0.001$) dose-dependent interactive effect of the *PRKAG2*-02 (rs4726050) with *MDM2* SNP309 on breast cancer survival. The better survival effect by *PRKAG2*-02 (rs4726050) was confined to *MDM2* SNP309 (TG/GG) carriers only, lending further support on the survival effect by *PRKAG2*-02(rs4726050). The production of MDM2 protein is increased by the *MDM2* SNP309 promoter variant, leading to the attenuation of the TP53. We have previously shown a statistically significant interaction of the *TP53* 72Pro variant and the GG genotype of *MDM2* SNP309 on worse breast cancer survival, consistent with the Pro variant having a role of enhanced TP53 DNA repair function combined with increased expression of the MDM2 protein and overall attenuation of the TP53 pathway in the tumor cells (6). The functional effects mediated by *PRKAG2*/AMPK are not completely understood but the results from this study could be taken to suggest that genetic variants affecting the energy metabolism and homeostasis

maintenance by AMPK/*PRKAG2*, in combination with *MDM2* SNP309, may have a differential effect on TP53 function on senescence vs. cell cycle arrest/DNA repair and thus cell survival in breast tumors especially under hypoxia and energy shortage.

We found suggestive evidence for *PPP2R2B* (rs10477313) rare A-allele (AA/AG) predicting better patient survival and this effect was specifically evident among ER/PR positive patients who received hormonal therapy but not among patients who had not received such treatment. The variant was an independent predictor of better outcome after hormonal therapy in multivariate model. Previously, Vasquez et al. had suggested the association of another SNP in the same gene, *PPP2R2B* (rs319217) with breast cancer recurrence in the group of patients who had received adjuvant hormonal therapy (12). Furthermore, our results on breast cancer survival by *PPP2R2B* RNA expression indicated a role also for this gene in breast cancer progression and patient outcome as such even though no result on patient survival by hormonal therapy could be obtained due to the small sample size. The results from the two studies together suggest an impact of *PPP2R2B* in response to hormonal therapy, with different germ line variants in the gene associating with enhanced or reduced survival of breast cancer patients after adjuvant hormonal treatment. *PPP2R2B* encodes the regulatory B subunit of the Ser/Thr protein phosphatase 2A (PP2A). PP2A is a tumor suppressor phosphatase that acts in a wide variety of signaling pathways associating with cancer progression (44). A recent integrated copy number aberration-expression analysis revealed somatic deletion events affecting *PPP2R2A* in primary breast tumors (13). PP2A has also been suggested as a determinant of ER expression in human breast cancer cells. The expression of PP2A appeared to correlate with ER expression and activity but it did not change with ER degradation suggesting PP2A might be an upstream regulator of ER (45, 46). PP2A dephosphorylates TP53 in response to DNA damage, resulting in modification of TP53 activity levels in cells (47). Likewise, cyclinG recruits PP2A in order to dephosphorylate MDM2 and thus regulate both MDM2 and TP53 (48). When PP2A is phosphorylated in its catalytic subunit at Y307 (tyrosine residue) it becomes inactivated (49). pY307-PP2A is highly increased in Her2 positive breast tumors and significantly associates with breast cancer progression (50).

In this study, we have found suggestive evidence for SNPs in two TP53 network genes affecting survival in breast cancer, especially in interaction with other TP53 network genes or after specific adjuvant therapy. A drawback of our study was that out of totally 1303 deceased patients, for 369 (28%) the cause of death were unknown. This may have caused some bias in the survival estimates with respect to mortality from breast cancer, but unlikely to the extent that this would change our results. Altogether, only one of the survival associations suggested by the discovery study (HEBCS) remained significant in the pooled analysis of the four participating studies, i.e. the association of the *PPP2R2B* (rs10477313) with outcome after adjuvant hormonal treatment, supporting the previous findings (12). In addition, while not individually significant, data from the four studies converged to consistent and significant association of the *PRKAG2*-01 (rs1029946) with better survival, with suggestive evidence also for *PRKAG2*-02 (rs4726050) which was especially significant in interaction with *MDM2* SNP309. Taken together, these results suggest a role for the *PRKAG2* gene in breast cancer prognosis. To the best of our knowledge, this is the first

study investigating *PRKAG2* germ line variation for breast cancer survival and further studies are needed to confirm these results. Larger materials will be needed also for a more in depth analysis of the genetic variants in the studied genes to fully assess their role in breast cancer survival, also after adjuvant chemotherapy. (12). Overall, the genetic variation in the TP53 related genes is likely to form a complex interactive network on breast cancer survival and with more in depth analyses of also other genes similar effects are likely to be discovered. In the near future, this may have also clinical significance for models of breast cancer prognosis or treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCAC	Breast Cancer Association Consortium
ABCS	Amsterdam Breast Cancer Study
HABCS	Hannover Breast Cancer Study
HEBCS	Helsinki Breast Cancer Study
PBCS	Polish Breast Cancer Study
ER	estrogen receptor
PR	progesterone receptor

T	tumore size
N	nodal status
M	metastasis at time of diagnosis

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Novelty & Impact Statements

This is the first study investigating *PRKAG2* germ line variation for breast cancer survival. Analysis in 4701 invasive breast cancer cases showed evidence of improved survival for carriers of rare alleles of *PRKAG2*-rs4726050 and *PRKAG2*-rs1029946, with a significant interactive effect of the *PRKAG2*(rs4726050) with *MDM2*-SNP309. We also demonstrated that *PPP2R2B* (rs10477313) A-allele predicts better survival after hormonal therapy, supporting also previous findings.

