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Original contributions

Changes in *BRCA1* gene in patients with familial breast cancer in the Warsaw region of Poland

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52 patient-probands with a family history of breast cancer and other tumours were selected from among 500 breast cancer patients treated at the Maria Sklodowska-Curie Memorial Cancer Centre and Institute of Oncology. We studied DNA isolated from peripheral blood. The PCR-SSCP procedure was used for seeking mutations in all protein coding exons of the BRCA1 gene. Fragments suspected of mutation presence were subjected to sequencing. In 36 probands of the BRCA1 gene 11 types of changes were found in exons and 5 types in introns. In 19 (36.5%) probands no changes were detected within the BRCA1 gene. Among exon changes 3 were ones generally regarded as pathogenic mutations: 5382insC (3 families; 6 cases), T300G (2 probands), 3819delGTAAA (1 proband). Among intron changes we suspect the IVS6 +28 (C→A) change to be pathogenic, since it is present in intron area participating in exons splicing. It was found in three sisters (two had breast cancer, one was healthy). The remaining 8 changes were disclosed in exons 11, 13 and 16 (A1186G, C2201T, T2430C, C2731T, A3232G, A3668G, T4427C, A4956G) and regarded as polymorphisms. One of them, A1186G, was, as a heterozygotic change, more frequent in breast cancer cases (18.8 %) than in healthy women (4%). We observed frequent presence of several types of polymorhisms (3-6) in BRCA1 gene exons. Two heterozygotic changes (T300G and A1186G) in the blood DNA of two unrelated patients lost their heterozygosity in tumour DNA.

Zmiany w genie *BRCA1* u chorych na rodzinnego raka sutka, leczonych w Centrum Onkologii w Warszawie

Spośród przeszło 500 kobiet chorych na raka sutka, leczonych w Centrum Onkologii w Warszawie, wyselekcjonowano 52 probantki, które były obarczone rodzinnym występowaniem kilku raków sutka i innych nowotworów. Materiałem badawczym było DNA, izolowane z leukocytów krwi obwodowej. Procedurą PCR-SSCP posługiwaliśmy się w celu poszukiwania mutacji we wszystkich kodujących białko eksonach genu BRCA1. Fragmenty podejrzane o obecność mutacji poddawano sekwencjonowaniu. U 36 probantek w genie BRCA1 wykryto 11 rodzajów zmian w eksonach i 5 rodzajów zmian w intronach. U 19 (36,5%) probantek nie wykryto w genie BRCA1 żadnych zmian. Wśród zmian eksonowych trzy z nich powszechnie uznawane są za mutacje patogenne: 5382insC (3 rodziny; 6 przypadków), T300G (2 probantki), 3819del5 (1 probantka). Spośród zmian intronowych zmiana IVS6 +28 (C→A) podejrzewana jest przez nas o patogenne właściwości, gdyż umiejscowiona jest w obszarze intronu, który bierze udział w składaniu eksonów. Występowała ona u trzech sióstr (dwie chore i jedna zdrowa). Pozostałych 8 zmian, wykrytych w eksonach 11, 13 i 16 (A1186G, C2201T, T2430C, C2731T, A3232G, A3668G, T4427C, A4956G), to polimorfizmy. Jedna z nich - A1186G występowała jako zmiana heterozygotyczna częściej u chorych na raka sutka (18,8%), niż u kobiet zdrowych (4%). Wśród badanych chorych zaobserwowaliśmy w eksonach genu BRCA1 częste nagromadzanie się u tej samej chorej kilku (3-6) polimorfizmów. Dwie heterozygotyczne zmiany (T300G i A1186G), znalezione w DNA z krwi dwóch nie spokrewnionych chorych, utraciło swą heterozygotyczność w DNA izolowanym z guza.

