Absence of BRCA/FMR1 Correlations in Women with Ovarian Cancers

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Abstract

Previously reported findings in Austrian BRCA1/2 mutation carriers suggested a possible dependency of embryos with BRCA1/2 mutations on so-called low alleles of the fragile X mental retardation 1 (FMR1) gene, characterized by less than 26 CGG repeats (CGGn<26). The hypothesis arose from a study reporting highly statistically significant enrichment of low FMR1 alleles, significantly exceeding low allele prevalence in a general population, suggesting embryo lethality of BRCA1/2 mutations, “rescued” by presence of low FMR1 alleles. Such a dependency would also offer an explanation for the so-called “BRCA-paradox,” characterized by BRCA1/2 deficient embryonic tissues being anti-proliferative in nature, but proliferative in malignant tumors, including breast and ovarian cancers. Follow up investigations by other investigators, however, at most demonstrated trends towards enrichment but, mostly, no enrichment at all, raising questions about the original observation and hypothesis. We in this study, therefore, investigated CGGn of the FMR1 gene of 86 anonymized DNA samples from women with various forms of ovarian cancer, and were unable to demonstrate differences in prevalence of low FMR1 alleles either between positive and negative ovarian cancer patients for BRCA1/2 or between ovarian cancer patients and reported rates in non-cancer populations. This raises further questions about a suggested dependency between BRCA1/2 and FMR1, but also raises the possibility that investigated Austrian BRCA1/2 carrier populations differ from those in other countries. Either only selected BRCA1/2 mutations, therefore, interact with low FMR1 alleles or the Austrian data reflect only coincidental observations.

Introduction

Austrian colleagues and we previously reported in an Austrian population of women with functional BRCA1 and BRCA2 mutations statistically highly significant enrichment with so-called low fragile X mental retardation 1 (FMR1) gene alleles [1,2]. Such low alleles are defined by less than 26 CGG repeats (CGGn<26), and have been associated with premature decline in functional ovarian reserve, also called premature ovarian aging (POA) or occult primary ovarian insufficiency (OPOI) [3].
Since BRCA1 mutations have also been associated with POA/OPOI [4], above described findings in Austrian BRCA1/2 mutation carriers led to the hypothesis that BRCA1 effects on ovarian function may actually reflect FMR1 effects. Under this hypothesis, BRCA1/2 mutations are, in principle, embryo-lethal [1], a suggestion supported by some homozygous BRCA1/2 mouse homologs, indeed, being embryo-lethal, though with considerable variability in phenotype and in rescue from lethality on a p53-null background [5]. Embryos so, potentially, destined for mortality, if also carrying low FMR1 alleles, would, however, be rescued, leading to the enrichment of low FMR1 alleles in now rescued carriers of BRCA1/2 mutations, as observed in Austrian women [1,2].

This hypothesis also, for the first time, offered an explanation for the so-called “BRCA paradox,” which received its name from the contradictory observations that BRCA1/2 deficient tumor cells very rapidly proliferate, while BRCA1/2-deficient embryos suffer from proliferation defects (and, possibly, therefore succumb to embryo lethality) [5]. In animal models, p-53-nullizygosity can rescue BRCA1 mouse mutant but, often, only delays lethality [6–10].

In humans, BRCA1/2 mutations are strongly associated with increased risk for malignancies, including breast and ovarian cancers [11]. Low FMR1 alleles were to be able to suppress anti-proliferative (and, therefore, embryo-lethal) effects of BRCA1/2 mutations, allowing carriers of low FMR1 mutations to escape embryo-lethality, only BRCA1/2 carrying embryos would be born. They also would carry a low FMR1 allele, and grow up with suppressed anti-proliferative effects (i.e., would express a proliferative phenotype) and, therefore, be at risk for BRCA1/2-associated cancers. The actual culprit for cancer risk under such a scenario would, therefore, actually be the suppressive effect of low FMR1 alleles on BRCA1/2, converting anti-proliferative into a proliferative phenotypes [1].

