Screening of a novel BRCA2 mutation by rapid *in-house* PCR-RFLP

Screening al unei noi mutații *BRCA2* prin tehnică rapidă PCR-RFLP elaborată *in-house*

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Abstract

BRCA1 and BRCA2 are major cancer predisposition genes, responsible for a large percentage of hereditary breast and ovarian cancer (HBOC) families. The distribution of BRCA mutations has been studied in many populations, though only recently so in Romania. We started the first local oncogenetic study, searching for mutations of BRCA genes in HBOC families, by using mutation-specific PCR-based methods and dideoxy sequencing. We investigated 104 samples from familial (29 HBOC) and non-familial cancer cases (50 breast, 25 ovary), from North-Eastern Romania. Among the diversity of BRCA deleterious mutations, a novel BRCA2 mutation in exon 21, c.8680C>T, was identified. The T for C substitution creates a stop codon at position 2894, truncating the last C-terminal 525 amino acids of BRCA2 protein. The mutation is not yet stored in international databases. The mutation was firstly identified in a breast cancer predisposition family. We imagined a simple and rapid screening for c.8680C>T identification. This substitution creates an additional TaaI restriction site, allowing a quick identification of the mutation by PCR-RFLP and agarose gel electrophoresis. Rapid screening allowed the identification of the same mutation in a sporadic ovarian cancer patient. Our results could open the way for a population study to determine the frequency of c.8680C>T, its possible recurrence, and an eventual founder effect in Romania.

Keywords: *Breast/ovarian cancer predisposition, HBOC families, BRCA genes, c.8680C>T mutation, PCR-RFLP.*

Rezumat

BRCA1 and BRCA2 sunt gene majore de predispoziție la cancer, responsabile de o proporție importantă a familiilor cu predispoziție ereditară la cancerul mamar și la cancerul ovarian (familii HBOC). Distribuția mutațiilor BRCA a fost studiată în numeroase populații, destul de recent și în România. Am început primul studiu local de oncogenetică, căutând mutații la nivelul genelor BRCA în familii HBOC, prin metode specifice bazate pe PCR și prin secvențierea ADN. Am investigat 104 probe ADN, provenind de la cazuri de cancer familial (29 cazuri HBOC) și nefamilial (50 cazuri de cancer mamar, 25 cazuri de cancer ovarian), în nord-estul României. Printre

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diversele mutaţii deletere BRCA, am identificat şi o nouă mutaţie la nivelul exonului 21 al genei BRCA2, c.8680C>T. Substituţia nucleotidului C cu un T generează un codon stop în poziţia 2894, ceea ce trunchiază proteina de ultimii săi 525 de aminoacizi C-terminali. Această mutaţie nu este încă repertoriată în bazele de date internaţionale şi a fost iniţial descoperită într-o familie cu predispoziţie la cancerul de sân. Am imaginat o metodă de screening simplă şi rapidă pentru identificarea c.8680C>T. Această substituţie creează un situs adiţional de restricţie pentru enzima TaaI, permiţând o identificare rapidă a mutaţiei prin PCR-RFLP şi electroforeză în gel de agaroză. Screeningul rapid a permis identificarea aceleiaşi mutaţii la o pacientă cu cancer ovarian sporadic. Rezultatele noastre ar putea deschide calea unui studiu populaţional în vederea determinării frecvenţei c.8680C>T, a recurenţei acestei mutaţii, şi al unui eventual efect fondator în populaţia României.

Cuvinte-cheie: predispoziţie pentru cancer de sân/ ovarian, familii HBOC, gene BRCA, mutaţia c.8680C>T

Introduction

Breast cancer is the most common malignancy among women in the Western world [1], with a lifetime risk of more than 10% [2], while ovarian cancer, with a lifetime risk of 1.8 %, is among the most lethal cancers and is the fourth cause of cancer mortality in women [3]. The Romanian population remains poorly studied, though both World Health Organization (WHO) [4] and Romanian League of Cancer [5] statistics suggest that incidence and mortality are increasing to Western European levels. Most breast and ovarian cancers are sporadic, but some are the result of inherited predisposition, principally due to mutations in the tumor suppressor genes *BRCA1* [6] and *BRCA2* [7]. Women with inherited mutations in *BRCA1* or *BRCA2* are at a significantly higher risk of developing breast and/or ovarian cancer than women in the general population, but the risk magnitude is controversial [8-13]. Lifetime risk in breast cancer is estimated at 65–80% for *BRCA1* and at 45–85% for *BRCA2* carriers, while the lifetime risk in ovarian cancer is about 37–62% for *BRCA1* and 11–23% for *BRCA2* [14]. Together, these genes account for ~30% of hereditary breast and ovarian cancer (HBOC) families in outbred populations, and up to 80% of HBOC families in isolated populations. In developing countries where the incidence of breast cancer is much lower, the proportion of cases attributable to hereditary factors may be significantly higher.