Key words: familial breast cancer, *BRCA1*, gene
Słowa kluczowe: rodzinny rak piersi, *BRCA1*, geny

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Introduction

Since the time of *BRCA1* and *BRCA2* gene cloning, lesions of which can predispose to breast cancer development in humans [1-4], new studies of these genes have sprung up throughout the world. Until now over 400 various changes were found in the sequences of each gene, such as missense mutations, nonsense mutation, deletions, insertions etc. [5-7]. Some of them are evidently pathogenic, while others are just polymorphic changes of low importance for clinical medicine. The initial analyses of the role of *BRCA1* gene lesions in the development of familial breast cancer had suggested that this role varies in a broad range from 9% to 79% [5]. Later these values were recognised as overestimated, since in certain population groups ethnic and social-cultural factors have lead to an accumulation of genetic traits determining predispositions to hereditary breast cancer. Recently it has been estimated that the role of the *BRCA1* gene in the aetiology and pathogenesis of familial breast cancer can be estimated at between 11% and 17% [6].

The first Polish study on the role of the *BRCA1* and *BRCA2* genes in hereditary breast cancer was carried out in the Poznań region [8]. In the following years similar studies were held in the Warsaw region [9, 10], in West Pomerania [11] and in Silesia [12]. These studies concentrate on gathering data concerning the frequency and quality of changes in the *BRCA1* and *BRCA2* genes in patients with familial breast cancer.

The subject of this paper is to present the results of our studies on *BRCA1* gene changes in 52 women with familial breast cancer, inhabitants of the Warsaw region. We are also conducting a *BRCA2* gene study in this same group of patients and intend to publish it separately. Finding the most frequent pathogenic mutations of *BRCA1* and *BRCA2* genes for the Polish population will allow to define basic founder mutations predisposing to breast cancer development and will contribute towards more efficient genetic counselling of patients.

Material and methods

Patients

From over 500 women treated during a period of 3 years at the Department of Breast Cancer and Reconstructive Surgery of The Maria Sklodowska-Curie Memorial Cancer Centre in Warsaw 52 patients (age: 24-72 years; median 48 years) were selected due to a history of breast cancer and other tumours among family members. Over 65% of these women were inhabitants of Warsaw.

In the families of the selected patient-probands the number of breast cancer cases veried between 1 and 6, within the first or second degree of kinships. In two families the number of breast cancer cases was 5 and 6; in 6 families – 4 cases in each family, in 14 families – 3 cases, in 19 families – 2 cases (Table I). Among the studied patients two were mother-daughter relatives (Table I, nos. 5 and 6), six were sister pairs (Table I patients no 1 and 1A, no 23 and 24, no 40 and 44, no 52 and 56, no 63 and 65, no 78 and 80). Seven patients had no family history of breast cancer. They were included into the study for the following reasons: very young age (<40 years), family history of ovarian or prostatic cancer, presence of bilateral breast cancer, or several neoplasms, in the same patient (e. g. patients no 22 and 26 had breast, ovarian and endometrial cancers). In the families of the selected patients the number of cancer cases other than breast cancer varied between 1 and 7. These included cancer of the ovaries, the prostate, the endometrium, the kidney, the pancreas, the gallbladder, the lungs, brain tumours, myeloma and lymphoma.

Methods

We studied DNA isolated from peripheral blood obtained from 52 patients with breast cancer and from 25 healthy persons who had no near relatives with malignant neoplasms. In several cases tumour tissue was used also for examination. Blood and tumour tissue was kept at -70°C until DNA isolation with the phenol--chloroform method using proteinase K. The isolation procedure was performed according to Sambrook et al. [13].

The PCR-SSCP procedure was used for seeking mutations in all protein coding exons of the *BRCA1* gene. Due to its excessive size exon 11 was amplified in 23 fragments. The primers for the PCR reaction were as proposed by Friedman et al. [14].

Polymerase chain reaction (PCR) of genomic DNA was carried out in 25 μl volume containing 200 ng (5 μl) of genomic DNA, 2.5 μl of PCR buffer (20mM tris/HCl pH 8.4, 50 mM KCl (Perkin Elmer – PE), 200 μM (1 μl) of each deoxynucleotide (dATP, dTTP, dCTP, dGTP), 0.5 μM (0.6 μl) of forward and reverse primers, 2.5 mM (2.5 μl) MgCl2, and 1.5 U (0.3 μl). Ampli Taq Gold polymerase (PE), and ddH_20 up to 25 μl of total volume. Amplification was performed in a Perkin Elmer 9600 DNA Thermal cycler.