The potential importance of this hypothesis for oncology attracted follow up by investigators in The Netherlands [12], Israel [13] and Italy [14]. All three studies, however, failed to confirm the Austrian observation of low FMR1 allele enrichment amongst carriers of BRCA1/2 mutations. As a possible explanation, we noted in an accompanying editorial to the Italian study that investigated BRCA1/2 mutations in Austrian and Italian study patients were completely different [15].

Divergent results between Austrian and Italian studies, therefore, could reflect different BRCA1/2 mutations with different degrees of embryo lethality. BRCA1/2 mutations in these two countries are, indeed, known to diverge [16]. Especially relevant to the Dutch study [12], Verhoog et al reported that even within The Netherlands, significant divergence in BRCA1/2 mutations is observed even within very small geographic areas [17]. Finally, the Israeli study involved exclusively BRCA1/2 founder mutations associated with cancer risk in Ashkenazi Jewish populations [13] and, therefore, was by definition different from BRCA1/2 mutations in Austrian populations.

The possibility that different BRCA1/2 mutations may exhibit different degrees of dependency with the FMR1 gene was potentially also supported by the trend towards enrichment with low FMR1 alleles among BRCA1/2 mutation carriers observed in the Italian study (32.6% vs. 23.1%) [14]. Speaking against such an explanation, a recent study, however, suggested other, non-FMR1-associated molecular mechanisms as causes for BRCA1-associated POA/OPOI [18].

With the issue still unresolved, we, therefore, decided to further explore it in women with ovarian cancer. The hypothesis of here presented study is that, since ovarian cancer risk is associated with BRCA1/2 mutations [11], if low FMR1 alleles, indeed, are causally related to proliferative BRCA1/2 cancer risks, (i) women with ovarian cancers, overall, should demonstrate a higher prevalence of low FMR1 alleles than has been reported in cancer-free populations; and (ii) BRCA1/2-positive ovarian cancer patients should demonstrate more low FMR1 alleles than BRCA1/2-negative patients.

Materials and Methods

The study population involved genetic materials from 86 ovarian cancer patients, for who cryopreserved DNA samples were stored at -80°C at the University of British Columbia, Vancouver, Canada.

IRB approvals and specimens’ origin

Material transfer agreements were executed between the University of British Columbia and the Center for Human Reproduction (CHR) in New York City, and approvals for the studies from both Institutional Review Boards (University of British Columbia, Vancouver, Canada, and The Center for Human Reproduction, New York, N.Y.) were separately obtained, including waivers from both IRBs to get individual informed consents from patients who were the source of the genetic materials investigated because samples were coded, before the specimens were shipped overnight on dry ice from Vancouver to New York City. Each specimen contained at least 100 ng of DNA in 5 to 10 μL volume.

Illumina sequencing of CCGn

The exonic and limited flanking intronic sequence of BRCA1/2 was determined from peripheral blood derived gDNA following amplification using RainDance technology and Illumina sequencing. The resulting sequences were aligned to the hg19 human genome reference using BWA (both aln and bwasw algorithms), and assembled with ABYSS. Variant calling was performed using the samtools mpileup (ABYSS, bwasm, and aln) and pindel (aln only) packages. Identified variants were submitted by report to CGL. CGL: Submitted variants were interpreted and annotated using HGVS nomenclature, using reference sequences NM_007294 for BRCA1, and NM_000059 for BRCA2. Pursuant to HGVS convention, cDNA numbering begins at the A of the initiating codon (ATG). Sequences of low coverage regions and ACMG category 1 and 2 mutation variants were confirmed by Sanger sequencing. This test was developed and its performance characteristics determined by the Centre for Clinical Diagnostic Genomics and further validated at the Cancer Genetics Laboratory (BCCA).

