J Cancer Res Clin Oncol (2009) 135:1159–1168
DOI 10.1007/s00432-009-0556-x

ORIGINAL PAPER

A polymorphism in the promoter region of *Ku70/XRCC6*, associated with breast cancer risk and oestrogen exposure

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Received: 10 July 2008 / Accepted: 26 January 2009 / Published online: 15 February 2009

Abstract
Purpose Polymorphisms in double strand break repair genes could be involved in genetic breast cancer predisposition as enhanced chromosomal radiosensitivity is a hallmark for breast cancer. Previously, the c.-1310 C>G SNP, located in the *Ku70* promoter, showed a significant odds ratio (OR) of 1.85 ($P = 0.048$) in sporadic, but not familial breast cancer patients, indicating that other factors besides genetic aptitude influence this association. As breast epithelium is exposed to endogenous oxidative stress through oestrogen exposure, the influence of hormone exposure was further examined.
Methods and results A significant OR (1.69, $P = 0.017$) was found for an enlarged patient population through PCR-RFLP assays in a case–control study in a Belgian population. After dividing the patient population according to oestrogen exposure, high and significant ORs were seen for patients with a longer oestrogen exposure (late age at menopause: OR = 1.96, $P = 0.029$).
Conclusion These results show that the variant allele of c.-1310 C>G, located in the Ku70 promoter, is a risk allele for breast cancer. Furthermore, the association of the c.-1310 C>G SNP with breast cancer risk was stronger in women with a long oestrogen exposure.

Keywords Double strand break (DSB) repair · Non-homologous end-joining (NHEJ) · Ku70/XRCC6 · Breast cancer · Hormone exposure · Single nucleotide polymorphism (SNP)

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Introduction

A family history of breast cancer is a well-known risk factor for the disease (Teare et al. 1994) and extensive epidemiological studies have identified a number of breast cancer susceptibility genes of which BRCA1 and BRCA2 are the best known (Dunning et al. 1999). However, the proportion of breast cancer cases caused by mutations in *BRCA1/2* in the general patient population is estimated to be only 5%, thus indicating that mutations in low penetrant genes or subtle defects arising from low penetrant variations in other highly penetrant genes may predispose to breast cancer (Peto et al. 1999; Rebbeck 1999; Nathanson et al. 2001; Fu et al. 2003). As the preservation of genomic integrity is essential in the prevention of tumour initiation and progression, mutations and variations in DNA repair genes may play a role in the genetic predisposition to breast cancer. One of the most detrimental forms of DNA damage is the double strand break (DSB), because the DNA loses physical integrity and information content on both strands.

Besides being the result of normal metabolic processes, DSBs can also be induced by carcinogenic or mutagenic agents such as ionizing radiation (Khanna and Jackson 2001; Valerie and Povirk 2003). The fact that lymphocytes of breast cancer patients are characterized by an enhanced in vitro chromosomal radiosensitivity (Jones et al. 1995; Scott et al. 1998, 1999; Baeyens et al. 2002, 2005), suggests that breast cancer can be driven by DSB-initiated chromosomal instability. This hypothesis is further supported by the involvement of *BRCA1* and *BRCA2* in DSB repair (Tutt and Ashworth 2002) and by several population-based case–control studies, showing a link between single nucleotide polymorphisms (SNPs) in DSB repair genes and breast cancer risk (Dunning et al. 1999; Kuschel et al. 2002; Fu et al. 2003; Bau et al. 2004, 2007; Zhang et al. 2006; Ralhan et al. 2007; Nowacka-Zawiszac et al. 2008; Willems et al. 2008). Repair of DSBs in mammalian cells occurs by two main pathways, homologous recombination (HR) and non-homologous end-joining (NHEJ) (reviewed in Valerie and Povirk 2003). HR is mainly used by simple eukaryotes, but can also be applied for DSB repair in multicellular eukaryotes, during late S and G_2 phases of the cell cycle. The missing information is copied from an undamaged homologous chromatid or chromosome, making HR an error-free pathway (Valerie and Povirk 2003). Conversely, NHEJ is an error-prone pathway as the broken DNA termini are first processed to make them compatible and then sealed by a ligation step which often results in the loss of a few nucleotides at the broken ends. Nonetheless, this pathway is considered to be the major repair pathway of DSBs in eukaryotic cells during most phases of the cell cycle, particularly during G_0 and G_1 (Pfeiffer et al. 2004), and is suggested to be the main mechanism through which DSBs induced by ionizing radiation are removed from the DNA of higher eukaryotes (Iliakis et al. 2004). The key protein components of NHEJ (reviewed in Lieber et al. 2003) include the catalytic subunit of DNA protein kinase (DNA-PK_{CS}), the two regulatory subunits of the DNA-PK complex Ku70 and Ku80, DNA ligase IV with its cofactor XRCC4 (the X-ray cross complementing group 4 protein) and the nuclease artemis. The Ku70/Ku80 (Ku) heterodimer is the first protein to bind to the damaged DNA ends. When bound to the DSB, Ku recruits and activates DNA-PK_{CS}. As these proteins play a prominent role in DNA DSB repair, they are substantial for genome stability and will act as tumour suppressors. However, either the DNA protein kinase complex, or its three subunits individually, can also act as oncogenes, depending on the compartment of the cell in which they are expressed and on the cell cycle phase (Downs and Jackson 2004; Gullo et al. 2006). Furthermore, expression of the Ku heterodimer on the cell surface seems to play a role in cell adhesion and invasion (Muller et al. 2005).

