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Founder mutations account for the majority of *BRCA1*-attributable hereditary breast/ovarian cancer cases in a population from Tuscany, Central Italy

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Abstract *Background* Germline mutations in the *BRCA1* and *BRCA2* tumour-suppressor genes predispose to early-onset breast and ovarian cancer. Although both genes display a highly heterogeneous mutation spectrum, a number of alterations recur in some populations. Only a limited number of founder mutations have been identified in the Italian population so far. *Objective* To investigate the spectrum of *BRCA1/BRCA2* mutations in a set of families originary from the Central–Eastern part of Tuscany and to ascertain the presence of founder effects. We also wanted to approximate the age of the most frequent *BRCA1* founder mutation. *Results* Overall, four distinct *BRCA1* mutations accounted for a large fraction (72.7%) of *BRCA1*-attributable hereditary breast/ovarian cancer in families originary from this area. We identified common

haplotypes for two newly recognised recurrent *BRCA1* mutations, c.3228_3229delAG and c.3285delA. The c.3228_3229delAG mutation was estimated to have originated about 129 generations ago. Interestingly, male breast cancer cases were present in 3 out of 11 families with the c.3228_3229delAG mutation. *Conclusions* The observation that a high proportion of families with *BRCA1* alterations from Central–Eastern Tuscany harbours a limited number of founder mutations can have significant impact on clinical management of at risk subjects from this area. In addition, the identification of a large set of families carrying an identical mutation that predisposes to breast and ovarian cancer provides unique opportunities to study the effect of other genetic and environmental factors on penetrance and disease phenotype.

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Introduction

Germline mutations in the *BRCA1* (MIM +113705) and *BRCA2* (MIM +600185) tumour-suppressor genes predispose to early-onset breast and ovarian cancer. Although many different pathogenic mutations have been identified throughout the whole coding sequence of both genes, a number of alterations recur in some ethnic groups. Well known examples of *BRCA1* and *BRCA2* founder mutations have been observed in Ashkenazi Jews, Icelanders, Norwegians, Swedes, Finns, French Canadians and Dutch (reviewed in [1]).

Compared to the above mentioned populations, the spectrum of molecular defects associated with genetic

diseases in countries with a long-standing history of ethnic admixture and migration, like Italy, is usually highly heterogeneous. Nonetheless, a few founder recurrent mutations in *BRCA1* [2–4] and *BRCA2* [5, 6] originated from different regions of Italy have been observed.

In our analysis of breast/ovarian cancer families from the Central–Eastern part of Tuscany, we found four recurrent *BRCA1* mutations, one of which was associated with a history of male breast cancer. Haplotype analysis of nine polymorphic markers flanking the *BRCA1* gene was performed for the two most frequent mutations identified. We found that both sequence variants were derived from common ancestors, and then estimated the number of generations elapsed since the origin of the most frequent mutation, c.3228_3229delAG. Overall, we demonstrate that the vast majority (72.7%) of *BRCA1*-attributable hereditary breast/ovarian cancer families that are originary from this part of Italy harbour a limited number of “regional” mutations.

Materials and methods

Subjects

Families were ascertained at the Medical Genetics Unit of the University of Florence and at the ISPO, Scientific Institute for Cancer Prevention of Tuscany, in the period 1999–2005. Pedigrees were reconstructed for at least three generations and demographic data were obtained to determine the geographic origin of both the paternal and the maternal family branches. Where possible, cancers were verified from cancer registries, hospital notes or death certificates. Probabilities of carrying a mutation in the *BRCA1* or *BRCA2* genes were estimated using the CaGene Software, derived from the BRCAPro model (URL: <http://astor.som.jhmi.edu/BayesMendel/brcapro.html>) [7]. Genetic testing was offered to affected probands with a calculated *BRCA* mutation probability >5%. A total of 104 unrelated probands from Florence and the surrounding provinces of Prato, Pistoia and Arezzo in Central/Eastern Tuscany, underwent *BRCA1/2* genetic testing. Two additional male breast cancer families from Tuscany, ascertained in a population-based study [8], were also included for haplotype analysis.

Blood samples from 18 nuclear cancer-free families, originary from Central–Eastern Tuscany, and from 15 non-carrier relatives belonging to Tuscan families segregating *BRCA1* recurrent mutations were used to estimate allele frequencies and control haplotypes. Informed consent was obtained from all study participants.

