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HUMAN GENETICS

Search for Frequent Mutations in Genes Predisposing to Breast Cancer

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Abstract—DNA of oncological patients, including Ashkenazi Jews and Slavs, living in St. Petersburg was collected, and the resultant collection was screened for three common mutations of genes *BRCA1* and *BRCA2* by means of heteroduplex analysis. The mutation 5382insC in exon 20 of the *BRCA1* gene was found in four unrelated patients, including three Slavs and one Ashkenazi Jew, with a positive family history of breast cancer. The mutations 185delAG and 6174delT in the *BRCA1* and *BRCA2* genes, respectively, which are typical of Ashkenazi Jewish patients with breast cancer, were not found in the patients of either ethnicity living in St. Petersburg, although the 6174delT mutation was found in the control group of Ashkenazi Jews. A new 12-nucleotide duplication g.71741ins12nt found in intron 20 of the *BRCA1* gene was described. The high frequency of the 5382insC mutation in the *BRCA1* gene in patients with familial breast cancer in both St. Petersburg and Moscow indicates that Russian families with the history of breast cancer should be primarily tested for this mutation.

INTRODUCTION

About 10% of females in developed countries have breast cancer. Studying of families with a positive history of breast cancer (with several cases in the family) made it possible to identify specific genes determining susceptibility to this disease. These genes were called *BRCA1* [1] and BRCA2 [2] (*BRCA* is an acronym for breast cancer). The estimates of risk of breast cancer in carriers of these mutations range from 90 [3] to 36% [4], respectively, which is three to ten times higher than the average population risk [4]. Mutations in these suppressor genes in the germ-line cells account for familial forms of breast cancer with autosomal dominant inheritance, with the aforementioned two genes accounting for as much as 80% of all cases of autosomal dominant breast cancer [3]. Mutations in either of the genes increase the risk of breast cancer, and mutations in *BRCA1* also increase the risk of ovarian cancer. Although only 5–10% of the cases of breast and ovarian cancers in the total population may be explained by the inherited mutations in the susceptibility genes [5], attempts have been made to identify the mutations in genes of the *BRCA* family due to a high penetrance of the disease. The researchers have always focused on families with multiple cases of cancer in the history. However, it has been demonstrated that a considerable proportion of breast cancer cases in women with moderate or no family history of breast cancer is related to mutations in the genes *BRCA1* and *BRCA2* [6–8].

More than 300 and more than 100 mutations of the *BRCA1* and *BRCA2* genes, respectively, have been described to date. Several *BRCA1* mutations, including a frequent mutation 5382insC, have been described in families from Russia [9]; *BRCA2* mutations have not been studied in Russia. Each population or ethnic group has its own specific spectrum of mutations in the *BRCA* genes. Ashkenazi Jews stand out in this respect: 1% of them carry the mutation 185delAG in the gene *BRCA1* and about 0.9%, the mutation 6174delT in the *BRCA2* gene [10, 11]. The mutation 5382insC is also characteristic of this ethnic group, although it is considerably less frequent (about 0.13%) [12].

The mutation 5382insC was found in some Moscow families with a positive history of ovarian cancer; therefore, we searched for this and the two other mutations in Ashkenazi Jew patients with breast cancer in St. Petersburg. We were also motivated to conduct this study by our finding that Jewish patients with familial hypercholesterolemia (a disease unrelated to cancer) living in St. Petersburg exhibit a high frequency of a mutation characteristic of Ashkenazi Jews, namely, 652delGGT (deltaG197) [13]. We used heteroduplex analysis to test the patients' DNA for the presence of two short deletions causing breast cancer [14–16]. For the first time in Russia, we found a mutation in the *BRCA2* gene and a new variant of the *BRCA1* gene con-

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taining a 12-nucleotide duplication. We also found that the mutations 185delAG and 6174delT are either absent or extremely rare in Slavic patients with familial breast cancer (at least when it is not accompanied by ovarian cancer) in St. Petersburg. In addition, we demonstrated that the mutation 5382insC in the gene *BRCA1* is frequent in Russian patients not only in Moscow, but also in St. Petersburg. Thus, the mutation 5382insC is found in Russia not only in families with multiple cases of ovarian cancer, but also in those with multiple cases of breast cancer not accompanied by ovarian cancer.