PCR amplification conditions were as follows: denaturing at 95°C for 10 min, (30 cycles of denaturing at 94°C for 20 s., annealing for different primer pairs at between 50°C and 62°C for 20 s. and extension at 72°C for 20 s.), extension at 72°C for 7 min.

SSCP analysis

Amplified samples diluted with formamide (1:1) were denatured at 95°C for 6 min. Next they were cooled rapidly to 4°C and held for at least 2-3 min. For each sample 5 μl was loaded onto the non-denaturing 12.5% polyacrylamide gel and run at 600 V voltage, 10-15°C temperature, 2 h 30 min. time. The electrophoregrams were stained with Plus One DNA Silver Staining Kit (Pharmacia). The results of electrophoretic separation of DNA fragments from the patients were compared with analogous samples from healthy persons. Fragments suspected of mutation presence were subjected to sequencing. However, some DNA samples, especially those from patients with high number of breast cancer cases in their families, were sequenced directly without prior SSCP analysis. Total sequencing material was 70%.

Sequencing

The amplified DNA samples were purified with Centricon-100 columns (PE) and were then subjected to sequencing PCR. Then 10 μl of volume containing 30 – 90 ng purified DNA, forward or reverse primer 1.6 pmol, 4 μl Big Dye Terminator Ready Reaction Mix (PE) were used for sequencing reaction. Cycling conditions of sequencing reaction were as follows: 95°C- 2min., (96°C-10 sec, temperature of primer annealing 50°C – 60°C – 5 sec., 60°C – 4 min. -25 cycles). Sequencing primers were the same as those used to amplify the template. The product of the sequencing reaction was purified in spin columns (Centri Sep PE). Sequence was run on 5% denaturing polyacrylamide gel (Long Range gel solution) at 2,4 kV voltage for 7 hours in ABI Prism 377 Perkin Elmer apparatus.

Table I. Germline mutations and polymorphisms in *BRCA1* gene found in 52 women with familial breast cancer

Number of patient	Tumours in family breast	other	No of exon – E; change and no of nucleotide, aminoacid change and no of codon; type of change									
			E – 5 T300G Cys61Gly MS#	E – 11 A1186G Gln356Arg P#	E – 11 C2201T Ser694Ser P#	E – 11 T2430C Leu771Leu P#	E – 11 C2731T Pro871Leu P#	E – 11 A3232G Glu1038Gly P#	E – 11 A3668G Lys1183Arg P#	E – 11 3819 del 5# stop in codon 1242; F#	E – 13 T4427C Ser1436Ser P#	E – 16 A4956G Ser1613Gly P#	E – 20 5382 insC; stop in codon 1829; F#
1	3	0											
1A	3	0											
2	4	0											
3	4	0	* ⊕	*									
4	1	0											
5	2	2											⊕
6	2	2											⊕
7	1	6				*	*						
8	2	2											
9	2	3				*							
10	1	2											
11	2	0			*		*				*	*	
12	3	0				*	*						
13	2	0		*									
15	3	1											
16	1	1											
17	3	0			**	*	*						
18	5	1	* ⊕	*			*						
19	3	0		*									
20	4	1				*	*						
21	2	2				*	*						
22	1	2											
23	2	1											
24	2	1											
26	1	2		*									
27	3	0			**	*	*						
28	2	7											

⊕- patogenic mutation, P#- polymorphism; MS#- missense, del# – deletion; F#-frameshift; *- heterozygosity; ** – homozygosity; open space – not found changes

Table I cont. Germline mutations and polymorphisms in BRCA1 gene found in 52 women with familial breast cancer