MLPA

The presence or absence of copy number differences in BRCA1/2 genes or portions thereof, were determined via Multiplex Ligation-dependant Probe Amplification (MLPA) according to the manufacturer’s protocol (P002-C1, P090-A3, MRC-Holland, Amsterdam). Analysis of the resulting amplification products was performed using an ABI 3730 DNA Analyzer and associated analysis software. Large scale insertions and deletions which lie outside the regions assessed by the individual MLPA probes are not detectable by this method. Genetic variants lying within individual probe binding sites may lead to false positive MLPA results. Single exon deletions are independently confirmed. BRCA1 reference sequence: NM_0007294. BRCA2 reference sequence NM_000059.
**Table 1.** Ovarian cancer patient characteristics.

| Characteristic                      | Detail          | n = 80<sup>1</sup> | Percent |
|-------------------------------------|-----------------|----------------------|---------|
| **FMR1**                            |                 |                      |         |
| norm                                |                 | 48                   | 60.0%   |
| het-norm/high                       |                 | 8                    | 10.0%   |
| het-norm/low                        |                 | 19                   | 23.8%   |
| hom<sup>*</sup>                      |                 | 5                    | 6.3%    |
| **Ovarian cancer diagnosis**        |                 |                      |         |
| High-grade serous                   |                 | 60                   | 75.0%   |
| Clear cell                          |                 | 9                    | 11.3%   |
| Endometroid                         |                 | 6                    | 7.5%    |
| Low-grade serous                    |                 | 5                    | 6.3%    |
| **Functional oncogenic BRCA**       |                 |                      |         |
| BRCA1                               |                 | 11                   | 13.8%   |
| BRCA2                               |                 | 4                    | 5.0%    |
| Negative                            |                 | 65                   | 81.3%   |
| **All BRCA mutations**              |                 |                      |         |
| BRCA1                               |                 | 21                   | 26.3%   |
| BRCA2                               |                 | 6                    | 7.5%    |
| Negative                            |                 | 53                   | 66.3%   |

<sup>1</sup>For 6 cancer patients no FMR1 data were obtainable from submitted samples;<sup>*</sup>hom sub-genotypes are not broken out; 4/5 contained low alleles.

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**Figure 1.** Distribution of CGG<sub>n</sub> for each ovarian cancer patient’s lower and higher *FMR1* Allele (A & B).

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This test was developed and its performance characteristics determined by MRC-Holland (Amsterdam). Furthermore, this test kit is labeled “For Research Purposes Only.”

Specimens were initially shipped anonymized with identifier codes. Once FMR1 testing results had been obtained, clinical information in regards to each sample was forwarded from Vancouver to New York, which included BRCA1 and BRCA2 status, type of ovarian malignancy and stage of disease.

Once specimens were received in New York, they were immediately stored at −80°C until assayed by commercial assay for CGGn of the FMR1 gene (LabCorp, Burlington, North Carolina), as previously reported [3]. In short, no interpretable results were obtained in 6/86 submitted samples, leaving 80 ovarian cancer patients in the study for analysis. CGGn was reported for both alleles. Individual mutations were described as previously reported based on a normal CGGn range of 26–34. Alleles below CGGn = 26 were, therefore, considered low [3]. Women with both alleles in normal range are considered normal (norm); those with one allele in normal and one outside normal range are heterozygous (het) and those with both alleles outside normal range are homozygous (hom). Genotypes are then further
sub-divided into sub-genotypes based on low or high (CGG₅₋₇₄) alleles.

We then established the prevalence of low FMR1 alleles for the whole ovarian cancer group and compared it to control populations without known malignancies, previously reported in the literature. In a second analysis we then compared the prevalence of low FMR1 alleles in ovarian cancer patients, either with or without BRCA1/2 mutations. And, in addition, repeated the analysis only for functionally oncogenic BRCA1/2 mutations.

Statistical analyses were performed using IBM SPSS statistics version 21. Continuous variables were expressed as means ± standard deviation. Categorical variables were expressed as counts (percentage). Results were cross-tabulated and Chi Square test was used to compare different distributions.

**Results**

Satisfactory FMR1 results were obtained from 80/86 samples. Table 1 summarizes patient characteristics for these 80 patients.

