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Short title: AMPK SNPs and breast cancer risk

Keywords: AMP-activated protein kinase, breast cancer, cancer susceptibility, body-mass index

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Abstract

Purpose AMP-activated protein kinase (AMPK) is an energy-sensing/signalling intracellular protein which is activated by an increase in the cellular AMP:ATP ratio after ATP depletion. Once activated, AMPK inhibits fatty acid synthesis and the Akt-mTOR pathway, and activates the p53-p21 axis. All these molecular mechanisms are thought to play a key role in breast carcinogenesis We investigated the genetic variability of four genes encoding AMPK (*PRKAA1*, *PRKAA2*, *PRKAB1* and *PRKAB2*).

Methods Using a tagging approach and selecting SNPs we covered all the common genetic variation of these genes. We tested association of tagging SNPs in our four candidate genes with breast cancer (BC) risk in a study of 1340 BC cases and 2536 controls nested into the European Prospective Investigation into Cancer and Nutrition (EPIC). Given the relevance of AMPK on fatty acid synthesis and the importance of body fatness as a BC risk factor, we tested association of SNPs and body-mass index as well.

Results and conclusion We observed no statistically significant association between the SNPs in the PRKAs genes and BC risk and BMI after correction for multiple testing.

Introduction

Hormonal and metabolic factors have been postulated to mediate the effects of a western nutritional lifestyle which is characterized by low rates of energy expenditure and a high-energy diet, rich in saturated fats, refined carbohydrates and animal protein on breast cancer risk [1-5]. One of the metabolic key players is AMP-activated protein kinase (AMPK), a heterotrimeric complex comprising one alpha catalytic subunit and non-catalytic (regulatory) beta and gamma subunits [6].

Known as the ‘‘fuel sensor of the cell’’, AMPK is an energy-sensing/signalling intracellular protein which is inactive unless it has been phosphorylated in response to cellular stresses that deplete cellular energy levels and increase the AMP/ATP ratio [7-9]. Once activated, AMPK switches on ATP-generating (catabolic) pathways and switches off ATP-consuming (anabolic) pathways, allowing the cell to restore its energy balance [7]. The unique ability of AMPK to directly sense cellular energy places it in an ideal position to ensure that cell division, which is a highly energy-consuming process, only proceeds if cells have sufficient metabolic resources to support cell proliferation [10].

AMPK activation overcomes the growth-stimulatory signalling via multiple mechanisms. First, AMPK activity inhibits fatty acid and cholesterol biosynthesis and promotes fatty acid oxidation, thereby opposing intracellular lipid accumulation and development of insulin resistance in non-adipose tissues [11]. Numerous studies have investigated the role of fatty acids, dietary fat, and obesity in cancer

development [12,4,2,13-22]. Second, AMPK inhibits mTOR signaling downstream of Akt, and inhibition of mTOR pathway has been reported to inhibit tumour growth *in vitro* and metastasis in experimental animals [23,24]. This makes AMPK activation a possible therapeutic target for cancers with activated Akt signaling pathway [25].

Finally, Igata and coworkers reported that AMPK activation results in cell cycle arrest at the G1 phase and inhibited cell proliferation via activation of the p53-p21 axis [26-28].

The above observations led us to hypothesize that AMPK may be centrally implicated in mammary gland carcinogenesis, and that polymorphic alleles of its encoding genes that modify its expression or activity confer altered BC susceptibility. In this report we investigated for the first time, the genetic variability of the genes encoding *PRKAA1*, *PRKAA2*, *PRKAB1* and *PRKAB2*. By using a tagging approach and selecting SNPs we covered all the common genetic variation of these genes. We have tested association of tagging SNPs in our four candidate genes with BC risk in a study of 1340 BC cases and 2536 controls nested into the European Prospective Investigation into Cancer and Nutrition (EPIC). Given the key role of AMPK in energy sensing and inhibition of fatty acid synthesis, we tested also association of SNPs with body-mass index (BMI).

Materials and Methods

The EPIC cohort

A fully detailed description of EPIC has been published elsewhere [29]. Briefly, the EPIC cohort consists of about 370,000 women and 150,000 men, aged 35-69, recruited between 1992 and 2005 in 10 Western European countries. Follow-up for cancer incidence was last performed in 2007.

The vast majority (>97%) of subjects recruited in the EPIC cohort are of European ('Caucasian') origin. All EPIC study subjects provided anthropometric measurements (height, weight, and waist and hip circumferences) and extensive, standardized questionnaire information about medical history, diet, physical activity, smoking, and other lifestyle factors. Women also answered questions about menstrual and reproductive history, hysterectomy, ovariectomy, and use of exogenous hormones for contraception or treatment of menopausal symptoms. About 260,000 women and 140,000 men provided a blood sample. Follow-up for cancer incidence was last performed in 2007.