Number of patient	Tumours in family breast	other	No of exon – E; change and no of nucleotide, aminoacid change and no of codon; type of change										
			E – 5 T300G Cys61Gly MS#	E – 11 A1186G Gln356Arg P#	E – 11 C2201T Ser694Ser P#	E – 11 T2430C Leu771Leu P#	E – 11 C2731T Pro871Leu P#	E – 11 A3232G Glu1038Gly P#	E – 11 A3668G Lys1183Arg P#	E – 11 3819 del 5# stop in codon 1242; F#	E – 13 T4427C Ser1436Ser P#	E – 16 A4956G Ser1613Gly P#	E – 20 5382 insC; stop in codon 1829; F#
29	2	3			**		**						
30	6	1		*		*	*						
31	2	2											
33	2	3				**	**				**		
34	2	1											
35	3	4				*	*				*		
36	3	1		*			*				*		
37	2	3											
38	4	0				*							
39	3	1					*				*		
40	4	2				*	*				*		
41	2	1											
42	4	2								⊕			
43	1	3					*				*		
44	4	2				**	**				**		
45	3	2											
48	2	2											
50	2	0											
51	2	0			**	**	**	**	**		**		
52	3	0		*		*					*	*	
56	3	0		*		*					*	*	
63	3	2		nd	nd	nd	nd	nd	nd	nd	nd	nd	⊕
65	3	2		nd	nd	nd	nd	nd	nd	nd	nd	nd	⊕
78	2	5		nd	nd	nd	nd	nd	nd	nd	nd	nd	⊕
80	2	5		nd	nd	nd	nd	nd	nd	nd	nd	nd	⊕
Frequency of changes			2/52 3.8 %	9/48 18.8 %	5/48 10.4 %	16/48 33.3 %	18/48 37.5 %	1/48 2.1 %	1/48 2.1 %	1/52 1.9 %	11/48 22.9 %	3/48 6.3 %	6/52 11. 5 %

⊕- patogenic mutation, P#- polymorphism; MS#- missense, del# – deletion; F#-frameshift; *- heterozygosity; ** – homozygosity; nd - not done, open space – not found changes

Results

In the group of 52 patients tested by the PCR-SSCP procedure or direct sequencing in 33 patients 11 types of changes were found in *BRCA1* gene exons. These changes present with a frequency from 1.9% to 37.5% of cases were found in exons 5 (T300G), 11 (A1186G, C2201T, T2430C, C2731T, A3232G, A3668G, 3819del5), 13 (T4427C), 16 (A4956G) and 20 (5382insC). In the remaining 19 (36.5%) patients no changes were detected. These results are presented in Table I and Figure 1. In two unrelated patients (nos. 3 and 18) the changes in positions T300G and A1186G were found in blood DNA as heterozygotic, but in their tumour DNA these changes lost their heterozygosity.

The starters used for sequencing on both sides of the exons overlapped intron fragments. This allowed for the detection of changes not only in exons, but also in intron fragments adjacent to initial or final parts of exons. We found 5 different intron changes which had heterozygotic or homozygotic character. These changes are presented on the Figure 1.

The most frequent intron changes were: IVS17 –67 (A→G), IVS18 +66 (G→A), and IVS8 –34 (C→T) which was found in 12, 10 and 8 patients respectively. The most infrequent were changes in IVS2 +80 (A→G) and IVS6 +28 (C→A) detected in 2 and 3 patients respectively.

The IVS6 +28 change (C→A) is located in the area which participates in exons splicing. It was detected in three sisters, two of whom had breast cancer (Table I no 23 and 24) and the third (not shown in Table I) was healthy. In all three sisters this change was present in both alleles in blood DNA. Although in each sister the whole protein coding part of *BRCA1* gene was sequenced, no changes other than IVS6 +28 were found in the gene.

Discussion

Among the DNA obtained from 52 studied women only 3 theoretically pathogenic mutations types were found. One of them was detected in nucleotide position T300G: amino acid effect – Cys61Gly, predictive protein effect – loss of zinc motive. We have disclosed this mutation only in 2 out of 52 patients, what is more – unrelated. An analogous defect was detected by Friedman et al. [14] and Górski et al. [11]. It has also been recorded in the Breast Cancer Information Core (BIC) [7] 44 times as missense mutation.

The second mutation found in our material was a deletion of 5 nucleotides in exon 11 in position 3819delGTAAA, the consequence of which is the stop signal in codon 1242. It was found only in one out of 52 patients. The same mutation was found twice in our country, by Górski et al. [11]. In the BIC it has been recorded 10 times.