![Table 1](https://example.com/table1.png)

The table demonstrates data for all BRCA1/2 mutation carriers, whether functionally oncogenic or not. In this group of ovarian cancer patients the prevalence of low FMR1 alleles was actually nominally higher in BRCA1/2-negative (18/57, 31.6%) than BRCA1/2-positive ovarian cancer patients (5/23, 21.7%; P = 0.43), though the difference did not reach statistical significance.

When the same analysis was repeated for only 15 functionally oncogenic BRCA1/2 mutations, outcomes were very similar, 2/15 (13.3%) low FMR1 alleles in BRCA1/2 mutation carriers and 21/65 (32.3%) in ovarian cancer patients without BRCA1/2 mutations (P = 0.21).

Both analyses, thus, demonstrate that the combined presence of BRCA1/2 mutations and low FMR1 alleles actually appears to be less commonly associated with ovarian cancer than absence of both these mutations in the same patient.

Table 2

| Ovarian cancer patients | BRCA1/2-negative | BRCA1/2-positive |
|-------------------------|-------------------|------------------|
| Austrian study [1]*     |                   |                  |
| Infertile female controls | 18/57            | 31.6             |
| BRCA1/2-positive         | 5/23              | 21.7             |
| Dutch study [12]**      |                   |                  |
| BRCA1/2-positive         | 35.0              |                  |
| Israeli study [13]      |                   |                  |
| BRCA1/2-positive         | 31.5              |                  |
| Italian study [14]      |                   |                  |
| BRCA1/2-positive         | 23.1              |                  |

*Reports only het-norm/low sub-genotype since did not separately evaluate low hom sub-genotypes. True prevalence of low FMR1 alleles was, therefore even a few percentage points higher.

** Percentage of control population only graphically reported.

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the Table 3, and also demonstrated no significant overlap with either Austrian or Italian studies.

**Discussion**

We in this study investigated in women with various forms of ovarian cancer whether the presence of BRCA1/2 mutations resulted in enrichment of low FMR1 mutations, which would suggest interplay between these two genes, in establishing oncogenic risk. We, however, were unable to detect any difference in distribution of low FMR1 alleles in comparison to reported distributions in normal infertile populations without known malignancies [1,12–14], nor were we able to demonstrate a relative increase in low FMR1 alleles in BRCA1/2 carriers with ovarian cancers in comparison to ovarian cancer patients who were not BRCA1/2 mutation carriers. Indeed, this study actually demonstrated the opposite, a normal-range prevalence of low FMR1 alleles in BRCA1/2 mutation-carrying ovarian cancer patients but a trend towards higher prevalence in ovarian cancer patients who were not BRCA1/2 carriers. Interestingly, a similar result was reported in the Israeli study [13], where BRCA1/2 mutation carriers, a large majority of them already diagnosed with breast cancer, demonstrated only in 24.8% low FMR1 alleles, while random controls demonstrated low FMR1 alleles in 31.5% of women.

Why here reported ovarian cancer patients without BRCA1/2 mutations and Israeli controls present with such an unusually high, and apparently elevated prevalence over average populations, of low FMR1 alleles is unclear. In a large majority, low FMR1 alleles represent het-norm/low FMR1 sub-genotypes. In a small minority they also can represent either hom-high/low or hom-low/low sub-genotypes. Combined, low alleles rarely represent more than approximately 25% of an infertile female population [3].

### Table 3. BRCA1/2 mutations in here presented ovarian cancer patients.

| BRCA1/2 mutations | HGVS | BIC |
|-------------------|------|-----|
| **BRCA1**         |      |     |
| Undefined 3       | 2250A>T |
| c.3302G>a         | 1048delA |
| c.422?            | 547+1del |
| c.4186?           | 4357+1dup |
| c.6406>T         | - |
| -                 | 1048delA |
| -                 | 3726C>T |
| -                 | 4184delTCAA |
| -                 | 185delAG |
| -                 | 4797G>T |
| -                 | 5370C>T |
| -                 | 546G>T |
| c.3758C>G   1^    | ? |
| c.1530A>C         | pending |
| c.5236C>G         | - |
| c.4812A>G         | - |
| c.4039A>G         | - |
| c.4883T>C         | - |
| c.548-17T>G       | 28146T>G |
| c.3328_3330delAAG | - |
| **BRCA2**         |      |     |
| c.2883G>A 1^     | ? |
| c.2808_2811delACAA | - |
| c.4848-4849delAA  | - |
| -                 | 5445delTTTAAGT |
| c.4715C>G         | - |
| c.7301A>C         | - |
| c.4314C>T 1^    | - |