Mutation analysis

DNA was extracted by standard phenol–chloroform procedures. All coding exons and flanking intron sequences of *BRCA1* and *BRCA2* were analysed by denaturing high performance liquid chromatography (dHPLC) or direct sequencing. dHPLC was carried out on an automated instrument (Transgenomic Inc., San Jose, CA); optimal temperatures for heteroduplex detection were determined using WAVEMAKER software (Transgenomic). *BRCA1* exons 9, 11, 13, 16 and *BRCA2* exon 22 were directly investigated by sequencing because of their high content of polymorphic variants. DNA sequencing was carried out on an ABI 310 DNA analyser (Applied Biosystems) using the ABI PRISM BigDye terminators cycle sequencing kit (Applied Biosystems).

Mutation nomenclature follows the Human Genome Variation Society (URL: <http://www.hgvs.org/mutnomen/>) recommendations. The DNA mutation numbering is based on the *BRCA1* and *BRCA2* cDNA sequences (reference sequences: NM_007296.2 and NM_000059.3, respectively) with the A of the ATG translation-initiation codon numbered as +1. Amino acid numbering starts with the translation initiator methionine as +1.

Microsatellite analysis

Thirty-eight individuals belonging to 14 families segregating two recurrent *BRCA1* mutations, c.3228_3229delAG ($n = 11$) and c.3285delA ($n = 3$) and 54 controls, were genotyped at nine short tandem repeat markers located within or adjacent to *BRCA1*. The following microsatellites, listed in order from centromere to telomere, were analyzed: *D17S1872*, *D17S250*, *D17S800*, *D17S1328*, *D17S1323*, *D17S1325*, *D17S902*, *D17S806* and *D17S588* (Fig. 1). PCR primer sequences were obtained from the Genome Database (URL: <http://www.gdb.org>). Forward primers were labelled with 6-FAM or TET fluorescent dyes and PCR products were analysed on a model 310 automated sequencer (Applied Biosystems). Product size determination was assessed with Genotyper (version 2.1) software (Applied Biosystems).

Haplotyping and estimate of mutation age

Haplotypes were constructed manually, assuming the least number of possible recombinations. Mutation age estimate was carried out with the DMLE+2.2 software program (URL: <http://www.dmle.org>) [9].

The method implemented in the DMLE+2.2 software is an extension of the Bayesian LD mapping method of Rannala and Reeve [10] that was initially designed for high-resolution mapping of a disease mutation based on the

Fig. 1 Haplotype branching trees derived from haplotype analysis. The most common haplotype is indicated in *bold* numbers. The markers used are shown together with their position in the Marshfield genetic map. Family haplotypes are indicated with the corresponding

family number (in *bold* and *italics*). **a** Haplotypes of families segregating *BRCA1* c.3228_3229delAG. **b** Haplotypes of families segregating *BRCA1* c.3285delA

observed linkage disequilibrium between the mutation and linked markers in DNA samples of disease chromosomes. It uses the Markov chain Monte Carlo methods to generate the marginal posterior probability density of mutation age, based on the following parameters: observed haplotypes or genotypes in normal and affected chromosomes, map distances between markers and mutation site, fraction of mutated chromosomes sampled, and estimated population growth rate.

We used haplotypes for the nine 17q21 microsatellite markers in affected and unaffected chromosomes, with the following parameters: (a) chromosome map distances derived from the Marshfield and/or Genéthon sex-average genetic maps (URL: <http://www.ncbi.nlm.nih.gov/mapview/>) and by Bergman et al. [11]; (b) population growth rate: 0.054; (c) proportion of mutation-carrying chromosomes sampled: 0.00698.

The population growth rate (r) was estimated from the equation: $N = N_0 e^{gr}$, in which N is the estimated present population size, N_0 is the estimated size of the population at reference time and g is the number of generations between these two time points. The population of the city of Florence currently comprises about 330,000 people. Historical and demographic data indicate that it included about 15,000 people in year 1200 [12] and 59,500 in year 1550; accordingly, the average growth rate of this population was estimated to be 0.085 between years 1200 and present time and 0.119 from 1550 to the present time, assuming 25 years/generation. There are no data regarding the population of the entire region of Tuscany before year

1550; in that year it was estimated to include about 912,000 individuals [12] and now comprises about 3,619,000 inhabitants; the approximate growth rate per generation is therefore 0.076. We then calculated the ratio between the growth rates of the city of Florence (0.119) and of the entire region, inclusive of rural areas (0.076), for the period 1550 to present. The value thus obtained, 1.57, was used to estimate the growth rate since 1200 of the whole region, based on the urban data only (0.085) for this period. We thus calculated a value of $r = 0.054 = (0.085 \times 1/1.57)$, which was subsequently used for mutation age estimates.