In recent years, DSB repair pathways have also been implicated in cancer treatment. In breast cancer patients carrying a BRCA1 or BRCA2 mutation, tumour cells usually lose the wild type allele, leading to a loss of function of the BRCA1/BRCA2 protein. These tumours generally show a significantly lower ability to repair DSBs then the normal tissue, which could indicate that DSB-inducing agents, used in radiation therapy and chemotherapy, selectively affect the BRCA-deficient tumour cells. Results of preclinical and clinical studies confirm that the loss of BRCA1 function through mutation, sensitizes the cell to DNA-damaging chemotherapy commonly used in breast and ovarian cancer (Kennedy et al. 2004).

The rationale described here can also be extended to sporadic cancers. The development of DSB repair inhibitors, could sensitize tumour cells to DSB-inducing radiation therapy and chemotherapy in cancer patients (Belzile et al. 2006). As DNA-PK is a central actor in NHEJ, its inactivation has been considered to have clinical potential (Salles et al. 2006).

In our previous study we investigated the association between SNPs in NHEJ genes and breast cancer susceptibility (Willems et al. 2008). A positive association was found between the variant allele of the c.-1310 C>G SNP (NCBI rs2267437)—located in the promoter region of *Ku70*, overlapping with a directionally divergent intronic sequence of the gene FAM152B—and breast cancer risk, with a significantly increased odds ratio (OR) observed in sporadic breast cancer patients. In familial breast cancer patients, this SNP did not significantly increase breast cancer risk, which could indicate that other factors besides genetic aptitude modify the association between the c.-1310 C>G SNP in the *Ku70* promoter and breast cancer (Willems et al. 2008).

Other important risk factors associated with breast cancer are an early age of first menarche, nulliparity or late first childbirth, and late menopause (McPherson et al. 2000). The major determinant common for these risk factors is the prolonged exposure to female sex hormones and these hormonal influences on breast cancer risk have been mainly attributed to exposure to elevated levels of oestrogens (reviewed in Yager and Davidson 2006). Three mechanisms have been considered to be responsible for the carcinogenity of oestrogens: (1) receptor-mediated hormonal activity (Clarke et al. 2004; Pearce and Jordan 2004), (2) a cytochrome P450 (CYP)-mediated metabolic activation (Roy et al. 2007) and (3) the induction of aneuploidy (Russo et al. 2003).

In this study, we investigated if prolonged oestrogen exposure modifies the association between the variant allele of the c.-1310 C>G SNP in the *Ku70* promoter and breast cancer in an enlarged group of sporadic breast cancer patients.

Table 1 Information on the patient population

	Patients (*N*)	Mean (years) ± SD	Median (years)
Age of the total patient population	206	53 ± 12.35	53.5
Known age at first menarche	96	13 ± 0.88	13
Known age at menopause	76	50 ± 5.41	50
Known exposure interval	96	35 ± 5.75	37
Known menopausal status	177		
Known pT pN pM[a]	161		
Known receptor status[b]	163		
Known HER2 expression	160		

[a] *pT* Tumour size, *pN* nodal status, *pM* metastases

[b] Oestrogen and progesterone receptor status

Materials and methods

Study population

Our population of female breast cancer patients (mean age = 53.4 ± 12.4 years; $n = 206$) comprises an unselected group of patients. Blood samples were consecutively acquired through collaboration with the Department of Gynaecological Oncology of the Ghent University Hospital, and the Middelheim Hospital in Antwerp as the patients presented themselves to the hospital for treatment. Patients were not screened for familial clustering, and as only 15% of the general patient population has a family history of the disease (Baeyens et al. 2005), this population is presumed to consist mainly of sporadic breast cancer patients. All the patients signed an informed consent. Information on menopausal status, age at fist menarche and menopause, tumour classification, receptor status and HER2 expression of the tumour was collected from the patient files. Not every patient file included all the information. Age at first menarche was known for 96 patients and age at menopause was known for 76 postmenopausal patients. For further statistical analysis, the mean age of the patients with a known age at first menarche or menopause was used for the patients for whom this information was not available. The menopausal status was known for 177 patients and the mean age of menopause, as calculated for the patients for whom age at menopause was known, was used to classify the other patients as pre- or postmenopausal (Table 1). Tumour characteristics were available for over 160 patients. Patients with an oestrogen receptor (ER) or progesterone receptor (PR) expression under 5% were considered to be ER(−) and PR(−), respectively.

The control population (mean age = 50.4 ± 14.5 years; $n = 171$) of healthy, female individuals included mainly staff members of Ghent University and Ghent University Hospital. Blood samples were obtained during the annual occupational medical examination. Additional samples of elderly healthy women were acquired during local senior club meetings. All healthy volunteers signed an informed consent.

