Analysis of the polymorphisms in non-homologous DNA end joining (NHEJ) gene Ku70 and Ligase IV in sporadic breast cancer in women

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Purpose: Breast cancer is one of the major killers worldwide. Aberrant double-stranded break (DSB) repair leads to genomic instability, which is a hallmark of malignant cells. Double-stranded breaks are repaired in two pathways: homologous recombination (HR) and non-homologous DNA end joining (NHEJ). It is not known whether these repair pathways are affected in sporadic breast tumours.
Material and methods: In the present work the distribution of genotypes and frequency of alleles of the Ku70, A46922G (rs132793) polymorphism and Ligase IV, A6008G (Ile591Val) (rs2232641) polymorphism in breast cancer women were investigated. The genetic polymorphism analysis was performed using a DNA ABI PRISM™ 377 sequence detection system (Applied Biosystems) in 135 sporadic breast cancer cases.
Results: The distribution of the genotypes of the A46922G polymorphism of Ku70 in patients differed significantly ($p < 0.05$) from those predicted by the Hardy-Weinberg equilibrium. There were significant differences in the frequencies of alleles between the breast cancer subjects and controls ($p < 0.05$). However, the distribution of the genotypes of the A6008G polymorphism of Ligase IV in both controls and patients did not differ significantly ($p > 0.05$) from that predicted by the Hardy-Weinberg distribution.
Conclusion: The results support the hypothesis that the A46922G polymorphism of the Ku70 gene may be associated with the incidence of breast cancer in women from the Lodz region of Poland.

Key words: Ku70, Ligase IV, NHEJ, breast cancer, gene polymorphism.

Introduction

Breast cancer is an increasingly important cause of illness and death among women. Breast cancer affects one out of every 10 women in industrialized countries, and is a leading cause of cancer morbidity and mortality in women.

Genomic rearrangements such as translocations, deletions, and duplications are extremely frequent in cancer cells, particularly, in breast cancer cells [1–4]. Genomic rearrangements are believed to result from the aberrant repair of DNA double-strand breaks (DSBs).

A double-strand break (DSB) is the most lethal of all DNA lesions. If unrepaired, a DSB leads to loss of chromosome segments and threatens the survival of the cell. Equally detrimental to the organism are misrepaired DSBs that destabilize the genome and lead

to genomic rearrangements. Genomic rearrangements become common in aging organisms, ultimately leading to deregulation of transcription and malignancies [5, 6]. DSBs in DNA are repaired by two major mechanisms: homologous recombination (HR) and non-homologous end joining (NHEJ) [7, 8].

During HR-mediated repair of DSB, the sister chromatid is used as a template to copy the missing information into the broken locus. In contrast, the NHEJ pathway simply fuses two broken ends with little or no regard for sequence homology. NHEJ starts with binding of Ku70/Ku80 heterodimer to the broken ends. Ku facilitates recruitment of Artemis-DNA-PKcs complex, which processes the ends to prepare them for ligation [9].

Next, the gaps are filled by DNA polymerase of the poIX family, and covalently joined by XRCC4-DNA ligase IV complex. NHEJ is rarely error-free, leading to deletions or insertions of filler DNA [10]. DSBs situated between two direct repeats can also be repaired by single-strand annealing (SSA), a highly mutagenic recombinational mechanism in which the sequence between the repeats is deleted.

Because single nucleotide polymorphism (SNP) is the most frequent and most subtle genetic variation in the human genome and has great potential for application to association studies of complex diseases [11], we used SNPs in the NHEJ genes *Ku70* and *Ligase IV* to define their tumourigenic contribution to breast cancer development.

In the present work the association between *Ku70*, A46922G polymorphism and *Ligase IV*, A6008G (Ile591Val) polymorphism and breast cancer risk in Polish women was investigated.

Materials and methods

Patients

Blood was obtained from 135 postmenopausal women with node-negative (n = 55) and node-positive (n = 80) ductal breast carcinoma treated at the Department of Oncology, Institute of Polish Mother's Memorial Hospital. No distant metastases were found in patients at the time of treatment. The patients ranged in age from 43 to 82 years (median age 58 years). Median follow-up of patients at the time of analysis was 39 months (range: 2-71 months). The average tumour size was 20 mm (range: 17-32 mm). All tumours were graded by a method based on the criteria of Scarf-Bloom-Richardson. There were 31 (23.0%) tumours of grade I, 81 (60.7%) of grade II and 23 (16.3%) of grade III in total. Steroid receptor status was not determined in the investigated group. Blood samples from age-matched healthy women (n = 60) served as the controls.