Figure 1. Kaplan-Meier plots of cumulative 10-year survival for breast cancer patients carrying a *PRKAG2* variant

(a) *PRKAG2-01* (rs1029946), HR 0.53, 95% CI 0.3–0.9; $p = 0.023$ for GG vs. AA/AG and

(b) *PRKAG2-02* (rs1029946), HR 0.85, 95% CI 0.7–0.9; $p = 0.049$ for AG/GG vs. AA.

Figure 2. Kaplan-Meier plots of cumulative 10-year survival for breast cancer patients carrying *PRKAG2* (rs4726050) and *MDM2*-SNP309 variants

Each figure shows Kaplan-Meier breast cancer survival curves of *PRKAG2* (rs4726050) genotypes within one group of *MDM2*-SNP309 genotype (a) *MDM2*-SNP309 TT genotype, $p = 0.924$ (b) *MDM2*-SNP309 TG, $p = 0.002$ and (c) *MDM2*-SNP309 GG, $p = 0.038$. Carriers of *PRKAG2*-02 (rs4726050) rare G-allele have better breast cancer survival among the *MDM2* SNP309 TG/GG carriers HR 0.45, 95% CI 0.2–0.7; $p = 0.001$ (for b and c combined).

Figure 3. Kaplan-Meier plots of cumulative 10-year survival for breast cancer patients carrying *PPP2R2B* (rs10477313) variant in
(a) all breast cancer patients, HR 0.82, 95%CI 0.6–0.9; $p = 0.018$ for GA/AA vs.GG **(b)** those who received hormonal therapy, HR 0.66, 95%CI 0.5–0.9; $p = 0.048$ for GA/AA vs.GG and **(c)** those who did not receive hormonal therapy, HR 0.87, 95%CI 0.7–1.1; $p = 0.200$ for GA/AA vs.GG. Carriers of *PPP2R2B* (rs10477313) rare A-allele (GA/AA) show better overall survival especially after hormonal therapy compared to GG genotype.

Table 1

Description of characteristics of the participating studies.

Study design	ABCS (n=1442)		HABCS (n=794)		HEBCS (n=2256)		PBCS (n=1540)	
	n	%	n	%	n	%	n	%
Hospital-based consecutive cases	42.8±5.2		58.0±10.9		56.4±12.7		55.3±9.8	
Age at Diagnosis								
Mean ± SD								
Grade								
1	450	35.2	48	7.4	561	26.2	299	21.3
2	440	34.4	343	52.9	960	44.9	716	50.9
3	390	30.5	257	39.7	619	28.9	391	27.8
missing	162		146		116		134	
Histopathological type								
Ductal	1080	74.9	557	79.8	1533	68.0	885	73.0
Lobullar	148	10.3	74	10.6	459	20.3	247	20.4
Medullar	3	0.2	12	1.7	29	1.3	11	0.9
Others	211	14.6	55	7.9	235	10.4	70	5.8
missing	0		96				327	
Tumor size								
1	666	62.9	337	60.4	1357	60.8	693	50.1
2	373	35.3	187	33.5	717	32.1	634	45.8
3	19	1.8	15	2.7	75	3.4	57	4.1
4	0	0.0	19	3.4	84	3.8	0	0.0
missing	384		236		23		156	
Nodal status								
Negative	739	52.1	472	66.6	1217	54.7	866	62.3
Positive	679	47.9	237	33.4	1008	45.3	525	37.7
missing	24		85		31		149	
Metastasis at diagnosis								
Negative	1406	98.3	693	97.0	2145	96.7	1330	98.8
Positive	24	1.6	21	3.0	72	3.7	16	1.2

Study design	ABCS (n=1442)			HABCS (n=794)			HEBCS (n=2256)			PBCS (n=1540)		
	n	%		n	%		n	%		n	%	
Hospital-based consecutive cases												
missing	12			80			39			194		
ER Status												
Negative	325	34.1		59	10.1		408	18.9		503	36.0	
Positive	627	65.9		523	89.9		1750	81.1		895	64.0	
missing	490			212			98			142		
PR Status												
Negative	451	47.6		72	13.0		744	34.5		682	48.9	
Positive	496	52.4		483	87.0		1412	65.5		712	51.1	
missing	495			239			100			146		
Her2												
Negative	691	75.9		NA	NA		1071	84.9		828	82.0	
Positive	220	24.1		NA	NA		190	15.1		182	18.0	
missing	531						995			530		
TP53 Status												
Negative	542	69.5		23	67.6		1322	78.2		523	68.6	
Positive	238	30.5		13	32.4		368	21.8		239	31.3	
missing	662			758			566			778		
years of Dig												
Range	1974–1994			1997–2003			1997–1998			2000–2003		
Follow up Mean ± SD	10.2±5.3			7.5±4.3			7.4±2.1			4.8±1.6		
Vital Status												
Alive	914	63.4		660	83.1		1851	82.0		1251	84.1	
Deceased: all-cause	528	36.6		134	16.9		405	18.0		236	15.9	
Deceased: BC-specific	468	32.5		112	14.1		301	13.3		53	3.4	
References	(21, 51)			(52)			(2, 28, 29)			(53)		