Appropriate medical follow-up, including early and more frequent mammography and pelvic examinations for the early detection of ovarian cancer, is therefore essential for *BRCA* mutations carriers. The emphasis is currently on early detection; preventive measures are mostly limited to prophylactic surgery, most notably annexectomy in post-reproductive women to reduce the risk of both ovarian and breast cancer. The identification of *BRCA1* and *BRCA2* mutation carriers and individualized risk assessment is an important procedure growing in clinical importance, since management protocols for mutation carriers become well established and proven life-saving, risk-reducing preventive medical interventions exist [15,16]. While the training of specialists in oncogenetics and the development of multidisciplinary biomedical networks to take charge of women at risk is becoming the standard of care in Western Europe, such services are only now becoming available in Romania.

Over one thousand small sequence variations have been reported in the Breast Cancer Information Core (BIC) database. More than half of these mutations cause the loss of function by premature protein synthesis termination [17], and around 60% are unique to a family. Not all variants can be considered pathological, notably missense alterations and intronic variants with unknown disease relevance. To date, 43.5% of *BRCA* variants are of uncertain clinical significance. The distribution of *BRCA* mutations has been studied in many populations, though only recently so in Romania. Different ethnic and geographical regions have different *BRCA1* and *BRCA2* mutation spectrum and prevalence. The knowledge of the genetic structure of particular populations is important for develop-

ing effective screening protocols and may provide more efficient approach for the individualization of genetic testing. Elucidating of founder effect in *BRCA1/2* genes can have an impact on the management of hereditary cancer families on a national and international healthcare system level, making genetic testing more affordable and cost effective.

Eastern Europe is mostly characterized by few recurrent mutations responsible for the majority of HBOC families, while vastly diverse unique mutations can be found in most outbred western populations [18]. We have recently performed the first description of genetic factors in Romanian population [19] within a lot of HBOC families and implemented oncogenetic follow-up in North-Eastern Romania [20]. We observed a variety of *BRCA* mutations, which may place the North-Eastern Romanian population somewhere between Western and Eastern populations. A recurrent "Eastern" *BRCA1* mutation was observed in two distinct predisposition families [21], while some other mutations seem to be novel, either local or unique to one family. When performing a systematic BRCA test in larger populations, prescreening PCR-based methods for the rapid and cheap identification of most common mutations can be particularly useful by limiting systematic BRCA gene sequencing, therefore reducing the time and costs of the analysis. Herein we describe a technique we developed *in-house* for screening of an apparently common *BRCA2* mutation.

Patients and methods

Patients

We investigated 104 samples from familial (29 HBOC) and non-familial (50 breast, 25 ovary) cancer cases, from North-Eastern Romania. The main recruitment criterion for HBOC families was three or more breast or ovarian cancer cases within the same family branch. The non-familial cases were considered especially if cancer was diagnosed before the age of 40, if breast and ovarian cases were observed in the same family, in male breast cancer, bilateral breast cancer, or medullary breast cancer. All patients agreed by written informed consent. Personal and familial cancer histories were obtained from patients and some participating relatives.

Molecular analysis

Genomic DNA was extracted from 10 ml peripheral blood using the Wizard™ Genomic DNA purification kit (Promega Inc, Madison, WI, USA). DNA concentration was estimated by spectrophotometry.