MATERIALS AND METHODS

Patients. Patients with breast cancer treated at the Petrov Research Institute of Oncology were proposed to be tested for the presence of three mutations in *BRCA* genes. After they gave their informed written consent, the family history was revised. We considered a patient to have a familial predisposition to breast cancer if there were at least three cases of breast cancer in the family, with the degrees of relatedness of the affected persons within the family being at least 25%. Otherwise, the case was considered sporadic, i.e., without a familial predisposition. The patients were interviewed to determine their ethnicity; a patient was assigned to Ashkenazi Jew if at least one of the patient's parents was a Jew. Finally, the patients were divided into the following groups: (1) Ashkenazi Jews with familial breast cancer; (2) Slavs with familial breast cancer; and (3) Ashkenazi Jews with sporadic breast cancer. The control group consisted of Ashkenazi Jews with familial hypercholesterolemia, which is not associated with breast cancer.

DNA isolation and analysis. To obtain the patient's DNA, we sampled about 3 ml of venous blood into a microcentrifuge test tube containing an anticoagulant (sodium citrate or EDTA). DNA was isolated from fresh or frozen blood by the method of Kunkel [17] modified by Bell [18] for small samples of blood. We usually used 1 ml of blood and stored the remaining 2 ml at –20°C. The concentration of the isolated DNA was estimated by electrophoresis in 0.8% agarose gel in the presence of DNA at the known concentration [19]; 20 ng of genomic DNA of each patient were used for polymerase chain reaction (PCR). The *Escherichia coli* strain DH5α was transformed by plasmids containing cloned nucleotide sequences of *BRCA* genes with the use of the calcium–chloride technique; we used the standard alkaline lysis method [19] to isolate the plasmids to be used in the PCR analysis. We obtained large numbers of copies of the required regions of the *BRCA1* and *BRCA2* genes by means of PCR using the following pairs of primers: 5'–GAA GTT GTC ATT TTA TAA ACC TTT–3' and 5'–TGT CTT TTC TTC CCT AGT ATG T–3' (for the amplification of exon 2 of the *BRCA1* gene and search for the 185delAG mutation); 5'–ATA TGA CGT GTC TGC TCC AC–3' and 5'–GGG AAT CCA AAT TAC ACA GC–3' (for the amplification of exon 20 of the BRCA1 gene and search for the 5382insC mutation) [20]; 5'–GTC TGG ATT GGA GAA AGT TT–3' and 5'–CTC TTG TGA GCT GGT CTG AA–3' (for amplification of exon 11 of the BRCA2 gene and search for the 6174delT mutation). The sizes of the amplified fragments were 258, 401, and 275 bp, respectively. Reaction mixtures for PCR were 30 μl in volume and contained 0.25 μM of each primer, 50 mM KCl, 10 mM Tris–HCl (pH 8.4 at room temperature), 1.5 mM $MgCl_2$, 200 μM of each deoxyribonucleotide triphosphate, and 1 U of *Taq* polymerase. When conducting PCR, we used the same temperature profile for all of the three pairs of primers after the initial denaturing of genomic DNA for 5 min at 94°C on a thermocycler: 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C. After 30 cycles were completed, the PCR samples were incubated for 9 min at 72°C. We used a Cyclotemp-2 thermocycler (CTM, Moscow, Russia). To obtain heteroduplexes, we mixed the PCR-amplified DNA fragments with control samples, denatured them on the thermocycler for 5 min, and gradually cooled them to room temperature using the thermocycler. The cooling had a step of 5°C, with the samples being kept at each fixed temperature for 1 min. The PCR products and heteroduplexes were identified by means of electrophoresis in 8% polyacrylamide gel (the acrylamide-to-bisacrylamide ratio was 29:1) buffered with 1× Tris–borate (TBE) [19]. The direct cloning of the products in the vector pCR™ 2.1 (Promega) was performed as recommended by the manufacturer. For the direct cyclic sequencing of the PCR products and the fragments cloned in the plasmids, a thermosequenase kit (Medigen, Russia) was used.

