FMR1 CGG allele length in Israeli *BRCA1/BRCA2* mutation carriers and the general population display distinct distribution patterns

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Summary

Premature ovarian failure and diminished ovarian reserve have been noted both in female *BRCA1/BRCA2* mutation carriers and in carriers of the Fragile X syndrome *FMR1* gene CGG repeat size premutation. Based on the observation that *BRCA* mutation carriers do not harbour long CGG repeats in the *FMR1* gene, it was hypothesized that BRCA-associated premature ovarian failure is mediated via *FMR1*. To test this notion, we evaluated the distribution of constitutional *FMR1* genotypes in 188 *BRCA1/BRCA2* mutation-positive Jewish Ashkenazi women and 15 708 female, mostly Ashkenazi controls in Israel. *BRCA1/BRCA2* mutation carriers displayed a unique distribution of *FMR1* genotypes compared with controls (p = 0·018) with a prominence of the shorter CGG alleles (<26 repeats). There was no allele size distribution differences within *BRCA* carriers when comparing cancer free (n = 95) and breast cancer affected women (n = 93) (p = 0·43). In conclusion, *BRCA* mutation carriers exhibit a distinct CGG *FMR1* repeat size pattern compared with the general population, but it is unlikely to account for the reported diminished ovarian reserve or act as a modifier breast cancer gene in *BRCA* mutation carriers.

1. Introduction

The fragile X mental retardation 1 (*FMR1*) gene, is localized to Xq27·3, and contains a repetitive DNA segment, the (CGG)n trinucleotide. Mutation status depends on the number of CGG repeats, women who carry 58–200 repeats and those who carry more than 200 repeats are designated premutation and full mutation carriers of the Fragile X syndrome (MIM # 300624), respectively (Willemsen *et al.*, 2011). One of the phenotypes reportedly associated with having a long CGG repeat, is premature ovarian failure, or primary ovarian insufficiency (Wittenberger *et al.*, 2007). Moreover, diminished ovarian reserve was also reportedly associated with a CGG repeat size that is not in the range of the "normal size repeat", defined as 26–34 repeats (Gleicher *et al.*, 2010 *a*, 2010 *b*). Diminished ovarian reserve was also noted in *BRCA1/BRCA2* mutation carriers (Oktay *et al.*, 2010). These phenotypic similarities in ovarian reserve, have led Weghofer and co-workers to analyze *FMR1* CGG repeat length in *BRCA1* and *BRCA2* mutation carriers (Weghofer *et al.*, 2012). They report that *BRCA1/BRCA2* mutation carriers almost exclusively show low CGG repeats, with significant allele distribution differences between mutation carriers and noncarrier infertile controls. They conclude that this unique *FMR1* CGG allele distribution in *BRCA1/BRCA2* carriers underlies many of the noncancerous ovarian phenotypes reported in mutation carriers. In addition, based on their results, Weghofer and co-workers hypothesized that the unique *FMR1* CGG repeat alleles may underlie the so called "BRCA paradox", where *BRCA* alleles in the embryo exert an anti-proliferative effect (that may account for embryonic lethality in BRCA null embryos) whereas in adult tissues lack of BRCA protein expression is associated with a strong proliferative effect that eventually

leads to tumor formation in the relevant tissues, e.g. breast and ovarian.

The present study was performed to validate and expand this previous report, targeting Jewish Israeli *BRCA1/BRCA2* mutation carriers and a large Jewish control population group.

2. Materials and methods

(i) *Study participants*

Individuals counselled and genotyped for *BRCA1/BRCA2* germline mutations or *FMR1* CGG repeat size at the Danek Gertner Institute of Human Genetics at the Sheba Medical Center, Tel-Hashomer, Israel from 1 January 2008–31 December 2012 were eligible. The Oncogenetics Unit recruited women who were counselled and genotyped for being at high risk for developing breast/ovarian cancer as determined by standard criteria (Laitman *et al.*, 2012) and were subsequently found to harbour either a *BRCA1* or *BRCA2* mutation (see below). The control group was composed of women who were genotyped for their *FMR1* CGG repeat length status in the context of prenatal genetic testing, offered in Israel. The study was approved by the ethics committee of the Sheba Medical Center and each participant gave an informed consent to participate in the approved study protocol.

(ii) *Analysis of the predominant Jewish mutations in the BRCA1/BRCA2 genes*

Mutational analyses for two of the three founder mutations (185delAG in *BRCA1* and 6174delT in *BRCA2*) were carried out by restriction enzyme digest of PCR products, and analysis of the digested PCR products on agarose gels as previously described by us (Laitman *et al.*, 2012). For each of these mutations, a known mutation carrier was used as a positive control in each experiment and each mutation was confirmed by sequencing.