Collection of blood samples

Heparinized blood samples of patients and controls were kept at room temperature. Lymphocyte separation was performed within 24 h after venepuncture, using Lymphoprep (Axis-shield, Lucron). Isolated lymphocytes were stored in liquid nitrogen until DNA-extraction was performed (QIAamp DNA Blood Mini Kit, Qiagen).

Genotyping of the c.-1310 C>G SNP in the Ku70 promoter

Polymerase chain reaction (PCR) was combined with restriction fragment length polymorphism (RFLP) analysis to genotype the c.-1310 C>G SNP (Willems et al. 2008). PCR products were amplified using 100–200 ng DNA in a 25 µl reaction containing 0.5 mM dNTP's (Amersham Bioscience), 1× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 1 mM forward and reverse primer (Invitrogen; F- CTTCAGACCACTCTCTTCTC, R- TCACCTCACAG TAGTCGTTG) and 0.6 U Platinum Taq polymerase (Invitrogen). DNA amplification was performed using a 35-cycle PCR program consisting of an initial denaturation step at 95°C for 5 min, 35 cycles of 3 min with a denaturation step at 95°C (1′), an annealing step at 58°C (1′), and an elongation step at 72°C (1′) followed by a final extension step of 10 min at 72°C. The efficiency of the PCR reaction was confirmed by gel electrophoresis on a 1.5% agarose gel and visualized under ultraviolet light after ethidium bromide staining (Fig. 1).

After DNA amplification, the PCR products were digested using the specific restriction endonuclease, *Hha*I (New England Biolabs) during 4 h at 37°C. Digested products were then analysed by gel electrophoresis on a 2% agarose gel and visualised under ultraviolet light after ethidium bromide staining (Fig. 2).

Statistical analysis

Statistical analysis of the data was performed using Microsoft office Excel 2007 and Statistical Package for Social Sciences (SPSS), version 15.0, software.

The observed genotype distributions were compared with those expected from Hardy–Weinberg equilibrium (HWE) using a standard χ^2 test.

The association of the c.-1310 C>G SNP in the *Ku70* promoter with breast cancer risk was evaluated by calculating

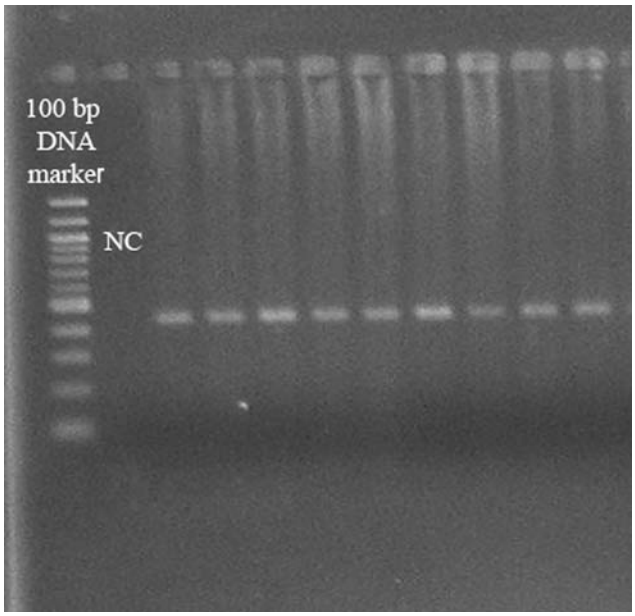

Fig. 1 Single banded PCR products of 438 basepairs. The negative control (*NC*) excludes possible contamination of the PCR reaction

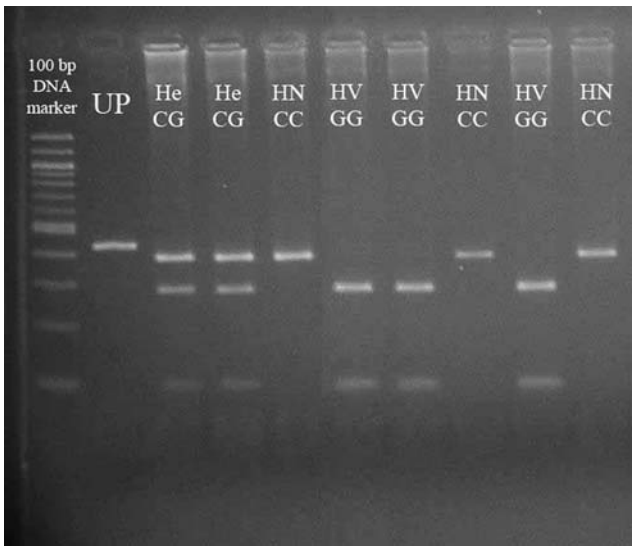

Fig. 2 Digest bands. Next to the DNA marker, the uncut PCR product (*UP*) is shown. After digestion, the C allele results in a visible band of 390 basepairs and the G allele results in two visible bands of 291 and 99 basepairs

crude ORs and 95% confidence intervals (95% CIs) of both the heterozygous (He) and homozygous variant (HV) genotypes using the homozygous normal (HN) genotype as reference. In order to improve the statistical power of the analytical work, especially for the rare HV genotype, we combined the He and HV genotypes in one group. The significance of the crude ORs was also assessed using a χ^2 test. Age corrected ORs were calculated using logistic regression. As information on menopausal status or age at menarche of the control population was not available, we evaluated the influence of these risk factors by comparing different patient groups to the entire control population.