Control DNA samples. The plasmids carrying the nucleotide sequences of the wild-type genes *BRCA1* (MDL11) and *BRCA2* (MDL21) and genes with the mutations 185delAG (MDL15) and 6174delT (MDL22) [16] were kindly provided by Dr. Mahesh Mansukhani (United States). To obtain the fragments of the *BRCA1* and *BRCA2* of these plasmids, we performed amplification as described in the previous section. DNA samples from patients with one of the mutations 185delAG, 5382insC (*BRCA1*), or 6174delT (*BRCA2*) in a heterozygous state were kindly provided by Raphael Catane, Israel).

RESULTS

We used blood samples of patients with breast cancer to create a DNA bank. The bank contained material from three groups of patients: 10 Ashkenazi Jews with a family history of breast cancer (multiple cases), 38 Slavic patients with familial breast cancer, and 16 Ashkenazi Jew patients with sporadic (nonfamilial) breast cancer. Thirty-eight Ashkenazi Jew donors without a family history of breast cancer served as a control group.

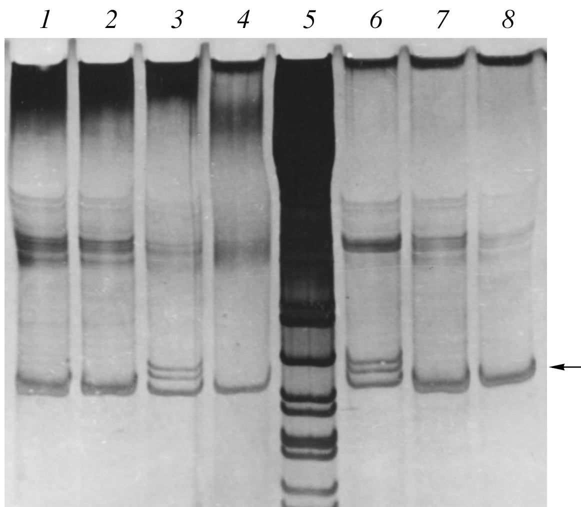

Fig. 1. Identification of the 6174delT mutation of the *BRCA2* gene by means of heteroduplex analysis: *1, 2*, and *4*, the amplification products of genomic DNA from patients without the mutation; *3,* the amplification product of genomic DNA of a patient heterozygous for the 6174delT mutation; *6*, the control for heteroduplex formation (denatured and annealed amplification products of the plasmids with fragments of the *BRCA2* gene carrying (*7*) the 6174delT mutation and (*8*) the wild-type *BRCA2* gene were used). The coincidence of the positions of heteroduplexes in the gel on lane *3* and *6* (shown by the arrow) indicates that the tested genomic DNA (lane *3*) contained the 6174delT mutation. *5*, the phage λ DNA completely hydrolyzed by *Pst*I endonuclease that served as a marker of molecular weight.

We used PCR to amplify exons 2 and 20 of the *BRCA1* gene and exon 11 of the *BRCA2* gene together with the adjacent regions of boundary introns and searched for the 85delAG and 5382insC mutations of the *BRCA1* gene and the 6174delT mutation of the *BRCA2* gene with the use of heteroduplex analysis. As a result, we found two of these three mutations in our DNA collection (Figs. 1–3).

Amplification and analysis of exon 20 of the *BRCA1* gene together with the adjacent regions of introns 19 and 20 and the subsequent heteroduplex analysis of the amplification products allowed us to find a new insertion (Fig. 4). When we sequenced the unusual cloned allele, we found that the 12-nucleotide insertion had resulted from duplication of 12 nucleotides of intron 20. According to the accepted nomenclature, we denoted the new insertion as g.71741ins12nt.

