Title: Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families

Authors:

Mateja Krajc (mkrajc@onko-i.si)
Erik Teugels (eteugels@uzbrussel.be)
Janez Zgajnar (jzgajnar@onko-i.si)
Guido Goelen (guido.goelen@uzbrussel.be)
Nikola Besic (nbasic@onko-i.si)
Srdjan Novakovic (snovakovic@onko-i.si)
Marko Hocevar (mhocevar@onko-i.si)
Jacques De Grève (jacques.degrev@uzbrussel.be)

Version: 2 Date: 25 June 2008

Author's response to reviews: see over
Dear Mr. Joseph Dunckley,

We should kindly ask you to consider the enclosed revised manuscript “Five recurrent BRCA1/2 mutations are responsible for breast cancer predisposition in the majority of Slovenian families” for the publication in the BMC Medical Genetics.

We addressed the comments in a revised manuscript and are attaching (in the continuation) answers by a point-by-point response to the concerns of the reviewers.

As suggested we copyedited the paper to improve the style of written English.

We look forward to your favorable reply,

On behalf of the authors,

Sincerely yours,

Mateja Krajc
(1) Reviewer's report

**Title:** Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families

Version: 1 Date: 14 May 2008

**Reviewer:** Giuseppe Palmieri

**Reviewer's report:**

In this study, Krajc and colleagues investigated about the prevalence of the BRCA1/2 mutations among Slovenian breast/ovarian cancer families. The entire work has been well-conducted and well-organized for both family selection (it was crucial to adopt the criterion of selecting probands on the basis of the presence of two or more first-degree relatives with breast and/or ovarian cancer in the family) and mutation analysis (it was correct to use two different testing approaches: full screening for significant families and PTT analysis for lower risk probands). Results of the study are well-described throughout the manuscript.

- **Major Compulsory Revisions**
  NONE

- **Minor Essential Revisions**
  NONE

- **Discretionary Revisions**
  In Methods (section "Patients and families"), Authors indicated that the "study was performed in families residing in Slovenia". Was the Slovenian origin ascertained in all cases through genealogical studies? If yes, it is much more appropriate to indicate that families were originated from Slovenia and not only resident in Slovenia.

  CA: The Slovenian origin was not ascertained in all cases through genealogical studies, therefore we used the term “residing”. This comment was added in the manuscript.

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests
Reviewer's report:

This report describes BRCA1 and BRCA2 mutation analyses in 145 Slovenian families with a personal and/or family history breast and/or ovarian cancer. Germline BRCA1/2 mutations were identified in 56 (~ 39%) families. The most interesting outcome of this study is the observation that 5 specific mutations (4 in BRCA1 and 1 in BRCA2) were found more than once, and accounted for 67% of the mutation-positive families. These results could significantly impact the genetic counseling of high-risk women in this population by facilitating mutation-detection of these very large and complex genes. These findings also add to the growing list of examples of recurrent BRCA1/2 mutations found in specific populations.

Major Compulsory Revisions

1. The study uses three specific inclusion criteria for mutation detection. Background section requires a clarification of rationale for the selection of specific individuals for mutation detection.

   CA: Inclusion criteria are described in detail in the reference 12 as also referred to in the manuscript. In the background section we formulate that minimal inclusion criteria were employed for cost-effectiveness reasons. We rewrote this part of the Methods section, since it was unclear. The reasons for choosing specific intake criteria are also described in the Discussion session.

2. In the Methods section, the inclusion criteria of cases tested for mutations requires further clarification and elaboration.

   CA: We further clarified inclusion criteria in the manuscript and in the additional file.

New text:

“Based on that information, probands were selected for screening according to liberal inclusion criteria adapted from Brussels [12]: (i) two or more first degree relatives with breast and/or ovarian cancer; if no ovarian cancer in the family, and only two breast cancers were diagnosed, one breast cancer had to be diagnosed before the age of 50. Individual patients with (ii) breast and ovarian cancer and (iii) patients with bilateral breast cancer or (iv) patients with breast cancer diagnosed before the age of 40 and (v) male breast cancer patients without any other cancers in the family were also included.”
Three specific inclusion criteria are indicated, but these include both female and male probands tested for mutations, as well as unaffected probands. It is difficult to sift through the additional files to appreciate the cases and family history in each of the categories listed for each inclusion criteria. Indicating the number of cases in each category would help clarify the situation. Moreover, what is the rationale for including ‘patients with bilateral breast with other cancer types in the family’? What are these other types of cancer?