The proportion of mutation-carrying chromosomes sampled was estimated from a population of 1,175,000 inhabitants in Florence and the surrounding provinces of Prato and Arezzo in Central–Eastern Tuscany assuming a prevalence of *BRCA1* mutation carriers of 0.014 (1:714). The latter represents the median value calculated from a meta-analysis of prevalence data available for populations of European ancestry [13–17], since estimates of *BRCA1* mutation prevalence are not available for the Italian population.

Results

Mutation analysis and phenotypic characteristics

Germline deleterious *BRCA1* and *BRCA2* mutations were identified in 22 and 13 out of 104 probands from Central–Eastern Tuscany, respectively. We found ten different

Table 1 *BRCA1* and *BRCA2* deleterious mutations in high risk families from Tuscany

Gene	Description of mutation	Effect of mutation	Number of families with the same mutation	BIC nomenclature ^a	Number of times recorded in BIC
<i>BRCA1</i>	c.3228_3229delAG	p.Arg1076fs	9	3347_3348delAG	24
	c.3285delA	p.Lys1095fs	3	3404delA	0
	c.1377_1378insA	p.Ile460fs	2	1499insA	12
	c.5062_5064delTGT	p.Val1688del ^b	2	5181_5183delTGT	6
	c.182_183delTG	p.Cys61fs	1	301_302delGT	0
	c.2155_2168del14	p.Lys719fs	1	2274_2287del14	0
	c.2296_2297delAG	p.Ser766fs	1	2415_2416delAG	3
	c.2405_2406delTG	p.Val802fs	1	2524_2525delTG	5
	c.4327C>T	p.Arg1443Stop	1	4446C>T	126
	c.5251C>T	p.Arg1751Stop	1	5370C>T	29
<i>BRCA2</i>	c.9117G>A	r.8954_9117del (Exon 23 skipping) ^c	2	9345G>A	14
	c.289G>T	p.Glu97Stop	1	517G>T	0
	c.632-2A>G	r.517_631del (Exon 7 skipping)	1	IVS7-2A>G	0
	c.2950G>T	p.Glu984Stop	1	3178G>T	0
	c.3860delA	p.Asn1287fs	1	4088delA	2
	c.5086_5087insA	p.Gly1696fs	1	5314insA	0
	c.5851_5854delAGTT	p.Ser1951fs	1	6079_6082delAGTT	11
	6468_6469delTC	p.Ser2156fs	1	6696_6697delTC	22
	c.7707delT	p.Gly2569fs	1	7935delT	0
	c.7963C>T	p.Gln2655Stop	1	8191C>T	0
	c.8878C>T	p.Gln2960Stop	1	9106C>T	10
	c.9501+3A>T	r.9257_9501del (Exon 25 skipping) ^d	1	IVS25+3A>T	15

^a BIC: Breast Cancer Information Core; note that sequence variants are designated differently in this study (for which the nomenclature recommendations of the Human Genome Variation Society were used) and in BIC (<http://research.nhgri.nih.gov/bic/>)

^b This variant is considered to be deleterious based on functional studies [4]

^c This variant is considered to be deleterious based on RNA studies [18]

^d This variant is considered to be deleterious based on RNA studies (this study; data not shown)

BRCA1 and twelve different *BRCA2* germline mutations (Table 1). Four of the ten *BRCA1* mutations and one of the twelve *BRCA2* mutations were observed in more than one family. The *BRCA1* c.3228_3229delAG and c.3285delA mutations were observed in nine and three families, respectively. Two additional *BRCA1* mutations, c.1377_1378insA and c.5062_5064delTGT and one *BRCA2* variant, c.9117G>A, were observed in two families each.

Haplotype analysis

Three families with the c.3285delA mutation and 11 families carrying the c.3228_3229delAG, as well as 18 nuclear families used as controls, were investigated.

Haplotype analysis using polymorphic markers spanning the *BRCA1* locus was performed on probands and, when possible, on additional family members. Two to seven family members were genotyped in 8 of the 11 families segregating the c.3228_3229delAG and in 1 of the 3

families with the c.3285delA. In the remaining families only the index case was available for genotyping.

Two distinct haplotypes were associated with each of the two recurrent *BRCA1* mutations, c.3228_3229delAG and c.3285delA (Fig. 1). These haplotypes were absent in non-carriers from the families segregating the two mutations and were not found in control chromosomes. In addition, in all families with only one individual available for analysis, the observed genotypes were compatible with the shared haplotypes identified in families with the same mutation.