DISCUSSION

We searched for the 185delAG and 5382insC mutations of the *BRCA1* gene and the 6174delT mutation of the BRCA2 gene with the use of a simple and inexpensive method of heteroduplex analysis according to the original method [16]. The PCR-amplified exons 2 and 20 of the BRCA1 gene and exon 11 of the *BRCA2* gene together with the adjacent regions of the boundary introns obtained from DNA of the patients with breast cancer were annealed with amplified control DNAs. The amplified DNA of patients that do not carry these mutations does not form heteroduplexes when annealed with amplified fragments of normal DNA; however, it forms heteroduplexes when annealed with amplified cloned DNA carrying the mutation. Conversely, amplified DNA of the patients heterozygous for the mutation forms heteroduplexes in both cases, i.e., whether it is annealed with amplified DNA from plasmids containing the mutant gene or with that from plasmids containing the wild-type gene. The positions of the heteroduplexes in polyacrylamide gel were characteristic of each specific mutation. Given an adequate control, i.e., annealed amplified DNAs of plasmids containing a fragment of the wild-type or mutant gene, we might judge on the presence or absence of the mutations in question in the genomic DNA tested. When amplifying one DNA sample from a control Ashkenazi Jew patient, we noticed formation of heteroduplexes in the amplification product itself, even before annealing with tester plasmid DNA products. Experiments on formation of heteroduplexes confirmed that this sample was heterozygous for the 6174delT mutation of the BRCA2 gene. Thus, the annealing of amplified DNAs with tester plasmids that was recommended by Mansukhani

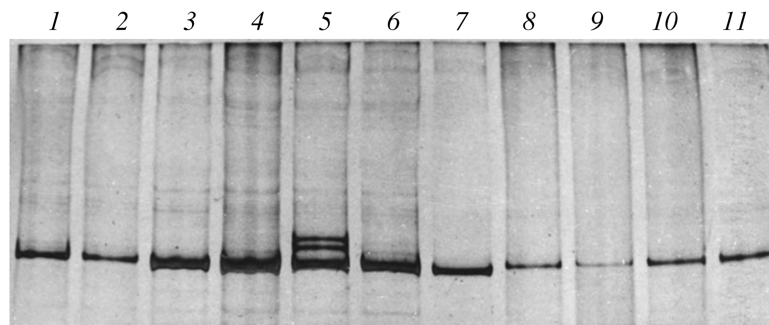

Fig. 2. Analysis of amplification products of exon 11 of the *BRCA2* gene in patients with familial breast cancer for the presence of the 6174delT mutation. *5*, a DNA sample from a patient heterozygous for the 6174delT mutation detected by additional heteroduplex bands: *1–4*, *6–11*, DNA samples from patients lacking mutation 6174delT.

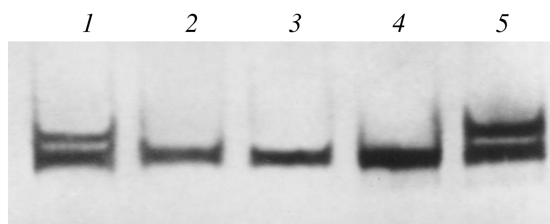

Fig. 3. Identification of the 5382insC mutation of the *BRCA1* gene by means of heteroduplex analysis: *1* and *5*, amplified DNA from patients carrying the 5382insC mutation in the *BRCA1* gene detected by an additional heteroduplex band; *2–4*, DNA from patients lacking mutation 5382insC.

et al. [16] is not a necessary step for detecting this mutation, which makes it possible to perform a rapid one-step screening test for the deletion. When we searched for the 5382insC mutation, an additional annealing of DNA also proved unnecessary. However, in the case of the 185delAG mutation in the *BRCA1* gene, heteroduplexes were only formed after gradual cooling of the denatured DNA samples and were formed ineffectively during the PCR temperature cycles themselves.

Although the number of the patients studied was small, the absence of two mutations tested, namely, 185delAG in the *BRCA1* gene and 6174delT in the BRCA2 gene in all groups of patients with breast cancer is noteworthy. This suggests that their contribution to familial forms of breast cancer in the St. Petersburg Slavic population is insignificant. The 6174delT mutation of the *BRCA2* gene was found in the control Ashkenazi Jew patients, rather than in the patients with breast cancer, which confirms that we used an appropriate approach to detecting this mutation. We found three cases of the 5382insC mutation in the *BRCA1* gene in families of Slavic patients with familial breast cancer. We also found this mutation in one out of ten studied Ashkenazi Jew families with familial breast cancer. We did not find the 5382insC mutation in either Ashkenazi Jews with sporadic breast cancer or in the group of 100 Slavs and 38 Ashkenazi Jews without breast cancer. Thus, the 5382insC mutation is found not only in patients with familial ovarian cancer [9, 15], but also (and frequently) in patients with familial breast cancer in samples of families not selected for ovarian cancer. The high frequency of the same mutation (5382insC) in the *BRCA1* gene in Russian families with hereditary cancer from Moscow [9] and St. Petersburg indicates that this mutation is common for Russian patients and should be tested for in families with positive history of breast and ovarian cancers. In Eastern Europe and the Baltic region, the 5382insC mutation of the *BRCA1* gene is also frequent. Further studies are required to determine whether there are other common mutations of the *BRCA1* and *BRCA2* genes in the Russian popula-