CA: Thank you for this remark – it was a typing error – it was meant to be: “patients with bilateral breast without any other cancer types in the family” – we rewrote the whole section and specified in more detail the inclusion criteria in the manuscript.

Unaffected probands were also screened for the presence of a BRCA1/2 mutation when there were more than two first-degree relatives with breast and/or ovarian cancer as described now in more detail in the inclusion criteria added in the Methods section.

---

3. The Methods section refers to an impressive cancer registry dating back to 1950. Were all of the cancers (breast and ovarian cancers as well as cancer at other sites) mentioned in the study, particularly those listed in the additional tables, recorded in the registry? Or were some of these cases self-reported?

CA: Where possible (majority of cases), reported cancers were cross-checked, depending on the accuracy of individual data provided (name, surname, birth date, etc.). Since the obligatory registration of cancer cases dates back to 1950, some of the cases diagnosed before 1950 are not in the registry.

---

4. The mutation detection rate at 39% appears impressive. Likewise the observation that 5 specific mutations accounted for about 67% of mutation-positive cases. Although the mutation analysis of BRCA1 and BRCA2 was extensive, it was not comprehensive, as the largest exons of these genes were screened by PTT assay; and families with only 2 cases of breast cancer may have been further restricted to screening for the recurrent mutations only (Please clarify statement in Methods section paragraph 4) It is possible that mutations may have been missed and that the mutation detection rate identified in the 145 families is an underestimate of the overall mutation rate. It is also possible that the Slovenian families are more genetically heterogenous than indicated by the present analysis. The possibility of missed mutations due to the methods used should be discussed. It is difficult to evaluate how many families have a high a priori probability of harboring a mutation because there is no adequate description of these mutation-negative families – only the cancer phenotypes of the positive families are shown in the additional tables. Is it not possible to summarize the phenotypes of the mutation-negative families according to the categories used in the inclusion criteria for testing.

CA: We added to the Discussion part of the manuscript the possibility of underestimation of the mutation detection rate due to the methodology employed. It is really not possible to include in the scope of the article the cancer phenotype of the families in which no mutation was found and we are not aware that this was done in other similar reports on BRCA1/2 mutations.
5. Further to the above comment, in the Discussion section cites mutation detection rates of between 15% and 37% from independent reports. However, were the cited studies using the same inclusion criteria? If not then it is difficult to compare studies.

CA: Adopted in the Discussion:

New text:

“Since we are often facing genetic counseling for members of small families, we applied minimal selection criteria before initiating a search for a BRCA1/2 mutations. Despite these liberal criteria the overall mutation detection rate (MDR) was 39 % (56/145 screened families), which is high when compared to what was previously reported for other populations where the MDR are between 15 % and 37% with usually more stringent selection criteria with regard to familial cancer phenotype than in our study [17] [18] [19] [20] [21]. The intake criteria we employed thus seem adequate and practical for further use in our population [12].”

6. The authors indicate in the Results section that the probability of finding a mutation ‘correlated with the number of affected patients’. However only percentages are given and no statistical analyses are included to support this statement.

CA: This part is rewritten – new text in the result section:

“Although more families need to be investigated to reach statistical significance, the probability to find a mutation correlates numerically with the number of affected patients in breast cancer only families: with 3 or less affected members mutations were found in 16/64 families (25%) compared to 10/21 (48%) when there were more than 3 affected family members. However, when also ovarian cancer was present in the family: a mutation was found in 24/ 46 (52%) with 3 or less affected family members and 7/14 (50%) when > 3 affected family members, indicating that the presence of ovarian cancer seems much stronger predictor for finding a BRCA1/2 mutation than the number of breast cancer cases in the family. The presence of recurrent mutations in this population permits the identification of cancer predisposing mutations in 67% of the BRCA1/2 mutant families by just analyzing 4 PCR fragments by DGGE. A screen restricted to these 4 fragments could therefore be performed on patients with a low probability for finding a BRCA1/2 mutation.”

7. The Results section describing the cancer phenotypes of families is the weakest part of the manuscript. It describes relative risk estimates and penetrance. Overall the analysis is interesting but is not necessarily valid given the biases inherent in the selection of individuals for mutation detection, the small sample size used in the calculations, and the possibility of missed mutations due to incomplete mutation analyses for all cases.