Age estimate of the *BRCA1* c.3228_3229delAG mutation

The DMLE+2 program was used to estimate the age of the c.3228_3229delAG mutation based on haplotype data from patients and controls. Using the median prevalence of 1:714 to estimate the number of mutation-carrying chromosomes sampled and considering a growth rate of 0.054,

Fig. 2 Plot of the posterior probability densities of mutation age expressed in number of generations estimated with DMLE software. Broken lines define the boundaries of the 95% confidence interval

the mutation age was estimated to be ~ 129 generations (95% credible set: 88–184) (Fig. 2).

Clinical characteristics associated with the *BRCA1* c.3228_3229delAG mutation

Ovarian cancer was recorded in 8 of the 11 families exhibiting the *BRCA1* c.3228_3229delAG mutation (Table 2). The age at first BC diagnosis ranged between 30 and 82 years (median 43.5 years). The age at first OC diagnosis ranged between 30 and 68 years (median

47.0 years). Interestingly, male BC cases were present in 3/11 families with the *BRCA1* c.3228_3229delAG mutation.

Discussion

In the present study, we have investigated high risk breast–ovarian cancer families referred to a single breast cancer family clinic of Central Italy in the period 1999–2005. The majority of the families investigated (104/185; 56.2%) originate from Florence and the surrounding provinces of Prato, Arezzo and Pistoia (Fig. 3). Of the 104 probands of Tuscan origin, 35 (35.65%) were heterozygotes for a *BRCA1/BRCA2* deleterious mutation. Although the *BRCA1/BRCA2* mutation spectrum was highly variable, two mutations, c.3228_3229delAG and c.3285delA, were present in 57% of all *BRCA1*-attributable families. The *BRCA1* c.3285delA has never been reported in the Breast Cancer Information Core (BIC) (URL: <http://research.nhgri.nih.gov/bic/>) and, to the best of our knowledge, in Italy. Two additional *BRCA1* mutations, c.1377_1378insA and c.5062_5064delTGT, were detected in two unrelated families each in our series. Both of these have been shown to be Italian founder mutations, originating from Western Tuscany [3] and North-Eastern Italy [4], respectively. Therefore, overall recurrent founder mutations accounted for 72.7% of *BRCA1* mutations in our series.

The *BRCA2* mutation spectrum was more variable. We found two unrelated families carrying the same *BRCA2* c.9345G>A mutation causing exon 23 skipping, already described in another patient from Pisa, Tuscany [18]. In

Table 2 Families with the *BRCA1* c.3228_3229delAG mutation

Family ID	Sex of proband	Phenotype and age at diagnosis ^b	Family history of breast and ovarian cancers and age of diagnosis ^c	Family history of other cancers ^d
F11	Female	BC42+44	M-BC39, MA-BC?	Colon
F41	Female	BC34	S-OC41, M-OC56, MA-OC68, MGM-OC43	Gastric, leukaemia
F61	Female	BC57	M-BC82, MA-BC45, MA-BC60	Colon, brain, pancreas
F74	Female	OC52, BC54	M-OC56	Cerebellar
F114	Female	OC42	MA-OC66	Gyn?
F187	Female	BC43	S-OC44, F-BC40, PGM-BC71, PGGM-BC42	Gastric, colon, lung
F188	Female	BC42	S-BC47, S-BC52, S-OC45, N-BC37, N-BC37	None
F223	Female	OC44	S-BC41, M-BC30, MA-OC50, MGM-OC49	None
F263	Female	OC53	M-OC43	Gyn?
F5 ^a	Male	BC60	S-BC45, S-BC41+OC41, PA-OC30, PC-OC57, PC-BC64	Leukaemia
F56 ^a	Male	BC55	MC-BC31, PA-BC70	Colon, prostate, stomach, liver

^a Families ascertained in a population-based study of male breast cancer (Ottini personal communication)

^b BC: breast cancer; OC: ovarian cancer

^c M: mother; F: father; S: sister; N: niece; PGM: paternal grandmother; PGGM: paternal grand-grandmother; MA: maternal aunt; MC: maternal cousin; MGM: maternal grandmother; PA: paternal aunt; PC: paternal cousin

^d Gyn?: undefined gynaecological cancer

Fig. 3 Geographic distribution (province of residence) of the families segregating founder *BRCA1* mutations in Tuscany. Each family is represented by the symbol corresponding to the observed mutation

addition, the *BRCA2* c.6468_6469delTC frameshift mutation, identified in a single family in this study, has previously been described in an unrelated male breast cancer patient from Florence [19].

The most frequent *BRCA1* germline mutations identified in our sample are c.3228_3229delAG and c.3285delA. Haplotype analysis of nine microsatellite markers, covering a region of ~9.14 Mb surrounding *BRCA1* on 17q21, was conducted on 14 families and the results obtained clearly indicate that both mutations can be traced back to common ancestors.

