SUPPLEMENTARY INFORMATION for Pölsler et al, Eur J Hum Genet

Supplementary Materials and Methods
Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplemental references

SUPPLEMENTARY MATERIALS AND METHODS

Patients

An interdisciplinary network for HBOC counselling, testing, and management was established in the Tyrol in 2009. Procedures are based on international guidelines[1] adopted by the Tyrolean Working Group for Oncology.[2] Between January 2009 and February 2013, index individuals from 130 seemingly unrelated families fulfilled the indication criteria for *BRCA1* and *BRCA2* genetic analysis and requested testing after genetic counselling. In addition, molecular studies were performed in 108 apparently independent index individuals who were referred to the Centre for Medical Genetics, Medical University Innsbruck, from other, mainly Western Austrian hospitals and clinics. Together these individuals form the *diagnostic cohort* of 238 families in this report.

Presence of *BRCA1* stop mutation c.4183C>T, p.(Gln1395Ter), was investigated in tumour samples from a *research cohort* of unselected Tyrolean individuals with breast cancer (n = 471) and ovarian cancer (n = 186). Samples had been collected prospectively in the biobank of the Department of Obstetrics and Gynaecology, Medical University Innsbruck, between January 2000 and July 2012. Two breast and two ovarian cancer samples were later excluded because of insufficient DNA quality. Information linked to the samples included the patients' home area code as well as clinical and pathological data on their tumours. Staging in ovarian cancer patients was performed in accordance with the International Federation of Gynaecology and Obstetrics (FIGO) classification system. Samples from mucinous ovarian cancers were excluded because *BRCA1*-associated ovarian cancers with this histology are rare.[3] Breast and ovarian cancer samples were selected to fall each into two matching sub-groups based on patients' home address: One sub-group comprised 219 breast and 59 ovarian cancer patients from the Lower Inn Valley (LIV) region (districts Schwaz and Kufstein). The other sub-group included 250 breast and 125 ovarian cancer patients from other districts of the North Tyrol (districts Landeck, Reutte, Imst, Innsbruck-Land,

Innsbruck and Kitzbühel). Numbers of analysed cases according to districts can be seen in Figure 1 A & B. Patients in the two sub-groups were matched for patient age, sample age, tumour histology, and (for breast cancers) tumour size (Supplementary Table S1 A & B).

Geographic region-specific incidence analysis included all breast and ovarian cancer cases diagnosed in the Tyrol between 2001 and 2010 affecting female patients below 50 years (breast cancer) and 65 years (ovarian cancer), respectively.

DNA Isolation

For comprehensive *BRCA1* and *BRCA2* genetic analysis in the diagnostic cohort, genomic DNA was extracted with standard procedures from peripheral blood lymphocytes.

For molecular studies in tumour samples, DNA was isolated from formalin fixed paraffin embedded (FFPE) tumour specimens using the DNeasy Blood and Tissue Kit according to manufacturer's instructions (Qiagen, Hilden, Germany).

BRCA1 genetic analysis

In the diagnostic cohort all coding exons including (where possible) at least 50 nucleotides of the flanking intron sequences were PCR-amplified. Primer sequences and amplification conditions for the 24 *BRCA1* and 27 *BRCA2* exons are available upon request. Subsequent bidirectional Sanger sequencing was performed using BigDye Terminator Cycle Sequencing chemistry (Applied Biosystems (ABI), Foster City, CA). Sequencing reactions were visualized on an automated capillary sequencer (ABI DNA analyser 3730) and analysed using SequencePilot (JSI Medical Systems, Kippenheim, Germany). Individuals in whom a mutation in one of the genes was identified were not further analysed. For all other individuals, MLPA analysis was subsequently applied to detect potential copy number changes, i.e. single and multiple exon deletions and duplications, using commercially available kits (SALSA P002 (*BRCA1*) and P045 (*BRCA2*), MRC-Holland, Amsterdam, The Netherlands). From January 2012 onward, index patients from the Lower Inn Valley region were first tested for *BRCA1* mutation c.4183C>T, and no further analyses were carried out if this mutation was detected.