CA: We added a comment in the discussion section.

The text added:

‘However, due to the small sample size these results should be confirmed in a larger sample size.”
a. In the first paragraph of this section, does the mean age of diagnosis of cancer include both breast and ovarian cancers? If so, why are they combined when the penetrance and age of diagnosis of breast cancer in mutation carriers is usually lower than ovarian cancer cases?

CA: *We adopted in the text this remark:*

*New text:*

“The mean age at breast cancer diagnosis in *BRCA1* mutation carriers was 42.9 years (CI: 40.1 – 45.8) and 48.71 years (CI: 43.4 – 54.0) for *BRCA2* mutation carriers, respectively. By using T test for equality of means, difference in mean age at breast cancer diagnosis was significant (p < 0.0001).”

b. Paragraph two of this section describes cancer phenotypes of all cases studied as shown in Table 1. What is the purpose of doing this? The inclusion criteria are complex. Again for reasons mentioned above, the frequency of mutation-positive cases per inclusion criterion maybe more meaningful. Also included are cancers at other sites. As mentioned above, were these cases verified by the cancer registry? If the authors argue the merits of including such a table why not also include similar data for the mutation-negative cases?

CA: *The purpose of this section was to reveal the other types of cancers found in mutation positive families and our next step will be collecting tumor blocks whenever possible and test for presence of a mutation. There are several inclusion criteria and the sample size seems too small to draw conclusions from the frequencies of mutation positive cases per inclusion criterion. It is a very good suggestion to implement it when we collect bigger sample size. Anyway we added the categorization of inclusion criteria in the additional file. The cases were, whenever possible, verified by the Cancer registry (already discussed above). Mutation negative cases are not in the scope of this manuscript.*

c. In paragraph four of this section, although controlled for mutation type, the calculations for relative risk of cancers is not convincing given the small number of cases used in the analysis. This perhaps explains the discordant observation with previous studies (as noted by the authors) where the analysis of IVS16-2A>G mutation was only reported in families with breast cancer unlike the present study which included families with ovarian cancer.

CA: *Remark is adopted in the text of the manuscript.*

d. Paragraph five of this section, describe penetrance estimates. This data too is not convincing given the small sample size, the possibility that not all cases within the family where tested for mutations, and biases inherent in the inclusion criteria.

CA: *Comment is added in the discussion.*
8. The cancer phenotypes other than breast an ovarian cancer are shown in the additional tables and referred to the Discussion section. Where these phenotypes also observed in the mutation-negative families? (Also see comment 7b above.)

CA: It is really not possible to include in the scope of this article the cancer phenotype of the families in which no mutation was found and we are not aware that this was routinely done in other similar reports on BRCA1/2 mutations.

Minor Essential Revisions

1. A lot of information about family history is presented in the additional tables that would be of particular interest to genetic counselors. It might be easier to study the information in the tables if the age(s) of diagnosis of the proband where put into a column separate from the cancer status. The penetrance for breast and ovarian cancer is much higher than any other type of cancer, the number of female first degree relatives (above age 18 years) would give a better indication of ‘penetrance’ than all such individuals.

As the authors have indicated in the Discussion, it is difficult to assess risk assessment of cancers at sites other sites. However, reporting cancers that occur in mutation-positive families from other sites is interesting. Could this information be separated from breast/ovarian cancer cases (with the exception of double primary cases)? As noted above (see item 6), the reliability of the data should be clarified.

CA: We redid the tables in the additional file and added two colons, we separated in the tables the age(s) of diagnosis of the proband as proposed and also we added the colon stating the inclusion criteria categorization.

2. In the Discussion section, the authors suggested that the level of participation is ‘higher’ than reported in the literature. Perhaps authors could clarify this statement, particularly for readers unfamiliar with participation rates reported in the literature.

CA: Remark adopted in the Discussion session.

New text:

“This level of participation is considerably higher than some report in the literature, where around 50% of eligible probands opt for screening [15].”