The third mutation was found in exon 20. It was 5382insC in codon 1755 (its consequence being a stop in codon 1829). We found it in 3 families (table I: mother-daughter Nos. 5 and 6, two sister pairs nos. 63 and 65,

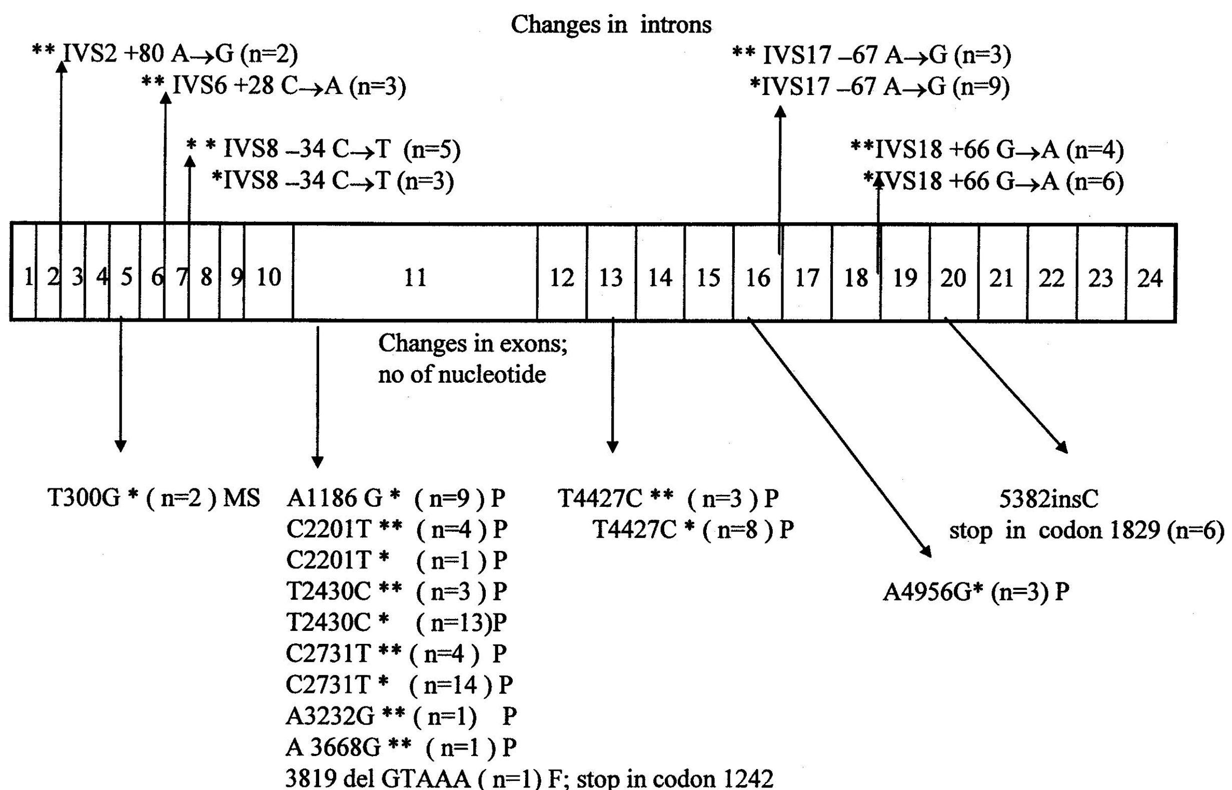

Figure 1. Germline changes found in *BRCA1* gene in patients with familial breast cancer in Warsaw District.
P – polymorphism; MS – missense; F – frameshift; del – deletion; ins – insertion; * – heterozygosity, ** – homozygosity, n – number of cases.
In the scheme of *BRCA1* gene the relation between dimension of exons is disregarded.

and nos. 78 and 80). In Poland this mutation was found earlier in Silesia, in the Poznań region [12] and in West Pomerania [11]. In the BIC it has been recorded 190 times [15].

Among 8 further changes detected in exons of the *BRCA1* gene, six were present in exon 11, one in exon 13 and one in exon 16. Three of them had no consequences in the changes in amino acid pattern of proteins (exon 11 – C2201T – Ser694Ser, T2430C – Leu771Leu, and exon 13 T4427C – Ser1436Ser). They were found also by Friedman et al. [14] and they are recorded in the BIC.

One of the heterozygotic changes is of interest – the A1186G with consequence – Gln356Arg, which was more frequent in patients with breast cancer (9/48 – 18.8%), than in healthy women (1/25 cases – 4%). The change in position A1186G has been recorded in the BIC only once and defined as missense change without any biological consequence known as yet.