The entire coding sequence of both genes, including exon/intron boundaries, was analyzed using amplification and dideoxy sequencing. PCR was performed in 20 µl containing 0.4 mM each dNTP, 0.8 µM of each primer (forward and reverse), 100 ng genomic DNA, and one unit of either AmpliTaq® or AmpliTaq®Gold Polymerase with appropriate 1X Buffer (Applied Biosystems Inc, Foster City, CA, USA). PCR cycling comprised an initial denaturation step at 94ºC for 5 min followed by 30 cycles of 94ºC for 20 sec, 54ºC for 20 sec and 72ºC for 30 sec, and a final extension of 7 min at 72ºC.

Amplicons were verified by gel electrophoresis, then purified by ExoSap® enzymatic digestion (Affymetrix Inc, USA), following the producer's instructions. The product was sequenced in forward and reverse reactions, using the BigDye® Terminator Cycle Sequencing Kit (Applied Biosystems), according to the manufacturer's instructions. Cycle sequencing consisted of an initial denaturation step at 94ºC for 11 min, followed by 25 cycles of 94ºC for 10 sec, 52ºC for 5 sec and 70ºC for 3 min. Sequence products were resolved on an ABI 3130XL apparatus (Applied Biosystems). Sequence analysis was performed using Seqman (DNA Star Inc, Madison, WI, USA) and CEQ8000 Investigator (Beckman Coulter) software.

Mutations were confirmed by sequencing a second, independent DNA sample. All mutations and sequence variants are described according to HUGO approved nomenclature [22]. The nomenclature used by the BIC is also indicated [17], with 229 additional nucleotides on the coding sequence. Reference sequences

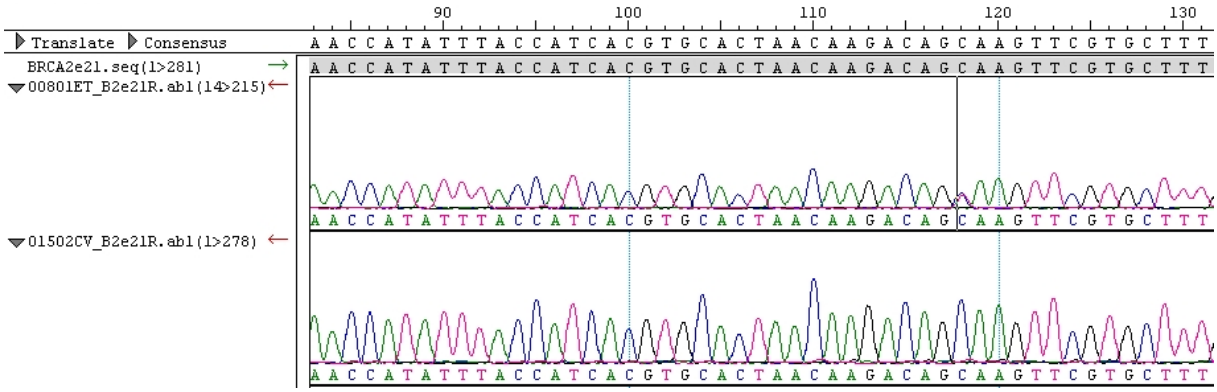

Figure 1a. Identification of C>T substitution by Sanger sequencing. Upper profile – heterozygous patient; downer sequence – normal wt patient

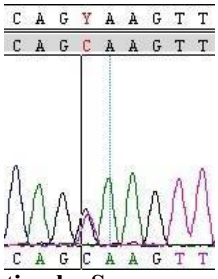

Figure 1b. Identification of C>T substitution by Sanger sequencing. Upper sequence – patient sample; downer sequence – consensus

for *BRCA2* were NM_000059.3 for coding, NC_000013.10 for genomic, and NP_000050.2 for protein sequences [23].

PCR-RFLP assay

BRCA2 exon 21 was amplified from 100 ng of template DNA, using the forward primer TTGGTTCTTTAGTTTTAGTTGCTTTTG and the reverse primer TATTCCTTCCTGTGATGGCC, at a final concentration of 0.4 µM in a volume of 20 µl, containing 1.25 units GoTaq Flexi DNA Polymerase (Promega™), with 1X appropriate buffer, 0.2 mM each dNTP, 1.5mM $MgCl_2$. Primers were designed using the PrimerExpress® software, Applied Biosystems. Positive and no-template controls were performed. The thermal cycling program for PCR consisted of an initial denaturation of 10 minutes at 95°C, followed by 30 cycles of denaturation (94ºC for 30 sec), annealing (55ºC for 30 sec), extension (72ºC for 45 sec), and a final extension of 10 minutes at 72ºC. 10 µl PCR reaction were digested for 1 hour with 10 units of TaaI restriction enzyme (Fermentas Inc, Maryland, USA), following the manufacturer's instructions. Digested products (10 µl) were electrophoresed alongside undigested amplicons (10 µl) in a 3% agarose TBE gel containing 0.5 µg/ml ethidium bromide. Migration was performed for 45 min at 5V/cm. Digestion of the wild-type amplicon generated fragments of 246 and 35 bp, while the c.8680C>T mutation generated 166, 80, and 35 bp fragments.