The association between the c.-1310 C>G SNP in the *Ku70* promoter and the different tumour characteristics were evaluated by a two-sided χ^2 test for trend.

Results

Comparison of the observed genotype distributions of the c.-1310 C>G SNP in the *Ku70* promoter with those expected from HWE shows no systematic deviation ($P > 0.05$). The variant allele frequency of our control population (0.37) is comparable with the values listed in NCBI (Global population: 0.28; European population: 0.412).

The first part of Table 2 lists the crude and corrected ORs for the whole patient population when comparing with the control population. In agreement with our previous study, we observe a positive and significant OR for the He genotype (age corrected OR = 1.68; $P = 0.027$) and the He + HV group (age corrected OR = 1.69; $P = 0.017$) in this expanded case–control study. For further analysis, the patient population has been divided in two groups based on age and menopausal status. Although the mean ages of patients and controls are comparable, correction for age is performed by logistic regression as the age distribution of the patient and control population differs. For the division based on age, a limit of 50 was used to obtain a sufficient number of patients in both groups. Furthermore, this is also the mean age at menopause, which allows us to compare the age influence with the influence of menopausal status. The patients of 50 years or more, who have the longest exposure to oestrogens and other environmental mutagens such as mutagenic pollutants and ionizing irradiation, show significant age corrected ORs ($OR_{He+HV} = 1.77$, $P = 0.044$) while the patients under 50 years of age display no significant results. Table 2 also shows the ORs for the patient groups, divided by menopausal status. As the menopausal status of the controls is not known, the complete control population was used to calculate the ORs. Significant, positive ORs (age corrected $OR_{He+HV} = 1.87$, $P = 0.025$) were found in the patient group which includes postmenopausal patients and patients who were going through menopause at the time of diagnosis (perimenopausal). With respect to female hormone exposure, this is the patient group with the longest exposure to oestrogens. As the period of oestrogen exposure will increase with age, both factors cannot be truly separated. However, when comparing the OR for the older patient group (≥50 years) with the peri- and postmenopausal patient group, we observe a more pronounced OR for the latter.

Table 2 Genotype frequencies of c.-1310 C>G (*Ku70* promoter) in the patient and control population with crude and age corrected ORs and the 95% CIs

	Genotype		Cases % (#)	Controls % (#)	Crude OR	95% CI		*P* (α = 0.05)	Corr OR	95% CI		*P* (α = 0.05)
All patients compared to all controls	HN	CC	28.64 (59)	41.52 (71)	Ref.				Ref.			
	He	CG	51.94 (107)	42.69 (73)	**1.76**	**1.12**	**2.78**	**0.020**	**1.68**	**1.06**	**2.67**	**0.027**
	HV	GG	19.42 (40)	15.79 (27)	*1.78*	*0.98*	*3.24*	*0.080*	*1.72*	*0.94*	*3.15*	*0.078*
	He+HV	CG+GG	71.36 (147)	58.48 (100)	**1.77**	**1.15**	**2.72**	**0.012**	**1.69**	**1.10**	**2.61**	**0.017**
Patients < 50 years Controls < 50 years	HN	CC	29.87 (23)	28.95 (29)	Ref.				Ref.			
	He	CG	49.35 (38)	50.00 (27)	1.77	0.85	3.71	0.179	1.53	0.70	3.34	0.288
	HV	GG	20.78 (16)	21.05 (13)	1.55	0.62	3.87	0.476	1.06	0.40	2.83	0.920
	He+HV	CG+GG	70.13 (54)	71.05 (40)	1.70	0.86	3.37	0.174	1.37	0.66	2.84	0.395
Patients ≥ 50 years Controls ≥ 50 years	HN	CC	28.35 (36)	41.18 (42)	Ref.				Ref.			
	He	CG	52.76 (67)	45.10 (46)	1.70	0.95	3.04	0.100	1.70	0.95	3.04	0.075
	HV	GG	18.90 (24)	13.73 (14)	2.00	0.90	4.43	0.128	1.99	0.90	4.42	0.090
	He+HV	CG+GG	71.65 (91)	58.82 (60)	*1.77*	*1.02*	*3.07*	*0.058*	**1.77**	**1.02**	**3.07**	**0.044**
Patients: Premenopausal All controls	HN	CC	32,56 (28)	41.52 (71)	Ref.				Ref.			
	He	CG	48,84 (42)	42.69 (73)	1.46	0.82	2.60	0.256	1.53	0.83	2.84	0.174
	HV	GG	18,60 (16)	15.79 (27)	1.50	0.70	3.20	0.390	1.54	0.69	3.43	0.289
	He+HV	CG+GG	67,44 (58)	58.48 (100)	1.47	0.85	2.53	0.209	1.54	0.86	2.74	0.147
Patients: Peri and postmenopausal All controls	HN	CC	25,83 (31)	41.52 (71)	Ref.				Ref.			
	He	CG	54,17 (65)	42.69 (73)	**2.04**	**1.19**	**3.49**	**0.013**	**1.83**	**1.02**	**3.26**	**0.042**
	HV	GG	20,00 (24)	15.79 (27)	*2.04*	*1.02*	*4.07*	*0.065*	*2.00*	*0.95*	*4.22*	*0.068*
	He+HV	CG+GG	74,17 (89)	58.48 (100)	**2.04**	**1.22**	**3.39**	**0.008**	**1.87**	**1.08**	**3.24**	**0.025**