(iii) *FMR1 CGG repeat analysis*

FMR1 analyses were performed as previously reported (Gleicher *et al.*, 2011). Briefly, the FMR1 repeat region is amplified using a fluorescent primer, the PCR product will then be mixed with Liz 500 internal size standard (Applied Biosystems Inc., Foster City, CA, USA), and analyzed on the ABI Prism 3100 genotyper. The GeneScan raw data were analyzed using the GeneMapper software (version 3.1) to obtain the allele repeat in base pairs.

(iv) *Statistical analyses*

Proportions of *FMR1* genotypes and sub-genotypes were compared between the two study groups using cross-tabulations and calculations of χ^2 and Cramer's V statistics. When comparing (CGG)n as a continuous function between the groups, nonparametric testing was used because (CGG)n in populations tends to be positively skewed. Mann–Whitney U tests were conducted to evaluate differences between the two groups in median change in (CGG)n of both alleles. All statistical calculations were performed utilizing SPSS, version 18 (Chicago, IL, USA).

3. Results

(i) *Participant characteristics*

(a) *BRCA1/BRCA2 mutation carriers*

Overall, there were 188 mutation carriers who participated in the study, 97 carried the *BRCA1**185delAG mutation and 91 carried the *BRCA2**6174delT mutation. There were 48 women who were diagnosed with breast cancer who harboured the 185delAG mutation and the mean age at diagnosis was $44{\cdot}05 \pm 12{\cdot}1$ years, and 45 breast cancer cases that carried the 6174delT mutation and the mean age at diagnosis was $44 \pm 12{\cdot}1$ years. All other *BRCA1/BRCA2* mutation carriers (n = 95) were cancer free (mean age at counselling and genotyping was 44 years for *BRCA1* carriers and 44·2 years for *BRCA2* mutation carriers).

(b) *Controls*

Overall 15 708 women underwent prenatal genetic testing to define the repeat length of the CGG trinucleotide repeat in the *FMR1* gene in order to evaluate the need for prenatal diagnosis of embryonic Fragile X syndrome (Toledano-Alhadef *et al.*, 2001; Berkenstadt *et al.*, 2007). The mean age at counselling and genotyping was $26{\cdot}7 \pm 4{\cdot}3$ (19–37) years and 59% were of Ashkenazi origin. Notably, these women did not undergo *BRCA1/BRCA2* mutational analysis.

(ii) *CGG FMR1 repeat length genotyping*

In the control, non-*BRCA* mutation carrier population, one *FMR1* allele repeat number range was 8–600 repeats, with a median allele size of 30 repeats, and the second allele repeat number range was 15–600 repeats (median allele repeat size 30 repeats). Notably, this *FMR1* CGG repeat length distribution was similar to that reported in other, ethnically diverse populations (Gleicher *et al.*, 2010 *b*; Gleicher *et al.*, 2011).

Amongst *BRCA* mutation carriers, one *FMR1* allele repeat number range was 14–40 repeats with a median allele of 29 repeats, and the second allele repeat size range was 23–92 repeats with the median allele repeat number of 30 repeats.

Table 1. *FMR1 genotypes and sub-genotypes distribution in BRCA1/2 carriers and the control population*

Genotypes and sub-genotypes*	BRCA1/2		Population	
	N	%	N	%
Normal	98	52·12	8804	56·04
Hom low	2	1·06	473	3·01
Hom high	1	0·53	131	0·83
Het low/high	8	4·25	431	2·74
Het normal/low	63	33·51	3841	24·45
Het normal/high	16	8·51	2028	12·91

* Low alleles (n < 26); normal alleles (n = 26–34); high alleles (>35). het, heterozygous; hom, homozygous.

FMR1 genotypes and sub-genotypes distribution in the control population is consistent with previous reports in the Israeli population (Toledano-Alhadef *et al.*, 2001; Berkenstadt *et al.*, 2007). *FMR1* genotypes and sub-genotypes distribution amongst *BRCA* carriers shows a distinct distribution compared with the control population (Table 1 and Fig. 1)

Comparison of genotypes distribution between the two study populations demonstrates a statistically significant difference ($\chi^2 = 13.612$, d.f. = 5, p = 0·018)

When comparing allele distribution of the first *FMR1* allele there is no difference in the distribution (Mann – Whitney p = 0·095). However, when comparing the allelic distribution of the second allele there is a marginally significant difference (Mann – Whitney p = 0·036) (Table 2). Notably, the rate of the normal/high alleles amongst heterozygote *FMR1* CGG allele in the control group (12·9%) did not show any significant difference compared with *BRCA* mutation carriers (8·5%) (p = 0·073). Yet, the rate of the normal/low alleles amongst heterozygote *FMR1* CGG allele in the control group (24·45%) showed a significant difference compared with *BRCA* mutation carriers (33·5%) (p = 0·004). There was no allele size distribution differences within *BRCA* carriers comparing cancer free (n = 95) and breast cancer affected women (n = 93) for the low allele (p = 0·19), for the high allele (p = 0·71) or for overall genotypes distribution (p = 0·43).