Cases of cancer occurring after recruitment into the cohort and blood donation are identified through local and national cancer registries in 7 of the 10 countries, and in France, Germany, and Greece by a combination of contacts with national health insurances and/or active follow-up through the study subjects or their next of kin. Follow-up on vital status is achieved through record linkage with mortality registries.

Selection of case and control subjects

Case subjects were selected among women who developed BC after blood collection. Control subjects (1-2 controls per case) were selected randomly by incidence density sampling, matching the cases for centre of recruitment, age at blood donation, duration of follow-up, menopausal status at the time of blood donation and use of exogenous hormones. This study did not include women who were using hormone replacement therapy at the time of blood donation. A total of 1340 invasive BC cases and 2536 controls were included in the present study. Each control was free of cancer up to the duration of follow up of the index case. The study was approved by the ethical review boards of the International Agency for Research on Cancer, and of the collaborating institutions responsible for subject recruitment in each of the EPIC recruitment centres.

Selection of tagging SNPs

We aimed at surveying the entire set of common genetic variants in the *PRKAA1, PRKAA2, PRKAB1* and *PRKAB2* genes.

We followed a SNP-tagging approach [30]. Gene regions were defined as the sequence between 5kb 5' of the beginning of the first known exon and 5kb 3' of the end of the last known exon. We included all polymorphisms in each gene region with minor allele frequency (MAF) ≥5% in Caucasians from the International HapMap Project (version 22; http://www.hapmap.org).

Tagging SNPs were selected with the use of the Tagger program within Haploview (http://www.broad.mit.edu/mpg/haploview/; http://www.broad.mit.edu/mpg/tagger/; [31]), using pairwise tagging with a minimum r^2 of 0.8. We used genotypes downloaded from the HapMap database as input for Tagger.

DNA extraction and genotyping

DNA was extracted from blood samples on an Autopure instrument (Qiagen, Hilden, Germany) with Puregene chemistry (Qiagen, Hilden, Germany). The order of DNAs from cases and controls was randomized on PCR plates in order to ensure that an equal number of cases and controls could be analyzed simultaneously.

26 SNPs were genotyped with the iPLEX Gold application (Sequenom, San Diego, USA), based on multiplexed PCR amplification of short amplimers surrounding the SNPs, single nucleotide primer extension reaction and separation of extension products by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF)[32], according to the manufacturer instructions.Plates were read on MassARRAY® system using the Typer software version 4.0 from Sequenom (San Diego, CA). The remaining 15 SNPs were performed using the Taqman assay. Pre-designed assays were purchased from Applied Biosystems (Foster City, CA). Genotyping was performed according to manufacturer's specifications, and PCR plates were read on an ABI PRISM 7900HT instrument using SDS software version 2.4 (Applied Biosystems).

Any sample where greater than 25% of the SNPs failed had all of the SNPs set to missing and these subjects were dropped from analysis. We then filtered data to remove poorly performing SNPs: all SNPs that failed on 25% of samples or more were set to missing, as were all SNPs that showed statistically significant ($p<10^{-3}$) deviations from Hardy-Weinberg equilibrium (HWE) among controls.

Repeated quality control genotypes (8% of the total) showed an average concordance of 99.50 %.

All genotyping was performed at the German Cancer Research Center (DKFZ). Since the genotyping was performed using two different techniques one of the SNPs (rs10074991) was typed on all subjects using both TaqMan and iPlex to ensure an internal quality control; concordance rate was 100%.

Statistical analysis

The frequency distribution of genotypes was examined for cases and controls. We used conditional logistic regression for multivariate analyses to assess the main effects of the genetic polymorphism on BC risk assuming dominant, recessive and codominant models of inheritance. The most common allele in the controls was held as the reference category in calculating the odds ratio (OR). Cases with carcinomas *in situ* (n = 84 cases and 148 matched controls) were excluded from these analyses. Subgroup analyses were performed based on menopausal status, age (with cutpoint at 50 or 55 years of age at diagnosis) and BMI (subjects

with BMI≥30 were categorized as obese, 25 ≤ BMI < 30 as overweight and <25 as normal weight). Additional analyses were performed by including cases of carcinoma *in situ*, by excluding cases diagnosed shortly (6 months, 1 year or 2 years) after blood drawing. Relationships of polymorphic gene variants with BMI were estimated by standard regression models and percentage of difference in geometric means in each genotype category compared to the major homozygote category was calculated. An additional analysis was performed by calculating odds ratios for obese or overweight vs. normal weight with unconditional logistic regression. All BMI analyses were adjusted for age at blood donation, center of recruitment and BC case-control status.