Both populations were divided in age groups and by menopausal status. Significant findings are highlighted

Table 3 shows the results of a more refined analysis in which the oestrogen exposure in patients was quantified through three parameters: (1) age at first menarche, (2) age at menopause and (3) exposure interval. An early menarche or late menopause will generally cause a longer oestrogen exposure of the breast tissue. To decide what is considered as 'early' and 'late', the median age was used (Table 1). Patients with an age of first menarche over 13 years are considered to have a late menarche, while patients who went into menopause before 50 years, are considered to have an early menopause. The exposure interval is calculated as follows: for premenopausal patients: exposure interval = age at first menarche to age at diagnosis; for perimenopausal and postmenopausal women: exposure interval = age at first menarche to age at menopause. The median is again used to define a 'short' (<37 years) and 'long' (≥37 years) exposure interval (Table 1). The complete control population is used to compute the association as information on menarche and menopause is not known for control individuals. The patient groups with a shorter oestrogen exposure show no significant results, while all patient groups with a longer oestrogen exposure show significantly positive ORs (Table 3; Fig. 3; patients with early menarche: corr $OR_{He+HV} = 1.84$, $P = 0.008$; patients with an age of ≥50 at menopause: corr $OR_{He+HV} = 1.96$, $P = 0.029$; patients with an exposure interval ≥37: corr $OR_{He+HV} = 1.81$, $P = 0.035$).

As Ku can also act as an oncogene and play a role in invasion, the patients of whom the information on tumour characteristics was available (Table 1) were also divided according to tumour size (pT), nodal status (pN), ER, PR and HER2 expression. As only three patients had metastases, this parameter was not used for further analysis. The results of this analysis are shown in Table 4 and Fig. 4. Although no significant results were found, we observed that the percentage of patients with nodal invasion (pN1+) and a HN genotype is higher then HN patients without nodal invasion (pN0). In addition, a higher percentage of the patients with a PR expression under 5% or no HER2 expression show a HN genotype when compared to patients with progesterone receptor or HER2 expression. As triple negative [triple(−)] tumours with an expression under 5% of ER, PR and no expression of HER2, are usually considered to be very aggressive (Reis-Filho and Tutt 2008), we also considered this group for further analysis. No significant *P* values were found, but patients with a triple(−) tumour showed a more pronounced difference in the percentages of the HN patients when comparing with non-triple(−) patients (Fig. 2, triple(−) HN: 43%; non-triple(−) HN: 29%).

Table 3 Genotype frequencies of c.-1310 C>G (*Ku70* promoter) in the patient and control population with crude and age corrected ORs and the 95% CIs

Oestrogen exposure	Genotype		Cases % (#)	Controls % (#)	Crude OR	95% CI		*P* (α = 0.05)	Corr OR*	95% CI		*P* (α = 0.05)
Patients: late menarche All controls	HN	CC	44.44 (8)	41.52 (71)	Ref.				Ref.			
	He	CG	44.44 (8)	42.69 (73)	0.97	0.35	2.73	0.833	0.95	0.34	2.68	0.925
	HV	GG	11.11 (2)	15.79 (27)	0.66	0.13	3.29	0.890	0.66	0.13	3.30	0.611
	He + HV	CG+GG	55.56 (10)	58.48 (100)	0.89	0.33	2.36	0.990	1.01	0.98	1.05	0.477
Patients: early menarche All controls	HN	CC	27.13 (51)	41.52 (71)	Ref.				Ref.			
	He	CG	52.66 (99)	42.69 (73)	**1.85**	**1.16**	**2.96**	**0.014**	**1.81**	**1.12**	**2.91**	**0.015**
	HV	GG	20.21 (38)	15.79 (27)	**1.94**	**1.21**	**3.11**	**0.008**	**1.91**	**1.01**	**3.54**	**0.040**
	He + HV	CG+GG	73.81 (137)	58.48 (100)	**1.91**	**1.23**	**2.97**	**0.006**	**1.84**	**1.17**	**2.87**	**0.008**
Patients: age at menopause < 50 All controls	HN	CC	31.58 (6)	41.52 (71)	Ref.				Ref.			
	He	CG	42.11 (8)	42.69 (73)	1.30	0.43	3.93	0.857	1.29	0.43	3.91	0.651
	HV	GG	26.32 (5)	15.79 (27)	2.19	0.62	7.78	0.375	2.20	0.62	7.82	0.223
	He + HV	CG+GG	68.42 (13)	58.48 (100)	1.54	0.56	4.24	0.554	1.54	0.56	4.23	0.407
Patients: age at menopause ≥ 50 All controls	HN	CC	25.49 (25)	41.52 (71)	Ref.				Ref.			
	He	CG	55.88 (55)	42.69 (73)	**2.14**	**1.20**	**3.80**	**0.013**	**1.95**	**1.03**	**3.67**	**0.039**
	HV	GG	18.63 (19)	15.79 (27)	2.00	0.95	4.20	0.100	1.99	0.87	4.52	0.101
	He + HV	CG+GG	74.51 (74)	58.48 (100)	**2.10**	**1.22**	**3.63**	**0.010**	**1.96**	**1.07**	**3.57**	**0.029**
Patients: exposure interval < 37 All controls	HN	CC	31.82 (28)	41.52 (71)	Ref.				Ref.			
	He	CG	46.59 (41)	42.69 (73)	1.42	0.80	2.55	0.295	1.47	0.79	2.71	0.222
	HV	GG	21.59 (19)	15.79 (27)	1.78	0.86	3.71	0.171	1.89	0.88	4.08	0.105
	He + HV	CG+GG	68.18 (60)	58.48 (100)	1.52	0.88	2.62	0.165	1.58	0.89	2.81	0.117
Patients: exposure interval ≥ 37 All controls	HN	CC	25.00 (31)	41.52 (71)	Ref.				Ref.			
	He	CG	55.15 (64)	42.69 (73)	**2.01**	**1.17**	**3.44**	**0.016**	**1.84**	**1.03**	**3.30**	**0.039**
	HV	GG	19.85 (21)	15.79 (27)	1.78	0.88	3.62	0.156	1.72	0.80	3.68	0.165
	He + HV	CG+GG	75.00 (85)	58.48 (100)	**1.95**	**1.17**	**3.25**	**0.015**	**1.81**	**1.04**	**3.14**	**0.035**