4. Discussion

In the present study, the *FMR1* CGG trinucleotide repeat length distribution among Jewish Israeli *BRCA1/BRCA2* mutation carriers was different from that exhibited by the general Israeli, mostly Ashkenazi, population. These *FMR1* CGG allele distribution differences results are in line with those of Weghofer *et al.* (2012) who previously reported a unique *FMR1* CGG allele distribution in *BRCA1/BRCA2* mutation carriers undergoing fertility treatment compared with noncarriers recruited from an infertility clinic. Yet, unlike the almost complete absence of long CGG repeat sizes (34 repeats and above) in the study by Weghofer and co-workers (2012), in the present study 8·5% of *BRCA1/BRCA2* carriers had at least one long CGG allele, a rate that was not statistically different to that of the control population (12·9%). The basic premise of the Weghofer *et al.* (2012) study was that the long CGG allele in the presence of a *BRCA* mutation may be embryonic lethal, and that rescue may be conferred by the existence of short (<26 repeats) alleles; however, our results do not support this notion. In line with our previous data, smaller studies published from Israel (Dagan *et al.*, 2014), Italy (Ricci *et al.*, 2014) and Holland (Brandão *et al.*, 2013) also failed to show the lack of long CGG *FMR1* alleles in *BRCA* mutation carriers. Another recent study that focused on ovarian cancer cases (n = 87) showed that ovarian cancer cases who are *BRCA* pathogenic mutation carriers (n = 15) exhibit a normal-range prevalence of *FMR1* CGG alleles, whereas ovarian cancer patients who are *BRCA* wild-type, display a trend (not reaching statistical significance) towards higher prevalence of low CGG repeats of the *FMR1* gene (Gleicher *et al*, 2014). Moreover, the data presented herein and the above mentioned previous reports (summarized in Table 3) also question the putative role attributed by Weghofer and co-workers (2012) to *FMR1* alleles in mediating premature ovarian failure in *BRCA* mutation carriers.

Several reasons may underlie the discrepancy in the results on the rates of the longer CGG alleles between the original Weghofer and co-workers (2012) study and the subsequent studies: the ethnicity of the genotyped groups in both Israeli studies was different, with mostly Ashkenazi Jewish women genotyped in these studies compared with mostly Caucasian European and American women in the original study. These ethnic differences are reflected in the location and type of mutations in both *BRCA1* and *BRCA2* genes, and given the genotype – phenotype correlations reported in *BRCA1/BRCA2* mutation carriers, the Weghofer and co-workers (2012) study results may be in part attributed to these genetic and ethnic differences. This is not a valid argument to account for the lack of replication in the Italian and Dutch studies. Another factor that may have led to the discordant results is the mode of recruitment and ascertainment of *BRCA* mutation carriers: a cancer and prenatal genetics clinic in the present study and the study by Dagan and co-workers (2014), a familial breast cancer clinic in the study by Ricci *et al.* (2014) and an infertility clinic in the Weghofer *et al.* (2012) study. However, this latter explanation is unlikely to account for
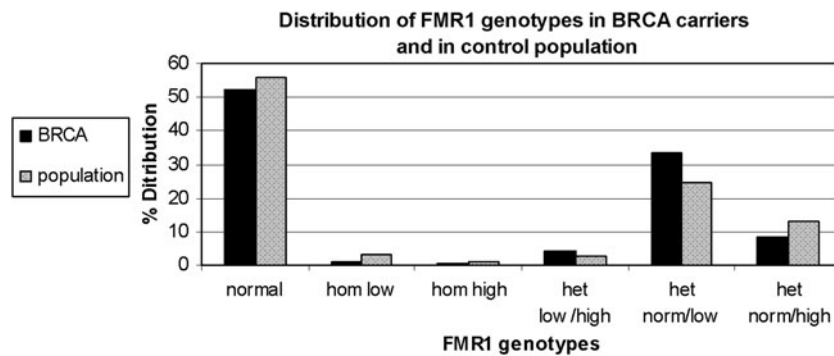
Fig. 1. FMR1 *genotypes and sub-genotypes distribution in* BRCA1/2 *carriers (black bars) and the control population (grey bars).*