Genotyping

We genotyped all patient and control samples for the listed polymorphisms (Table I) using a Big Day™ Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems) and the DNA ABI PRISM™ 377 sequence detection system (Applied Biosystems). TaqMan primers and probes were designed using Primer Express Oligo Design Software v1.0 (Applied Biosystems). Assays (15 μl) were carried out on 20 ng genomic DNA according to the manufacturer's instructions. Amplifications were carried out on the GeneAmp 2400 PCR System (Perkin Elmer) at the annealing temperatures given in Table 1. Plates were read post-PCR on the ABI PRISM 377 sequence detector using the Big Day™ Terminator Cycle sequencing Ready Reaction Kit (Applied Biosystems).

The results were analysed by Sequencing Analysis Software™ ver. 3.4.1 (Applied Biosystems) and Factura™.

Statistical analysis

For each polymorphism, deviation of the genotype frequencies in the controls from those expected under the Hardy–Weinberg equilibrium was assessed using χ^2-test. Genotype frequencies in cases and controls were compared by χ^2-tests. P-values < 0.05 were considered to be significant.

Results

Table II shows the genotype distribution of *Ku70* gene polymorphism between breast cancer patients and controls. It can be seen from the table that there were significant differences ($p < 0.05$) between the two investigated groups. The frequencies of the G and A alleles were 0.65/0.35 in patients and 0.54/0.46 in controls. In patients the observed frequencies of the G/G, G/A and A/A genotypes differed significantly ($p < 0.05$) from the distribution expected from the Hardy–Weinberg equilibrium.

Distributions of the A/A, A/G and G/G genotypes of the *Ligase IV* gene as well as the frequencies of the A and G alleles for breast cancer subjects and controls are displayed in Table III. It can be seen from the table that there were no significant differences between these two groups in both genotype distribution and allele frequencies ($p > 0.05$).

Dependencies of the distribution of genotypes and frequencies of alleles of both investigated polymorphisms on the tumour grade evaluated according to Scarf-Bloom-Richardson criteria in patients with breast cancer are displayed in Tables IV and V, respectively. There were no significant differences between distributions of genotypes in

subgroups assigned to histological grades and the distribution predicted by the Hardy–Weinberg equilibrium ($p > 0.05$). There were no differences in frequencies of any of the alleles between subgroups either ($p > 0.05$).

Discussion

DNA is a precious molecule. It encodes vital information about cellular content and function. Damage to DNA can lead to cell cycle arrest, cell death or mutation.

In the NHEJ pathway, *Ku70* and *Ku80* then bind the DSB, followed by recruitment and activation of DNA-protein kinase (DNA-PK). *XRCC4* and ligase IV (LIG4) are recruited by the DNA-PK holoenzyme, activated by DNA-PK-mediated phosphorylation, and then repair the break. Common variants in genes involved in DNA DSB repair are good candidates for low-penetrance breast cancer susceptibility. The aim of this study was to test the hypothesis that variants in genes *Ku70* and *LIG4* involved in DNA DSB repair confer increased susceptibility to breast cancer.

Kuschel *et al*. suggest that variants in *XRCC2* and *LIG4* alter breast cancer risk, together with stronger evidence that variants of *XRCC3* are associated with risk. The variants in *XRCC2* increase and those in *LIG4* decrease breast cancer risk [12].

The variant alleles of 1310C>G (*Ku70*) and 2099-2408G>A (*Ku80*) are risk alleles for breast cancer as well as chromosomal radiosensitivity CRS. The homozygous variant genotype of 1781G>T (*Ku70*), on the other hand, seems to protect against breast cancer and ionizing radiation induced micronuclei [13].

The data show that two SNPs in *Ku70* and *XRCC4* may be associated with breast cancer risk. The increased risk of developing breast cancer was found in women harbouring a greater number of putative high-risk genotypes of NHEJ genes [14].

Fu *et al*. genotyped 30 SNPs in all five NHEJ genes (*Ku70*, *Ku80*, *DNA-PKcs*, *Ligase IV*, *and XRCC4*) in primary breast cancer patients. Two SNPs in *Ku70* and *XRCC4* were associated with breast cancer risk; this study provides new insights to suggest the role of the NHEJ pathway in breast cancer development and supports the possibility that breast cancer is initiated by oestrogen exposure, which causes DNA DSBs [14].

In premenopausal breast cancer in women four variants were associated with breast cancer risk: two in the XPF gene and two in the *XRCC3* gene [15].