ABCS=Amsterdam Breast Cancer Study, HABCS=Hannover Breast Cancer Study, HEBCS=Helsinki Breast Cancer Study, PBCS=Polish Breast Cancer Study, NA=Not Available

Table 2

Genotype distribution of the studied SNPs among the breast cancer patients by study

	ABCS (n=1442)		HABCS (n=794)		HEBCS (n=925)		PBCS (n=1540)	
	n	%	n	%	n	%	n	%
<i>PRKAG2-01(rs1029946)</i>								
AA	1112	78.1	598	75.7	715	77.9	1035	70.3
AG	278	19.5	178	22.5	190	20.7	393	26.7
GG	33	2.3	14	1.8	13	1.4	44	3.0
missing	19		4		7		68	
<i>PRKAG2-02(rs4726050)</i>								
AA	NA	NA	296	37.5	468	50.8	519	34.9
AG	NA	NA	366	46.4	373	40.5	697	46.8
GG	NA	NA	127	16.1	81	8.8	272	18.3
missing			5		3		52	
<i>PRKAG2-03(rs6464153)</i>								
GG	1211	84.7	664	83.7	791	86.3	1190	78.5
GA	204	14.3	125	15.8	122	13.3	306	20.2
AA	14	1.0	4	0.5	4	0.4	19	1.3
missing	13		1		8		25	
<i>PRKAG2-04(rs7789699)</i>								
GG	447	31.3	191	24.1	248	27.0	NA	NA
GA	610	42.8	408	51.4	457	49.7	NA	NA
AA	369	25.9	195	24.6	215	23.4	NA	NA
missing	16		0		5			
<i>PPP2R2B(rs10477313)</i>								
GG	1041	73.7	610	77.0	747	81.1	1200	79.6
GA	265	18.8	170	21.5	161	17.5	287	19.0
AA	106	7.5	12	1.5	13	1.4	21	1.4
missing	30		2		4		32	
<i>MDM2(rs2279744) *</i>								
TT	483	41.7	325	41.6	776	34.6	628	41.9

	ABCS (n=1442)		HABCS (n=794)		HEBCS (n=925)		PBCS (n=1540)	
	n	%	n	%	n	%	n	%
TG	517	44.6	351	44.9	1110	49.4	702	46.8
GG	158	13.6	105	13.4	360	16.0	170	11.3
missing	284		13		10		40	
<i>TP53(rs1042522)*</i>								
GG (Arg/Arg)	651	53.4	424	54.2	807	53.6	759	53.2
GC (Arg/Pro)	468	38.4	297	37.9	591	39.2	558	39.1
CC (Pro/Pro)	101	8.3	62	7.9	109	7.2	109	7.6
missing	222		11		749		114	

* n=2256 in HEBCS

Table 3

Univariate and multivariate Cox's regression analysis by PRKAG2-01 (rs1029946), PRKAG2-02 (rs4726050) and PPP2R2B (rs10477313) for 10-year overall survival and by PPP2R2B (rs10477313) stratified by hormonal therapy in the pooled data.

Variable/Analysis	HR _{non-adjusted} (95% CI)	p	HR _{adjusted} (95% CI)	P
PRKAG2-01(rs1029946)/Overall survival				
GG vs AA/AG	0.53 (0.3–0.9)	0.023	0.57* (0.3–0.9)	0.044
PRKAG2-02(rs4726050)/Overall survival				
AG/GG vs AA	0.85 (0.7–0.9)	0.049	0.85* (0.7–1.1)	0.211
PPP2R2B(rs10477313)/Overall survival				
GA/AA vs. GG	0.82 (0.6–0.9)	0.018	0.83* (0.7–0.9)	0.034
PPP2R2B(rs10477313)/Hormonal treatment				
GA/AA vs. GG	0.66 (0.5–0.9)	0.048	0.48** (0.2–0.8)	0.014
PPP2R2B(rs10477313)/No Hormonal treatment				
GA/AA vs. GG	0.87 (0.7–1.1)	0.200	-	-

* Adjusted for grade, tumor size, nodal status, primary metastasis, estrogen receptor, progesterone receptor, study and age of diagnosis (and for TP53 status in *PRKAG2-01*(rs1029946) model).

** Adjusted for grade, tumor size, nodal status, adjuvant chemotherapy, study and age of diagnosis