Table 2. *FMR1 allelic distribution in BRCA1/2 carriers and the control population*

	First allele				Second allele			
	BRCA		Population		BRCA		Population	
CGGn allele count	N	%	N	%	N	%	N	%
Low (n < 26)	73	38·8	4745	30·20	2	1·1	473	3·01
Normal (26 < n < 34)	114	60·6	10 832	68·95	161	85·6	12 645	80·5
High (34 < n < 55)	1	0·5	118	0·75	24	12·8	2251	14·3
Pre (55 < n < 200)*	0	0	9	0·05	1	0·5	329	2·09
Full (n > 200)	0	0	4	0·02	0	0	10	0·06

* Although the definition in Israel of permutation is currently 58 repeats, we elected to use the 55 repeats figure to facilitate comparison with the other studies.

Table 3. *Summary of the studies focusing on CGG FMR1 alleles and BRCA mutation carriers*

Reference	Ethnicity	BRCA1/2 (n)	Population (n)	P value
Gleicher *et al.* (2010 *b*)*	BRCA – Austrian and American population	99	410	Genotype p = 0·0001 Low allele p = 0·001 High allele p = 0·07
Toledano-Alhadef *et al.* (2001)	Dutch	60 females 29 males	325	No association *P* not mentioned
Petek *et al.* (1999)	Ashkenazi Jewish	125	368	Normal or high vs. at least one low allele p = 0·156
Ricci *et al.* (2014)	Italian	43	104	Genotype p = 0·078 median/mean low allele p = 0·997
Current study*	Israeli Jewish	188	15 708	Genotype p = 0·018 Low allele p = 0·095 High allele p = 0·036

* Statistical analyses were performed using the same definitions and analysis tools.

for the inconsistent results, as previous studies reported a similar *FMR1* CGG repeat length in both fertile and infertile women of diverse ethnicities (Toledano-Alhadef *et al.*, 2001; Berkenstadt *et al.*, 2007; Gleicher *et al.*, 2010 *b*; Gleicher *et al.*, 2011).

Unique ovarian-reproductive phenotypes have previously been reported in *BRCA1* and *BRCA2* mutation carriers, in addition to ovarian cancer the following have been observed: an increased risk for recurrent miscarriages (Gal *et al.*, 2004), a skewed ratio of male to female offspring (de la Hoya *et al.*, 2003). The mechanisms for these noncancerous phenotypes and the pathways that underlie them are presently unclear. It is possible that the unique *FMR1* CGG allele size, as reflected by the different allelic distribution between cases and controls in our study,

plays a role in these additional phenotypes, but currently this possibility remains speculative at best.

The data presented also make it unlikely that the *FMR1* CGG repeat length size plays a significant role in affecting breast cancer penetrance in *BRCA1* or *BRCA2* mutation carriers, as the allele distribution was similar in breast cancer affected women and in ethnically matched cancer-free mutation carriers. This is further emphasized by the results of Gleicher and co-workers (2014) in *BRCA* carriers diagnosed with ovarian cancer, displaying a normal range of *FMR1* CGG alleles. It is noteworthy that the present study and all previous studies are underpowered to detect any subtle effect of the *FMR1* CGG genotype on *BRCA1/BRCA2* penetrance.

In conclusion, the CGG trinucleotide repeat in the *FMR1* gene in Jewish Israeli women, predominantly of Ashkenazi origin, shows a distinct distribution among *BRCA1/BRCA2* mutation carriers compared with the general population, but these differences are unlikely to account for the common phenotype of premature ovarian failure.

Declaration of interest

None.

References

Berkenstadt, M., Ries-Levavi, L., Cuckle, H., Peleg, L. & Barkai, G. (2007). Preconceptional and prenatal screening for fragile X syndrome: experience with 40,000 tests. *Prenatal Diagnosis* **27**, 991–994.

Brandão, R. D., van Roozendaal, K., Tserpelis, D. & Blok, M. J. (2013). *FMR1* low sub-genotype does not rescue *BRCA1/2*-mutated human embryos and does not explain primary ovarian insufficiency among *BRCA1/2*-carriers. *Human Reproduction* **8**, 2308–2311.