The patient population was divided according to oestrogen exposure. Significant findings are highlighted

Discussion

Genes involved in NHEJ such as *Ku70*, *Ku80* and *DNA-PK*$_{CS}$ are considered to be essential for genome stability and consequently for cell survival. Severe defects in these genes would result in cell death triggered by cell cycle checkpoint surveillance. However, small genetic variations such as SNPs might escape cell checkpoint surveillance. These variations can lead to suboptimal DNA repair which would allow DNA damage to accumulate and this could trigger tumour initiation (Fu et al. 2003). Breast tissue is subjected to enhanced oxidative stress through the exposure to oestrogens. Metabolic activation of oestrogens, through various cytochrome P450 complexes, generates reactive intermediates such as oestrogen quinones. These quinones can form oestrogen–DNA adducts that may form depurinating adducts when not properly repaired. During the metabolic redox cycling, between the quinone and hydroquinone forms of oestrogen, reactive oxygen species such as superoxide radicals and hydroxyl radicals are generated that cause oxidative DNA damage (Roy et al. 2007). DNA depurination and oxidative DNA damage can result in clustered sites of DNA damage including DSBs. If this induction of DSB is combined with a suboptimal repair of DSBs, the risk of breast carcinogenesis could vary in women with different profiles of oestrogen related risk factors. Cheng et al. (2005) found variations in oestrogen metabolizing genes that are associated with an increased breast cancer risk. When combining putative high-risk polymorphisms in oestrogen metabolizing genes with putative high-risk polymorphisms in DSB repair genes, a joint effect on breast cancer risk was seen for HR genes. This shows that oestrogen exposure may initiate breast cancer by causing DSBs. Our results support this hypothesis as the variant allele of c.-1310 C>G SNP in the *Ku70* promoter displays a significant positive correlation with breast cancer risk in peri- and postmenopausal women, while the correlation with breast cancer risk is not significant in premenopausal women (Table 2). Furthermore, patients with a prolonged oestrogen exposure, measured by several parameters such as age at

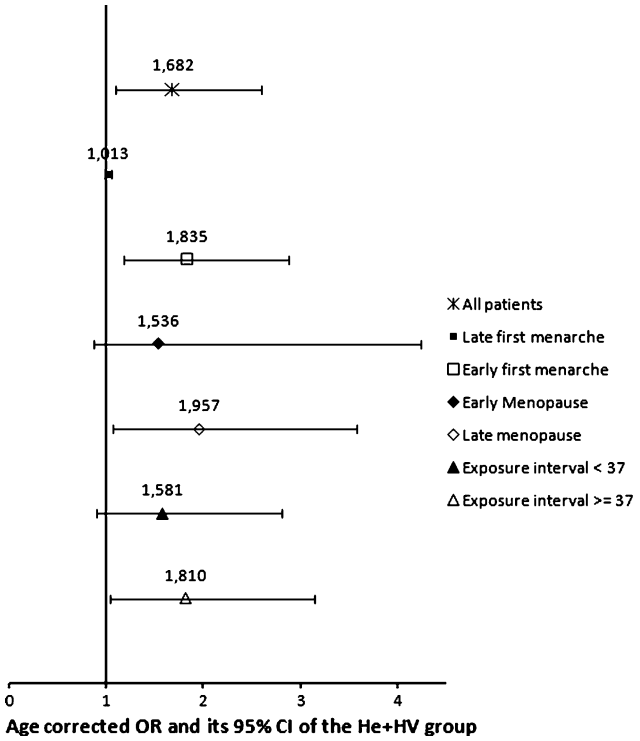

Fig. 3 Patient population divided in groups based on oestrogen exposure. Patient groups with a short oestrogen exposure: 1. Patients with late first menarche, 2. Patients with early menopause, 3. Patients with an exposure interval shorter then 37 years. Patient groups with a long oestrogen exposure: 4. Patients with early first menarche, 5. Patients with late menopause, 6. Patients with an exposure interval of 37 years or more

first menarche, age at menopause and exposure interval, resulted in higher, significant ORs compared to lower, non-significant ORs for patients with a shorter oestrogen exposure (Table 3; Fig. 1). When interpreting these results, one should still be cautious as they are based on relatively small populations. However, the fact that the influence of c.-1310 C>G (in the *Ku70* promoter) on breast cancer risk is still seen after doubling the patient population size, adds a great deal of reliability to the results.