We then used DMLE+2.2 software to estimate the age of the *BRCA1* c.3228_3229delAG; the c.3285delA mutation was not investigated because the number of families in which it was identified was considered inadequate to estimate its age.

The estimate of the number of generations since the origin of the *BRCA1* c.3228_3229delAG mutation was 129 (95% credible set: 88–184) generations, corresponding to ~3,225 years (95% confidence intervals: 2200–4600). Therefore its origin in Tuscany should date back to the Villanovian and Etruscan periods, indicating that it is one of the oldest founder mutations so far described in breast-ovarian cancer predisposing genes (Table 3). However, it is worth nothing that the DMLE+2.2 method estimates the time of origin of the mutation, which may be older than that of the most recent common ancestor estimated by the other methods used in the studies reported in Table 3 [10, 20]. In addition, DMLE+2.2 estimates are very sensitive to the population growth rate; i.e. using a growth rate of 0.085, calculated only on the Florentine population, the

estimated number of generations since the origin of the mutation was 92 (95% credible set: 65–127).

Interestingly, male breast cancer was present in three out of the eleven families segregating the *BRCA1* c.3228_3229delAG mutation. It is well known that male breast carcinoma is associated with deleterious germline mutations in *BRCA2*, and the estimated cumulative risk of breast carcinoma by age 70 years is 6.3% [21] to 6.8% [22] among *BRCA2* mutation male heterozygotes. However, the relationship between male breast carcinoma and deleterious germline mutations in *BRCA1* is not as well established. *BRCA1* mutations have been detected at very low frequency in series of unselected male breast cancer patients [19, 23–25]. On the other hand, a lifetime risk of male breast cancer of 5.8% in *BRCA1* mutation carriers has been calculated, but this estimate was based on data from high-risk families and was not adjusted for ascertainment [26]. More recently, the cumulative risk of breast cancer by age 70 years in *BRCA1* male carriers was estimated at 1.2% in a very large US cohort of *BRCA1/BRCA2* families [22]. The high number of *BRCA1* c.3228_3229delAG families with male breast cancer cases might be a specific phenotypic effect of the mutation itself; however, there are 24 previous reports of *BRCA1* c.3228_3229delAG in BIC and, to our knowledge, no association with male breast cancer has been pointed out. Therefore, it is possible that the genetic background of Tuscan male carriers influences phenotypic expression of this *BRCA1* variant. The presence of mutations in other breast cancer predisposing genes or in modifier genes might increase the risk of breast cancer among male heterozygotes for this specific mutation.

Table 3 Age estimates for *BRCA1* and *BRCA2* founder mutations

Gene	Mutation according to BIC nomenclature (number of families)	Mutation age in generations (confidence interval)	Country or population	Reference
<i>BRCA1</i>	3347_3348delAG (11)	129 (88–183)	Tuscany, Italy	Present study
	1499insA (14)	30 (22–56)	Tuscany, Italy	[3]
	G1738R	11 (4–24)	Greece	[27]
	3171ins5 (18)	50	South Sweden	[11]
	3744delT (8)	23–36	Finland	[28]
	4216-2A>G (9)	<10	Finland	[28]
	2804delAA (19)	32 (15–49)	Netherlands	[29]
	185delAG (17)	46 (23–80)	Ashkenazi Jews	[30]
	4184del4 (6)	170 (70–350)		[30]
	5382insC (19)	38 (18–68)	European, mixed	[30]
	Intron 5 splice (5)	75 (19–165)		[30]
	1294del40 (6)	9 (1–45)		[30]
<i>BRCA2</i>	156-157insAlu (14)	120–130	Portugal	[31]
	9346-2A>G (9)	7–11	Finland	[28]
	7708C>T (8)	10–20	Finland	[28]
	8555T>G (5)	7–9	Finland	[28]

The observation that a high proportion of families with *BRCA1* alterations from central–eastern Tuscany harbours a limited number of founder mutations has significant impact on clinical management of at risk subjects. Direct screening for these mutations could be undertaken as a first step for molecular analysis, at least in individuals of ascertained Tuscan ancestry. The identification of a large set of families carrying an identical mutation that predisposes to breast and ovarian cancer will provide unique opportunities to study the effect of other genetic and environmental factors on penetrance and disease phenotype. Should an increased risk of male breast cancer associated with the c.3228_3229delAG sequence variant be confirmed, it would be appropriate to consider offering appropriate surveillance to male carriers of this mutation.

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