Results

1. The mutation

In Sanger sequencing of *BRCA2* exon 21 (*Figure 1a*), one can observe the apparition of a "doubled" nucleotide in the upper profile compared to the normal wild-type down, sign of heterozygosity in the concerned patient. The

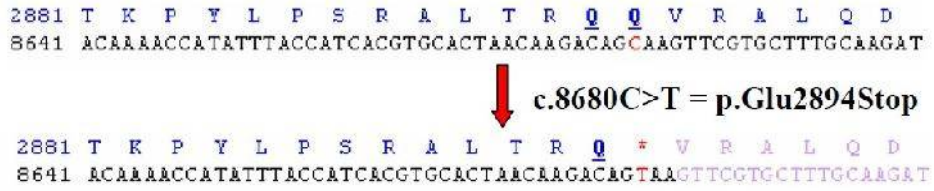

Figure 2. Probable effect of c.8680C>T mutation on BRCA2 protein

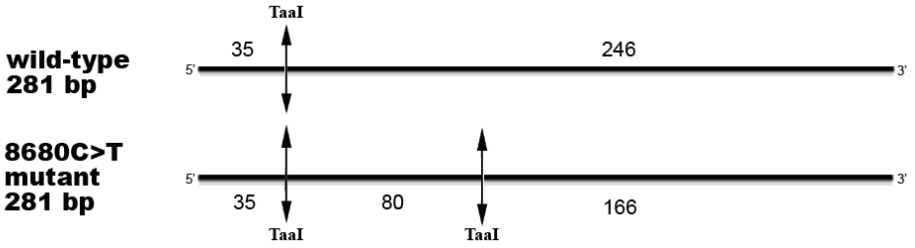

Figure 3. Restriction sites for TaaI on a BRCA2 281bp amplicon of exon 21, for the wild-type allele (top) or c.8680C>T mutant allele (bottom). Numbers correspond to the length of products in base pairs (bp).

sequence variation was confirmed in forward and reverse verification sequencing, the obvious C and T presence being clearer on a zoom (*Figure 1b*).

The genetic variant is in fact a point substitution of the C nucleotide in the 8680 position of the *BRCA2* coding sequence (NM_000059.3) with a T in the altered allele. The HUGO approved nomenclature for this mutation is c.8680C>T. The same alteration would be called 8908C>T in the ancient nomenclature still used by the BIC database. Using the reference protein sequence of *BRCA2* (NP_000050.2), we can observe (*Figure 2*) that substituted nucleotide is the first of codon 2894, CAA coding for glutamine. Its substitution with a T generates a TAA ochre stop codon and the end of protein synthesis message. We can suspect such a modification of truncating the *BRCA2* protein of its last C-terminus 525 aminoacids, although this would need a functional test assay to be clearly demonstrated [24]. This C-terminal domain is responsible for molecular interactions with *Rad51*, and it is involved in nuclear localization of *BRCA2*. Its deletion can be considered responsible for functional loss of the *BRCA2* tumor suppressor in cancer cases.

2. PCR-RFLP screening for BRCA2 c.8680C>T

As *BRCA* entire gene sequencing is complex, time-consuming and seriously expensive, developing of rapid and cheap pre-screening methods for targeting each identified mutation offers the advantage of quick diagnosis, avoiding entire gene sequencing. We imagined a simple and rapid screening for c.8680C>T mutation in large lots of patients by PCR-RFLP and agarose gel electrophoresis followed by UV visualization and interpretation.