3. The report requires careful editing throughout. Specific examples are included below:

CA: We edited the text and also included examples described below.

a. Methods section: Patients and families

i. Delete ‘and’ in first sentence of paragraph one

ii. Something is missing in last sentence of paragraph one
iii. Add ‘to the’ between ‘according’ and ‘liberal’ in first sentence of paragraph two
iv. First sentence of paragraph 4: “Both genes were screened in full, except in families with only 2 breast cancer cases.” rather than as written.
v. First sentence of paragraph 5: “After a mutation was found in the proband…” rather than ‘family’.

b. Results section: BRCA1 mutation analysis

i. Rewrite the sentences describing repeatedly observed mutations: “the throughout the world widely reported”.

iii. First sentence in the third paragraph requires editing (“… a search for BRCA1/2 mutations” rather than as written.) [This paragraph nicely describes the overall rationale for the study and could also be iterated in the Background section.] The sentence “However, in the Slovene breast cancer only families we found much more mutations than corresponding Belgian families” requires editing to read “However, more Slovene breast cancer only families were found mutation-positive as compared with comparable…”
iv. In paragraph seven, correct spelling of cysteines and add database to ‘BIC” database.
v. Paragraph 8 : clarify what is meant by ‘double rate of ovarian cancers’?

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Needs some language corrections before being Published

Statistical review: Yes, and I have assessed the statistics in my report.

CA: Comment adopted in the manuscript.

Declaration of competing interests:
I declare that I have no competing interests
(3) Reviewer's report

Title: Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families

Version: 1 Date: 26 May 2008

Reviewer: Arto Mannermaa

Reviewer's report:

The manuscript Mateja Krajc, Erik Teugels, Janez Zgajnar, Guido Goelen, Nikola Besic, Srdjan Novakovic, Marko Hocevar and Jacques De Grève BMC Medical Genetics Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families represents an interesting investigation on the frequency and importance of BRCA1/2 mutations in Slovenian breast cancer families. BRCA1/2 are at the moment the most important cancer susceptibility genes for inherited breast cancer / breast and ovarian cancer. More than 3700 mutations have been found in various populations around the world. There are no specific mutational hotspots in the genes, but some population specific founder mutations still exist. The importance of this manuscript lies behind the fact that identification of possible population specific (founder) mutations could help in genetic counselling of the cancer families and/or promote more cost-efficient laboratory diagnostics. The paper is concisely and clearly written and data sufficiently presented. I have some comments that need to be replied by the authors. In general, the manuscript requires minor revision before being accepted.

Comments:

Major Compulsory Revisions:

The critical point of the manuscript is the fuzzy description of patient material. The number of probands with two or more first-degree relatives, individual patients without family history and bilateral breast cancer patients with other cancer types should be included in the section Methods, Patients and families. Are all 145 families of high risk with two or more breast and/or ovarian cancer cases among first-degree relatives?

CA: We further clarified inclusion criteria in the article and all 145 families were selected according to these criteria.

New text:

“Based on that information, probands were selected for screening according to liberal inclusion criteria adapted from Brussels [12]: (i) two or more first degree relatives with breast and/or ovarian cancer; if no ovarian cancer in the family, and only two breast cancers were diagnosed, one breast cancer had to be diagnosed before the age of 50. Individual patients with (ii) breast and ovarian cancer and (iii) patients with bilateral breast cancer or (iv) patients with breast
cancer diagnosed before the age of 40 and (v) male breast cancer patients without any other cancers in the family were also included.«

The authors should consider to exclude the subject of patients’ and their relatives’ interest to genetic counseling from the aims of the study. This was poorly described in both methods and results section.

CA: From the “aims” listed in the “Background” section we deleted the subject “patients’ and their relatives’ interest to genetic counseling” as this was not the primary purpose of the work. However, the data collected on this topic were kept in the manuscript since they are of interest for persons involved in the counseling.

Minor comments:

Methods:
- Describe how many families were fully screened and how many partially screened by PTT and recurrent mutation screening... One can not deduce this now.

CA: We considered that in the text.

- How was MLPA test for BRCA1 performed? Describe especially the data analysis.

CA: Data obtained after running samples from “MLPA” reactions on a fragment analyzer (from an ABI in our case) need to be analyzed (normalization and equalization of the crude data). A few “programs” (excel spreadsheets) are available on the internet for this purpose. For practical reasons we have preferred to develop our own spreadsheet. We run our MLPA reactions in badges of 8 samples, each obtained from a cancer patient belonging to a different high- risk family, but do not include control samples for normalization. As we consider that in a set of 8 such samples we will not encounter 2 patients with a deletion/duplication of the same exon(s), we use the data from 6 patients (after having excluded the 2 extreme values) for normalization. At the request of the referee we adapted the manuscript, but did not include a detailed description of the program, as this is also not the case (or rarely?) in comparable articles. However, if required, we don’t have any objection to include this information in the text, and eventually mention that the spreadsheet is available on request.