1Two patients were carriers of BRCA1 and BRCA2 mutations;
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Here reported findings, however, do offer some potentially important answers: They make the hypotheses increasingly unlikely that (i) all BRCA1/2 mutations in humans are to a significant degree embryo-lethal; (ii) low FMR1 alleles rescue embryos from BRCA1-lethality and (iii) the FMR1 gene offers a final solution to the “BRCA paradox.”

Considering that hundreds of BRCA1/2 mutations have been reported, amongst which only few are functionally associated with increased cancer risks, even considering here presented study results, one, however, still cannot preclude that the previously suggested hypothetical interplay between BRCA1/2 and FMR1 genes, similarly, may be only restricted to selected BRCA1/2 mutations.

Such an explanation would suggest that the Austrian study, which so strongly suggested an embryonic selection process for low FMR1 alleles, disproportionally reflected a selective embryo-lethal BRCA1/2 population, favoring interaction with the FMR1 gene. Otherwise, this study of Austrian patients would have to be considered a statistical coincidence, though conducted in blinded fashion, with all BRCA and FMR1 assays performed in Austria by well established genetic laboratories in academic centers, while statistical analysis of assay data was, independently, performed in the U.S. [1].

While here reported study, therefore, further diminishes the likelihood that the BRCA and FMR1 genes interact in their effects on embryo survival and oncogenic risk, the study does not preclude the possibility that selected embryo-lethal oncogenic mutations of BRCA1/2, indeed, are rescued by low FMR1 alleles.

In this context, it is interesting to note that a variety of genome-wide association studies of BRCA1/2 mutation carriers recently identified some genetic loci, which affect BRCA1/2-associated cancer risks for breast and ovarian cancers [19–21]. The thought that specific mutations of the FMR1 gene may, selectively, affect BRCA1/2, therefore, is conceivable.

BRCA is generally considered a genetic repair gene, which, when mutated, amongst other negative effects, can also affect X-chromosome inactivation [22]. Skewed activation in women with breast and ovarian cancers, at least in part, has been attributed to BRCA1 and to a lesser extend BRCA2 mutations [23].

One also can further hypothesize about potential bi-directional effects of these two genes on each other. For example, certain BRCA1/2 mutations could affect the FMR1 gene, located at Xq27.3, via X-chromosome inactivation and methylation of FMR1. The FMR1 gene, in turn, could rescue, as previously hypothesized [1], selected embryo lethal BRCA1/2 mutations. Such interactive effects between the two genes would, of course, result in much more complex clinical phenotypes. Studies like this or previously reported studies by others [12–14], therefore, likely would not be able to discover such interactions between the two genes.

An FMR1 interaction as explanation of the “BRCA paradox,” therefore, appears increasingly unlikely but still cannot be completely excluded.

This study for the first time investigated the alleged BRCA1/2 interaction with low FMR1 mutations in an ovarian cancer model. All prior studies were conducted in breast cancer patients. The use of another BRCA1/2 associated cancer model, and the quite large number of available patient samples represent the strengths of this study. Somewhat of a weakness lies in the absence of racial data on investigated patients since FMR1 mutation prevalence to a degree is racially defined [24]. Ontarian law, however, does not allow for maintenance of such data in association with genetic studies.

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Author Contributions

Conceived and designed the experiments: NG DHB VAK. Performed the experiments: JNMA CBG HJL YGW ELT. Analyzed the data: DHB. Contributed reagents/materials/analysis tools: JNMA CBG HJL YGW ELT. Contributed to the writing of the manuscript: NG VAK DHB. Contributed patient samples and patient data: JNMA CBG. Coordinated between USA and Canada-based investigators: NG JNMA.

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