The T for C substitution creates an additional digestion site for the TaaI restriction enzyme (*Figure 3*). Therefore, digestion on a homozygous wild-type amplicon will generate fragments of 246 and 35 base pairs, while digesting a mutant heterozygote will generate 246, 35, 166 and 80 base pairs fragments. Since the 35 bp fragment will be hardly detectable in a gel, the difference between normal and mutant individuals should be highlighted by the additional 166 and 80 base pair fragments issued from the mutant allele, with the 246 fragment being common to all individuals.

There is an important difference in digestion electrophoretic profiles between c.8680C>T

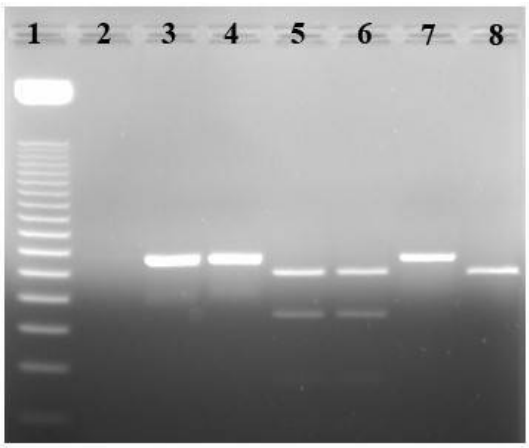

Figure 4. Detection of BRCA2 c.8680C>T mutation by a simple and rapid PCR-RFLP standard electrophoresis gel method. Lane 1: 50 bp step ladder; lane 2: no template control; lane 3: mutant heterozygote undigested amplicon (positive control); lane 4: patient 1 undigested amplicon; lane 5: positive control TaaI digestion; lane 6: patient 1 TaaI digestion; lane 7: patient 2 undigested amplicon; lane 8: patient 2 TaaI digestion. Patient 1 is c.8680C>T heterozygote carrier and patient 2 is wild-type homozygote.

heterozygote carriers (lanes 5&6, *Figure 4*) and wild-type homozygotes (lane 8). This allows a rapid discrimination of c.8680C>T carriers by simple PCR-RFLP followed by sequencing confirmation, avoiding entire gene sequencing. Hence, our technique can be rapidly performed on large lots of patients or in the general population.

Discussion

Oncogenetic testing of *BRCA* status is becoming a powerful therapeutical predictive tool. It is now obvious that in a near future the uptake and demand for rapid *BRCA1/2* mutations testing will increase and more flexible genetic counseling strategies will be needed. Currently, most laboratories performing diagnostic analysis of the *BRCA* genes use PCR of exons and intron-exon boundaries coupled to a prescreening step to identify anomalous amplicons. However, a full *BRCA1* and *BRCA2* gene screening still remains a labor and time consuming challenge due to large genes size, diverse mutations or variants of unknown significance (VUS) and complexity of large genomic rearangements (LGRs), requiring special technical approach. This procedure still remains too complex and expensive to cover a broader target (e.g. all breast or ovarian cancer patients and their first degree relatives) and cannot be routinely applied in less privileged countries.

We identified by sequencing a novel *BRCA2* mutation in exon 21, c.8680C>T. As full sequencing of the *BRCA* genes is complex, time-consuming and expensive, developing rapid and cheap pre-screening methods for targeting common mutations offers the advantage of quick and less expensive diagnosis. This strategy can be applied to populations with strong founder effects, where a limited number of mutations are to be screened. The PCR-RFLP assay presented here represents one such method for such a large-scale study.

We firstly identified the *BRCA2* c.8680C>T mutation in a breast cancer family with five early onset cancer cases. This mutation is novel and not yet stored in the BIC database. While screening by the in-house PCR-RFLP technique a sporadic lot of 50 breast and 25 ovarian cancer cases, unselected for familial history, we identified once again the *BRCA2* c.8680C>T mutation in a ovarian cancer sporadic case. This could open the hypothesis of a Romanian mutation, therefore strengthening the necessity of screening in a larger lot of patients.

Our data represent one early contribution in understanding the Romanian population genetic characteristic concerning *BRCA* genes and predisposition to breast and ovarian cancer. Research should be further developed in order to elucidate a mutation spectrum at a population level. Still, our first results open the way to targeted studies on most common or "local" mutations. This study also opens a way to investigate *BRCA* status in Eastern populations more in detail, with the aim

to compare groups within the Eastern European region or with Western populations.

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