Polymorphisms selected as tagging SNPs had a very high coverage of the total linkage disequilibrium of the gene region (on average r^2=0.943 for the *PRKAA1* gene, r^2=0.907 for *PRKAA2,* r^2=0.959 for *PRKAB1* and r^2=0.988 for *PRKAB2*), therefore we assumed that haplotypes were adequately captured by our tagging SNPs, and we did not attempt a haplotype analysis. In this study we tested SNPs using a study-wise statistically significant threshold of 0.05/39=0.0013. Thus p-values will be interpreted in light of the multiple comparisons.

STrengthening the REporting of GeneticAssociation studies (STREGA) criteria were used to report the data.[33]

Results

This study explored the association between 41 DNA polymorphisms in four key genes encoding AMPK and BC risk, as well as with BMI.

A total of 3876 women, 1340 BC cases and 2536 matched controls were included in the analysis. Table 1 summarizes the baseline characteristics of cases and controls. The genotype distributions at all SNPs typed in the EPIC samples were in Hardy-Weinberg equilibrium in controls, with non-significant χ^2 values with the exception of rs3805486 (PRKAA1) and rs11584787 (PRKAB2) which were excluded from further analysis. The average call rate was 92% (80%-100%).

The frequencies and distribution of the genotypes and the odds ratios for the association of each polymorphism with BC risk and BMI level are described in Supplementary table 1 and in Supplementary table 2 respectively. In table 2 and 3 we show for each gene the SNP with the most significant association respectively for BC risk and BMI. The strongest association we observed was for the heterozygous carriers (C/T) of the SNP rs17159882, of the PRKAB2 gene, which was associated with an increased risk of BC: OR=1.50 (95% CI 1.11-2.02) P=0.009. Since the MAF of this polymorphism is 0.056 the homozygous carriers were too few (1 case; 3 controls) to meaningfully test the possible association between this genotype and risk.

No other statistically significant association was observed between the remaining SNPs in the PRKAs genes and BC risk overall and stratifying for age or BMI. We also performed analysis dividing the subjects according to

their menopausal status, although the number of pre-menopausal women was fairly small (323 cases 723 controls). We did not find any significant association between any of the polymorphisms and BC in the strata considered.

Additional analyses performed by including cases of carcinoma *in situ*, excluding cases diagnosed shortly after blood drawing or dividing in subgroups for menopausal status showed essentially the same results as using all cases and controls (data not shown).

Discussion

We postulated that genetic polymorphisms in *PRKAA1*, *PRKAA2*, *PRKAB1* and *PRKAB2* might play an important role in development of BC given their key function in the regulation of whole-body energy metabolism and the response to acute changes in energy levels in individual cells. AMPK activity inhibits fatty acid and cholesterol biosynthesis and promotes fatty acid oxidation, thereby opposing intracellular lipid accumulation and development of insulin resistance in non-adipose tissues. It overcomes the growth-stimulatory signalling also by inhibiting the mTOR pathway and activating the p53-p21 axis [34,35,25].

Moreover, recent discoveries that a tumour suppresor, LKB1, is present upstream and two distinct tumour suppressors, p55 and TSC2 lie downstream, have provided novel evidence that AMPK may function as a suppressor of cell proliferation [25].

Recently metformin (an AMPK activator) which is increasingly being considered and tested for treatment of breast cancer; has been shown to reduce aromatase expression in breast adipose stromal cells, suggesting that AMPK activation could also reduce breast cancer risk by down-regulating local estrogen production [36,37].

We tested in a large-scale association study, nested within the EPIC cohort, the involvement in BC risk of common polymorphisms of the four genes. We did not observe any significant association between any polymorphisms in the PRKAs genes and an increased risk of cancer with

the exception of one SNP, rs17159882, which belongs to the *PRKAB2* gene locus. This SNP is situated at 1620bp 5' of the gene. So one can postulate that the polymorphism might lie in the gene promoter impairing its function and thus increasing the risk of BC. However, since we tested 39 SNPs a rigorous study-wise statistically significant threshold is 0.05/39=0.0013. Thus after correction for multiple testing, this finding is not significant. One likely explanation might be chance finding.

Body fatness directly affects levels of many circulating hormones, such as insulin, insulin-like growth factors, oestrogens and inflammatory factors, which are all well known risk factors for BC risk [36,18,19,38-40], thus creating an environment that encourages carcinogenesis and discourages apoptosis. For these reason we performed also a regression analysis to investigate the possible impact of PRKA gene polymorphic variants on BMI. We have not found any statistically significant association with BMI. In this study we had sufficient power (0.80 for codominant model) to detect OR=1.25 at alpha=$1.2x10^{-3}$ (study-wide significance p-threshold) for a SNP with a MAF of 0.30, or to detect OR=1.40 for a SNP with a MAF of 0.10 at the same level of alpha.