In the manuscript text:

“A multiplex Ligand probe assay (MLPA) for the BRCA1 gene was performed with probe set P002 and confirmed with probe set P087 (MRC-Holland, Amsterdam, The Netherlands), each time on a batch of 8 samples. Data analysis (normalization and equalization) was performed using a self designed Excel spreadsheet.”

Results:
- After identification of the sequence variants from BRCA genes, were any control material tested to reveal the frequencies of the mutations at the population? This is especially important in the case of novel missense mutations with possible reduced penetrance.
CA: We did not test control samples for the presence the BRCA1/2 mutations described in this article. Protein truncating mutations are generally considered as pathogenic, the effect IVS16-2A>G has been investigated at the mRNA level (see ref. 10) and missence mutations removing one of the crucial Cysteines in the RING finger domain are also considered as pathogenic. We describe one missence mutation never reported before (300T>A; C61S). However, this mutation affects the same “cysteine” as another missence mutations reported worldwide as cancer predisposing (300T>G; C61G, see BIC database). According to us, there is no evidence to doubt about properties of this mutation.

**Figure 1.**
Figure 1 is poorly drawn, please redraw. Breast /Breast and ovarian cancer families are now impossible to identify.

_**CA:** We redrew figure 1 – now it can be printed also in black and white version._

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests
(4) Reviewer's report

**Title:** Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families

Version: 1 Date: 27 May 2008

**Reviewer:** Jan Lubinski

**Reviewer's report:**

The manuscript entitled „Five recurrent BRCA1/2 mutations are responsible for cancer predisposition in the majority of Slovenian breast cancer families presents spectrum of BRCA1/2 mutations in Slovenian families with familial aggregations of breast/ovarian cancers. The authors demonstrate very interesting results. Five highly recurrent specific mutations were identified (1806C>T, 300T>G, 300T>A, 5382insC in the BRCA1 gene and IVS16-2A>G in the BRCA2 gene). They observed an exceptionally high frequency of 4 different pathogenic missense mutations, all affecting one of the cryptic cysteine residues of the BRCA1 Ring Finger domain. The results will contribute to a growing literature concerning the roles of BRCA1/2 mutations in breast/ovarian cancer predisposition.

**Major Comments**

The study group is mixed and not well defined. Probands were selected according „liberal intake criteria”. Authors should clearly summarize study group.

*CA: We further clarified inclusion criteria in the article.*

**New text:**

“Based on that information, probands were selected for screening according to liberal inclusion criteria adapted from Brussels [12]: (i) two or more first degree relatives with breast and/or ovarian cancer; if no ovarian cancer in the family, and only two breast cancers were diagnosed, one breast cancer had to be diagnosed before the age of 50. Individual patients with (ii) breast and ovarian cancer and (iii) patients with bilateral breast cancer or (iv) patients with breast cancer diagnosed before the age of 40 and (v) male breast cancer patients without any other cancers in the family were also included.«

**Minor Comments**

DGGE and especially PTT are methods with mutation detection sensitivity below 100%. This question should be discussed.

*CA: This question is addressed in the Methods and Discussion section. There is not one method that can claim to have a mutation detection sensitivity of 100%, even not direct sequencing. The DGGE and especially the PTT techniques are very well known by those involved in a BRCA1/2...*
screen. PTT misses all the missense mutations. However, missense mutations reported as pathogenic that are located in the DNA fragments covered by the PTT are extremely rare. On the other hand, it is not excluded that much more BRCA1/2 mutations are actually missed because they are localized in regions not covered by the actual screening strategies (e.g., located in promoter or other non-coding sequences, or due to large insertions, cf. Teugels E et al. De novo Alu element insertions targeted to a sequence common to the BRCA1 and BRCA2 genes. Hum Mutat. 2005; 26: 284.). We think it is not in the scope of this article to cover this aspect of the BRCA1/2 screen.

BRCA1 mutations 5382insC and 300T/G (detected by authors in 12 Slovenian families) are common Slavic ancestry mutations (many papers from Czech Republic, Lithuania, Poland, Slovakia, Russia …..) and should be properly presented in discussion.

CA: This remark is adopted in the Discussion section and references are added.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**
I declare that I have no competing interests