Dagan, E., Cohen, Y., Mory, A., Adir, V., Borochowitz, Z., Raanani, H., Kurolap, A., Melikhan-Revzin, S., Meirow, D. & Gershoni-Baruch, R. (2014). *BRCA1/2* mutations and *FMR1* alleles are randomly distributed: a case control study. *European Journal of Human Genetics* **22**, 277–279.

de la Hoya, M., Fernández, J. M., Tosar, A., Godino, J., Sánchez de Abajo, A., Vidart, J. A., Pérez-Segura, P., Díaz-Rubio, E. & Caldés, T. (2003). Association between *BRCA1* mutations and ratio of female to male births in offspring of families with breast cancer, ovarian cancer, or both. *Journal of the American Medical Association* **290**, 929–931.

Gal, I., Sadetzki, S., Gershoni-Baruch, R., Oberman, B., Carp, H., Papa, M. Z., Diestelman-Menachem, T., Eisenberg-Barzilai, S. & Friedman, E. (2004). Offspring gender ratio and the rate of recurrent spontaneous miscarriages in Jewish women at high risk for breast/ovarian cancer. *American Journal of Human Genetics* **74**, 1270–1275.

Gleicher, N., McAlpine, J. N., Gilks, C. B., Kushnir, V. A., Lee, H. J., Wu, Y. G., Lazzaroni-Tealdi, E. & Barad, D. H. (2014). Absence of *BRCA/FMR1* correlations in women with ovarian cancers. *PLoS One* **9**, e102370.

Gleicher, N., Weghofer, A. & Barad, D. H. (2010 *a*). Ovarian reserve determinations suggest new function of *FMR1* (fragile X gene) in regulating ovarian ageing. *Reproductive Biomedicine Online* **20**, 768–775.

Gleicher, N., Weghofer, A., Lee, I. H. & Barad, D. H. (2010 *b*). *FMR1* genotype with autoimmunity-associated polycystic ovary-like phenotype and decreased pregnancy chance. *PLoS One* **5**, e15303.

Gleicher, N., Weghofer, A., Lee, I. H. & Barad, D. H. (2011). Association of FMR genotypes with *in vitro* fertilization (IVF) outcomes based on ethnicity/race. *PLoS One* **6**, e18781.

Laitman, Y., Simeonov, M., Herskovitz, L., Kushnir, A., Shimon-Paluch, S., Kaufman, B., Zidan, J. & Friedman, E. (2012). Recurrent germline mutations in *BRCA1* and *BRCA2* genes in high risk families in Israel. *Breast Cancer Research and Treatment* **133**, 1153–1157.

Oktay, K., Kim, J. Y., Barad, D. & Babayev, S. N. (2010). Association of *BRCA1* mutations with occult primary ovarian insufficiency: a possible explanation for the link between infertility and breast/ovarian cancer risks. *Journal of Clinical Oncology* **28**, 240–244.

Petek, E., Kroisel, P. M., Schuster, M., Zierler, H. & Wagner, K. (1999). Mosaicism in a fragile X male including a *de novo* deletion in the *FMR1* gene. *American Journal of Medical Genetics* **84**, 229–232.

Ricci, M. T., Pennese, L., Gismondi, V., Perfumo, C., Grasso, M., Gennaro, E., Bruzzi, P. & Varesco, L. (2014). The *FMR1* CGG repeat test is not a candidate prescreening tool for identifying women with a high probability of being carriers of BRCA mutations. *European Journal of Human Genetics* **2**, 280–282.

Toledano-Alhadef, H., Basel-Vanagaite, L., Magal, N., Davidov, B., Ehrlich, S., Drasinover, V., Taub, E., Halpern, G. J., Ginott, N. & Shohat, M. (2001). Fragile-X carrier screening and the prevalence of premutation and full-mutation carriers in Israel. *American Journal of Human Genetics* **69**, 351–360.

Weghofer, A., Tea, M. K., Barad, D. H., Kim, A., Singer, C. F., Wagner, K. & Gleicher, N. (2012). *BRCA1/2* mutations appear embryo-lethal unless rescued by low (CGG n < 26) *FMR1* sub-genotypes: explanation for the "BRCA paradox"? *PLoS One* **7**, e44753.

Willemsen, R., Levenga, J. & Oostra, B. A. (2011). CGG repeat in the *FMR1* gene: size matters. *Clinical Genetics* **80**, 214–225.

Wittenberger, M. D., Hagerman, R. J., Sherman, S. L., McConkie-Rosell, A., Welt, C. K., Rebar, R. W., Corrigan, E. C., Simpson, J. L. & Nelson, L. M. (2007). The *FMR1* premutation and reproduction. *Fertility and Sterility* **87**, 456–465.