In conclusion, we have exhaustively investigated the complete genetic variation in *PRKAA1*, *PRKAA2*, *PRKAB1* and *PRKAB2* in relation with BMI and BC risk, in a large study of almost 4000 women. We did not find any SNP that emerged at the study-wise level of $p<0.0013$ and thus we can confidently exclude a major role of these SNPs in BMI alteration and in BC risk.

Competing interests

The authors declare that they have no competing interests

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Supplementary material

Supplementary table 1. Main effects of 39 SNPs genotyped in the study on breast cancer risk. Columns in this table show: gene name; NCBI dbSNP rs number; the three possible genotypes; numbers of cases for each of the three genotypes; controls for each of the three genotypes; odds ratios for heterozygotes and odds ratios for homozygotes for the rare allele with 95% confidence interval (referred to the homozygotes for the common allele); associated p-value; p-value of the trend test.

Supplementary table 2. Main effects of 39 SNPs genotyped in the study on BMI. Columns in this table show: gene name; NCBI dbSNP rs number; possible genotypes; numbers of subjects for each of the three genotypes; BMI mean for each of the three genotypes with 95% confidence interval; p-value of the trend test.

Table 1. Baseline characteristics of BC cases and control subjects.

Variable	***Cases***	***Controls***
Number	1340[a]	2536[a]
Women with carcinoma *in situ*	84[a]	148[a]
Pre-menopausal women	323[a]	723[a]
Peri-menopausal women	116[a]	211[a]
Post-menopausal women	901[a]	1602[a]
Mean age at blood donation	55.49 (40.6-67.5)[b]	55.0 (40.1-67.9)[b]
Mean age at diagnosis	57.8 (44.0-70.0)[b]	-
Height	161.8 (151.0-172.0)[b]	160.7 (150.0-172.0)[b]
Weight	67.5 (51.8-89.0)[b]	67.2 (51.0-89.4)[b]
Body mass index	25.8 (20.2-34.3)[b]	26.0 (20.1-34.9)[b]

[a]Number of subjects

[b]Mean (5th - 95th percentiles)

Table 2. Associations between SNPs in candidate genes and breast cancer risk. For each gene, the SNP with the lowest p-value is reported.

Gene	***tagSNP***	***Chr***	***Position***	***Cases/Controls[a]***			***OR Mm vs MM***	P_{value}	***OR mm vs MM***	P_{value}	p_{trend}
	rs_number			***M/M[c]***	***M/m[c]***	***m/m[c]***	***(95% CI)[b]***		***(95% CI)[b]***		
PRKAA1	rs10053664	5	40,821,568	392/680	579/858	219/321	1.13 (0.96-1.33)	0.146	1.12 (0.90-1.40)	0.293	0.197
PRKAA2	rs2796516	1	56,903,911	866/1386	297/438	24/31	1.11 (0.93-1.32)	0.247	0.81 (0.43-1.51)	0.629	0.228
PRKAB1	rs4767830	12	118,519,607	397/557	568/894	211/378	0.90 (0.76-1.06)	0.216	0.81 (0.66-1.00)	0.055	0.051
PRKAB2	rs17159882	1	145,091,689	1099/1756	90/99	1/3	1.50 (1.11-2.02)	0.009	0.56 (0.06-5.69)	0.621	0.017

[a] Numbers may not add up to 100% of subjects due to genotyping failure.

[b] OR: odds ratio: estimated by conditional logistic regression with matching of cases and controls for age at blood donation and centre of recruitment; CI: confidence interval

[c] M/M : Homozygous for the major allele; M/m: Heterozygous individuals; m/m: Homozygous for the minor allele

Table 3. Associations between BMI and SNPs belonging to candidate genes. For each gene, the SNP with the lowest p-value is reported.

Gene name	***rs#***	***Homozygotes common allele***		***Heterozygotes***		***Homozygotes rare allele***		P_{trend}
		N[a]	***BMI (95%CI)***	***N***	***BMI (95%CI)***	***N***	***BMI (95%CI)***	
PRKAA1	rs10074991	2039	25.86 (25.66-26.05)	1517	25.99 (25.76-26.23)	308	26.14 (25.64-26.64)	0.2131
PRKAA2	rs2796516	2854	25.98 (25.81-26.15)	922	25.83 (25.54-26.12)	72	25.56 (24.52-26.59)	0.2591
PRKAB1	rs1541345	3477	25.90 (25.74-26.06)	393	26.17 (25.73-26.61)	4	28.29 (23.91-32.68)	0.1881
PRKAB2	rs2304893	3141	25.87 (25.71-26.04)	549	26.18 (25.80-26.56)	22	27.43 (25.55-29.30)	0.0510

[a] Numbers may not add up to 100% of subjects due to genotyping failure

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