In 2 unrelated patients (Table I no 3 and 18) 2 heterozygotic changes – T300G and A1186G found in blood DNA and in tumour DNA occurred lost of their heterozygosity. The fact of above described coexistance of these changes in two unrelated patients with breast cancer suggest an interrelationship between these changes.

One of the most frequent changes (37.5%) in our material was a change in the nucleotide C2731T (Pro871Leu), which was also found in 46% of healthy women. We could not find this change in the BIC records.

We found the change A3232G (Glu1038Gly) in only one case; in the BIC it has been recorded 4 times. The biological effect of this change remains unknown. The next exon change – A4956G (Ser1613Gly) was found 3 times in our material, while in the BIC it is recorded twice as a polymorphism. The last exon change – A3668G (Lys1183Arg) found only once in our material has been recorded 26 times in the BIC as a polymorphism.

We have detected 5 types of intron changes in the form of homozygotes or heterozygotes with a predominance of the former ones (Figure 1). Presently we cannot express any opinion on the participation of respective intron changes in the pathogenesis of breast cancer. However, it should be stressed that intron change (C→A) in position IVS6 +28 is located in the area participating in exons splicing. This was the only change in the *BRCA1* gene found in blood DNA of the previously mentioned three sisters, and therefore its possible biological consequence should be studied.

Reviewing the studied material one can see that pathogenic mutations occurred in patients with a family history of 2 to 5 cases of breast cancer and 1 to 5 other neoplasms. However, 19 patients with no pathogenic changes detected had families with 1 to 5 breast cancer cases in addition to 1 to 7 other tumours. It would be interesting to know what are the reasons for not detecting pathogenic mutations in such a considerable number of our patients (36.5%). One of the reasons may be overlooking pathological mutations due to methodological grounds. PCR- SSCP technique does not detect all changes. As it has been mentioned above, only 70% of our material was sequenced.

Polymorphisms A1186G and C2731T were more frequent in patients with higher numbers of breast cancer cases in the family. Are the supposedly polymorphisms really so neutral with respect to breast cancer pathogenesis or whether their frequent cumulation in one person is an evidence of an "unrest" occurring in genome DNA? Among our patients we have observed cases with 3 to 6 various polymorphisms. Friedman et al. [3] have reported that the presence of disequilibrium between polymorphisms has led them to finding 10 families with pathogenic mutations.

Assuming that there may occur a typical 185delAG Jewish mutation in the Polish population, we have examined all our patients DNA by means of sequencing second exon. The mutation, however, has not been found. 185 del AG mutation is a change commonly found throughout the world, and it has also been detected in several centers in Poland [12].

5382insC change, commonly known throughout the world as founder mutation, is observed among Ashkenazi Jews [16] and in Eastern Europe [17]. It is the most frequently observed change in our material (6/9 pathogenic mutations – 66.7% – Table I).

As in our study we have found mutations generally regarded as pathogenic in 9 cases we have calculated that in the Warsaw region the *BRCA1* gene changes connected with breast cancer occur in 17.3% cases. This value lies within the range estimated for the world population i. e. 11-17% [6].

Of the three types of pathogenic mutations found in our material the most frequent was 5382insC (3 families), less frequent was the T300G mutation (2 families) and the least frequent was 3819del5 mutation (in one family). Intron change IVS6 +28 (C→A) was present in one family. All these changes, except IVS6 +28 (C→A) intron mutation, were found also in the West Pomeranian and Silesian regions [12]. As for the changes described by Sobczak et al. [8] in the Poznań region, i.e. exon 11 – codon/nucleotide 1345/4153, exon 22 – 1782/5465, exon 7 – 105/433 and double insertion of GTATTCCACTCC fragment in intron 20 we did not come across them in the Warsaw region subpopulation.

So far research on the *BRCA2* gene mutation conducted on the same human material has not revealed any serious mutations in the *BRCA2* gene, apart from some nonpathogenic polymorphisms. Considering the occurrence of tumours linked to mismatch repair genes (*hMLH1* and *hMSH2*) or *TP53* gene in our patients' families, research is also being carried out in this direction.

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