In the present work we did not find any correlation between occurrence of cancer and *LIG4* gene polymorphism in Polish women. However, we detected a significant difference in distribution

Table I. Primers, probes and PCR conditions for genotyped single-nucleotide polymorphisms

Polymorphism	Forward primer(5'–3')	Reverse primer(5'–3')	Concentration (nM)	Vic-probe	Concentration (nM)	Fam-probe	Concentration (nM)	Anneal temperature
Ku70	TCAGTCCTGGAA GTGCTTGGT	AGGCCTGCCG GGCT	900	CTGCTTCTTCAGC CCACTCTTCAGC	100	TCCTGCTTCTTCAGA CCACTCTTCAGC	100	64°C
Ligase IV	TGAACCTTGTAA TTCTGTCATTG TTCA	TCAATTCGTGGA AAACGCAA	300	ATCGTACCCAGTG ACATGTATAAA ACTGGCTG	200	ATCGTACCCAGT GATATGTATAAA ACTGGCTGC	50	60°C

Table II. Distribution of A/A, G/A and G/G genotypes and frequencies of the A and G alleles (Ku70) in patients with breast cancer (n = 135) and controls (n = 60)

	Breast cancer patients		Controls	
	Number	Frequency	Number	Frequency
G/G genotype	70	0.52	15	0.25
G/A genotype	35	0.26	35	0.58
A/A genotype	30	0.22	10	0.17
χ^2	25.15[a]		1.83[b]	
G allele	175	0.65	65	0.54
A allele	95	0.35	55	0.46

[a] *$p < 0.05$ as compared with Hardy-Weinberg distribution*
[b] *$p > 0.05$ as compared with Hardy-Weinberg distribution*

Table III. Distribution of A/A, A/G and G/G genotypes and frequencies of the A and G alleles of *Ligase IV* in patients with breast cancer (n = 135) and controls (n = 60)

	Breast cancer patients		Controls	
	Number	Frequency	Number	Frequency
A/A	40	0.30	18	0.17
A/G	75	0.56	58	0.55
G/G	20	0.15	30	0.28
χ^2	2.50[a]		1.261[b]	
A allele	155	0.57	94	0.44
G allele	115	0.43	118	0.56

[a] *$p > 0.05$ as compared with Hardy-Weinberg distribution*
[b] *$p > 0.05$ as compared with the controls*

Table IV. Dependency of genotypes and frequencies of the alleles of *Ku70* gene polymorphism on the tumour grade in patients with breast cancer[a]

Grade[b]	I		II		III	
	Number	Frequency	Number	Frequency	Number	Frequency
G/G	20	0.65	56	0.69	12	0.52
G/A	10	0.32	22	0.27	8	0.35
A/A	1	0.03	3	0.04	3	0.13
G allele	50	0.81	134	0.83	32	0.70
A allele	12	0.19	28	0.17	14	0.30
χ^2	0.03[c]	0.20[c]	0.73[c]			

[a] *n = 135*
[b] *according to Scarf-Bloom-Richardson criteria*
[c] *$p > 0.05$ as compared with Hardy-Weinberg distribution*

frequency of A and G alleles of *Ku70* gene polymorphism between patients and controls ($p < 0.05$). The distribution of the genotypes G/G, G/A and A/A in patients differed from the one expected from the Hardy-Weinberg equilibrium, with an overrepresentation of G/G homozygotes. It is possible that the presence of the G allele is in linkage disequilibrium with another (so far unknown) mutation located outside the coding region in the *Ku70* gene, which may be important for the *Ku70* concentration in plasma.

On the other hand, we haven't detected any significant difference between the genotypes in subgroups assigned to histological stages, which suggests a lack of association between the *Ku70* polymorphism and breast cancer invasiveness.

Table V. Dependency of genotypes and frequencies of the alleles of *Ligase IV* gene polymorphism on the tumour grade in patients with breast cancer[a]

GRADE[b]	I		II		III	
	NUMBER	FREQUENCY	NUMBER	FREQUENCY	NUMBER	FREQUENCY
A/A	19	0.61	41	0.51	21	0.87
A/G	9	0.29	32	0.39	2	0.09
G/G	3	0.10	8	0.1	1	0.04
A allele	47	0.76	114	0.71	32	0.91
G allele	15	0.24	48	0.30	14	0.81
χ^2	1.34[c]	0.22[c]	4.7[c]			

[a] *n = 135*
[b] *according to Scarf-Bloom-Richardson criteria*
[c] *p > 0.05 as compared with Hardy-Weinberg distribution*

Our study implies that polymorphism of the *Ku70* gene may be associated with the occurrence of breast cancer in women from the Lodz region of Poland. Further studies, conducted on a larger group, are required to clarify this point.

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