Our data are in agreement with the results of Fu et al. (2003) where a similar result was observed when combining the effect of nulliparity in conjunction with putative 'high-risk' genotypes of five NHEJ genes. The combination of both, is associated with a greater risk of breast cancer.

The c.-1310 C>G SNP is adjacent (…[C>G]CG **CCACCC**…) to the first putative CACCC box of the *Ku70* promoter, as described by Hosoi et al. (2004). Because CACCC consensus sequences are usually extended to their 4–5 upstream nucleotides (Hasan and MacDonald 2002), the c.-1310 C>G SNP may even lay within- or one base before- the CACCC consensus sequence. CACCC boxes are known binding sites for Sp1 and other Kruppel-like transcription factors like KLF4, KLF6 and KLF5, the latter

Table 4 Association of the c.-1310 C>G SNP in the *Ku70* promoter with tumour characteristics such as tumour size (pT), nodal status (pN), oestrogen receptor (ER) progesterone receptor (PR) and HER2 expression

	pT1	pT2+	pN0	pN1+	ER(+)	ER(−)	PR(+)	PR(−)	HER2(+)	HER2(−)	Non-triple(−)	Triple(−)
Total #	92	69	90	71	124	39	103	59	29	131	137	23
Variant allele frequency %	0.45	0.44	0.48	0.40	0.43	0.46	0.44	0.43	0.45	0.44	0.44	0.41
HN # (%)	29 (31.52)	20 (28.99)	24 (26.67)	25 (35.21)	39 (31.45)	11 (28.21)	29 (28.16)	21 (35.59)	7 (24.14)	43 (32.82)	40 (29.20)	10 (43.48)
He # (%)	43 (46.74)	37 (53.62)	45 (50.00)	35 (49.30)	63 (50.81)	20 (51.28)	57 (55.34)	25 (42.37)	18 (62.07)	62 (47.33)	73 (53.28)	7 (30.43)
HV # (%)	20 (21.74)	12 (17.39)	21 (23.33)	11 (15.49)	22 (17.74)	8 (20.51)	17 (16.50)	13 (22.03)	4 (13.79)	26 (19.85)	24 (17.52)	6 (26.09)
P ($\alpha = 0.05$)		0.658		0.336		0.893		0.281		0.355		0.128

Triple(−) tumours, which are ER(−), PR(−) and HER2(−) were also further examined as a separate group. Patient numbers and percentages for the different genotypes (HN, He and HV) are given. The P values were calculated using a two-sided χ^2 test for trend

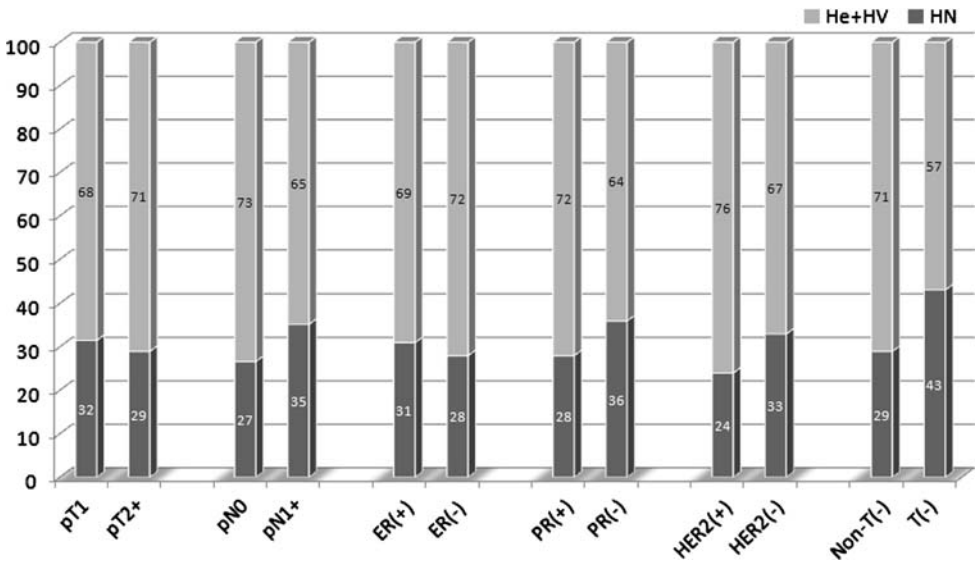

Fig. 4 Association of the c.-1310 C>G SNP in the *Ku70* promoter with tumour characteristics such as tumour size (*pT*), nodal status (*pN*), oestrogen receptor (*ER*) progesterone receptor (*PR*) and HER2 expression. Triple(−) (T(−)) tumours, which are ER(−), PR(−) and HER2(−) were also further examined as a separate group. The patient percentages of the HN and He + HV genotypes are given for the different tumour characteristics

having been described as a likely tumour suppressor in breast cancer (Chen et al. 2002; Rozenblum et al. 2002). Because it has been demonstrated that not only single nucleotide substitutions within Sp1/Kruppel-like binding sites, but also changes in adjacent sequences have a profound effect on the binding/activity of these transcription factors (Hasan and MacDonald 2002), we are currently investigating whether the "G" variant of the c.-1310 C>G polymorphism can influence the expression profile of Ku70 in normal and tumour cells.