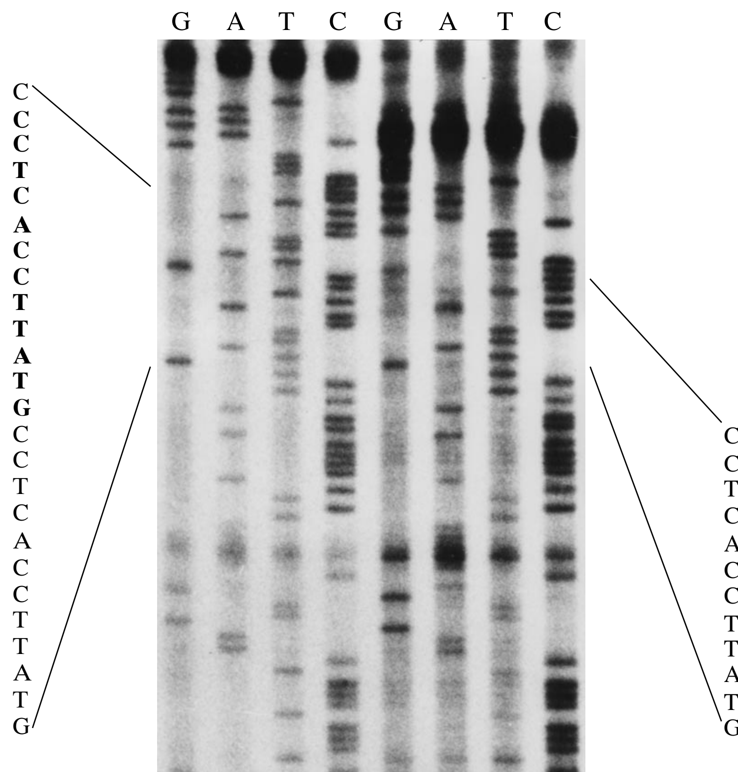

Fig. 4. Identification of the g.71741ins12nt insertion by means of direct sequencing of the amplified DNA. The first four lanes on the left correspond to the DNA sample containing the insertion; on the right, the DNA sample without the insertion.

tion. Apparently, new variants of the *BRCA* nucleotide sequences will be found in Russia, some of them being unique for this population. One example is the perfect 12-nucleotide duplication in intron 20 of the *BRCA1* gene (g.71741ins12nt) described by us; this duplication has not been found elsewhere. This duplication is apparently a silent mutation; however, we expect that mutations of the *BRCA1* and *BRCA2* genes that cause breast cancer and are specific for the Russian population will also be found.

When dealing with the gene of the receptor for human low density lipoproteins, which is a very variable gene, we found a Russian-specific mutation (C139G) in two unrelated families with familial hypercholesterolemia (the total number of families was 100; therefore, only 2% of the probands had this mutation). Conversely, in the cases of phenylketonuria and cystic fibrosis, there are mutations that predominate in Russian patients. These are the mutations R408W and deltaF508, respectively, which account for at least 50% of mutant alleles [21]. Regarding hereditary breast cancer in Russia, it is obvious that the 5382insC mutation alone must account for a considerable percentage of cases (definitely more than 2% but less than 50%). Studying individual exons of the genes of the *BRCA* family by means of single strand conformation polymorphism analysis followed by sequencing of some exons would allow us to determine whether there are frequent mutations other than 5382insC (the *BRCA1* gene) in *BRCA* genes in the Russian population and to estimate the contribution of these mutations to the hereditary predisposition to breast cancer determined by the *BRCA1* and *BRCA2* genes.

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