An alternative hypothesis for a functional role of the c.-1310 C>G polymorphism may be represented by its association with other mutations associated by a strong linkage disequilibrium. Examples of these mutations have been previously described (Willems et al. 2008).

It must be taken into consideration that the sequence harbouring this mutation overlaps with a long intronic region at the beginning of FAM152B, a gene not yet characterized, encoded in reverse orientation. Thus, the polymorphism studied in our investigation, rather than in Ku70 expression/activity, might be functionally related to LOC27351, the hypothetic protein encoded by FAM152B.

Besides acting as tumour suppressors through their role in DNA repair, the Ku70/Ku80 heterodimer and DNA-PK_{CS} also act as oncogenes (Gullo et al. 2006). The cytosolic expression of Ku 70 can bind and inhibit the pro-apoptotic protein BAX and consequently prevents apoptosis, this might allow a cell with a compromised genome to survive (Downs and Jackson 2004). Although the Ku heterodimer has a positive role in telomere maintenance, it is also involved in mediating disastrous chromosomal fusions by its NHEJ capacity when telomeres are dysfunctional in mammalian cells (Downs and Jackson 2004). These opposing functions of Ku in the formation of gross chromosomal rearrangements are also seen in yeast (*Saccharomyces cerevisiae*). It is said that different cell cycle phases and/or different modifications of Ku could influence in which direction Ku will play a part (Banerjee et al. 2006). Increased Ku expression has been associated with the progression of certain tumour types such as gastric cancer (Gullo et al. 2006) and modulation of Ku DNA-binding activity in human neoplastic breast tissues is possibly related to tumour progression (Pucci et al. 2001). Furthermore, expression of Ku on the cell surface of normal cells seems to be correlated with cell adhesion, migration and invasion (Muller et al. 2005).

Analyses of the c.-1310 C>G SNP in patient groups differing in their tumour characteristics (Table 4; Fig. 4) are supportive for a link between the "C" allele and tumour progression. Patients with low aggressive tumours (pN0) show a higher percentage of genotypes with at least one variant allele of c.-1310 C>G in the *Ku70* promoter, while patients with more invasive tumours (pN1+) are more likely to express the HN genotype. Aggressive triple(−) tumours have the lowest percentage of patients with a variant allele (Fig. 4). While the variant allele of c.-1310 C>G in the *Ku70* promoter is associated with an enhanced breast cancer risk, it could promote less aggressive breast tumours. Possibly, the variant genotype could be linked with a phenotype with suboptimal DSB repair which allows accumulation of mutations and promotes chromosomal instability and ultimately cancer development. Defects in DNA repair might contribute to the early steps of tumourigenesis, but they are not beneficial to the long-term progression of cancer and will sensitize the tumour cells to DNA damage inducing chemo/radiotherapy (Belzile et al. 2006). Sporadic breast cancer patients with a low DNA-PK activity measured in the peripheral blood lymphocytes were

reported to display enhanced chromosome instability and a more aggressive cancer phenotype (Someya et al. 2007). Several studies also correlated Ku70 expression with clinical outcome after treatment. Low expression of Ku70 was correlated with a better survival in patients with cervical carcinomas, suggesting that the lack of Ku leads to increased radiosensitivity (Wilson et al. 2000). Good local control rates after radiotherapy correlates with a low proportion of Ku70-expressing tumour cells in nasopharyngeal carcinomas (Sang-Wook et al. 2005). As cytosolic expression of Ku70 was implicated in apoptosis through inactivation of the pro-apoptotic BAX, cells that were induced to overexpress BAX were highly sensitized to the chemotherapeutic agent curcumin when Ku70 was down regulated (Karunagaran et al. 2005). Our results indicate that, whereas normal Ku70 will protect genome stability during tumour initiation, it will play an opposite role in established tumour tissue by promoting tumour progression.

In conclusion, the data presented here show that the variant allele of the c.-1310 C>G SNP in the *Ku70/XRCC6* promoter is a risk allele for breast cancer and the presence of the variant allele of c.-1310 C>G, in combination with prolonged oestrogen exposure, shows a more pronounced elevation in breast cancer risk. The combination of a SNP in NHEJ genes and a hormonal factor, possibly reflecting susceptibility to oestrogen exposure, is associated with increased breast cancer risk. Furthermore, the c.-1310 C>G "G" allele might promote the development of less aggressive breast carcinomas.

Acknowledgments The work was supported by a grant of the "Bijzonder Onderzoeksfonds" (Gent University, No B/05780/01). The authors declare that we have no conflict of interest. We wish to thank all the patients and volunteers who participated in this study.

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