All identified *BRCA1* and *BRCA2* mutations and associated phenotypes were submitted to the ClinVar and LOVD databases (www.ncbi.nlm.nih.gov/clinvar, study name BRCA-Tyrol, and http://databases.lovd.nl/shared/variants/BRCA1, study name Pölsler et al. 2015). Mutations are named according to HGVS nomenclature using the reference transcript sequences NM_007294.2 for *BRCA1* and NM_000059.3 for *BRCA2* with the A of the start codon ATG being position c.1. Exons are numbered according to the most widely used numbering system where exons are named with consecutive numbers

(without letter extensions) and the start codons of *BRCA1* and *BRCA2* are each located in the second exon (exon 2) of the respective transcript. According to this nomenclature the *BRCA1* reference transcript is lacking an exon 4. Hence, the coding sequences are contained in the *BRCA1* exons 2, 3 and 5-24 and *BRCA2* exons 2-27.

Haplotypes associated with the *BRCA1* mutation c.4183C>T in carrier families were deduced from the genotype of four single nucleotide variants (SNV) c.2082C>T, c.2311T>C, c.2612C>T, and c.3113A>G, and the three intragenic polymorphic microsatellite repeats D17S1323, D17S1322, and D17S855. The SNVs c.2082T, c.2311C, c.2612T, and c.3113G belong to the haplotype B described by Frosk *et al.* [4] SNV genotypes were obtained through diagnostic or targeted sequence analyses. Primer pairs used to PCR amplify the three microsatellite repeat markers are given in Supplementary Table S2 A. Fragment analysis was conducted on an ABI Genetic analyser 3130xl and evaluated using the program GeneMarker from SoftGenetics LLC (State College, PA, USA).

In the research cohort *BRCA1* stop mutation c.4183C>T was tested with the TaqMan allelic discrimination technique on an ABI PRISM 7000 instrument according to manufacturer's instructions using 10ng of template DNA (Assay Nr. 5016693; ID 1266982). Mutation-positive samples were sequenced to confirm the presence of the mutation and to determine the SNV haplotypes; PCR-primers used to generate small amplicons from FFPE-tissue-extracted DNA that contain the *BRCA1* mutation c.4183C>T as well as the four SNV defining the B-allele are provided in Supplementary Table S2 B.

Statistical analyses

For testing associations between clinicopathological features and *BRCA1* mutation status the Chi-square test and between age and mutation status the Mann-Whitney-U-Test were used.

A possible influence of *BRCA1* mutation c.4183C>T prevalence on geographic region-specific breast and ovarian cancer incidence was evaluated using clinical and residential information from the Tyrolean Cancer Registry.[5] Incidence data have been previously published.[6] The expected number of cases was calculated given the incidence risk in the North Tyrol excluding the Lower Inn Valley as a standard and adjusted for age. The Lower Inn Valley region was further subdivided into the Zillertal (a major side valley, denoted LIV-Z) and the main Lower Inn Valley (denoted LIV-M). Age-adjusted standardized incidence ratio (SIR) was defined in the classical way as observed cases divided by expected cases. Confidence intervals were computed using an exact method.[7] The cumulative risk (CUM risk) for being diagnosed with cancer was computed following accepted methods in cancer epidemiology.[8]

Supplementary Table S1:

Clinicopathological features of research cohort patients.

(A) Breast cancer patients, (B) ovarian cancer patients.

A

	All analyzed breast cancer patients	
	Lower Inn Valley (Schwaz, Kufstein) (n = 219)	**Remaining Tyrol** (n = 250)
Patient age at diagnosis (years)		
Median	56.2	55.7
Range	22.3 - 84.0	24.2 - 82.3
Sample age (years)		
Median	4.8	5.0
Range	1.4 - 11.1	1.1 - 11.3
Tumour size		
T1	157	183
T2	53	58
T3	3	3
T4	5	6
unknown	1	0
LN		
neg	126	162
pos	89	85
unknown	4	3
Tumour grade		
I	28	29
II	164	181
III	26	38
unknown	1	2
Histology		
invasive lobular carcinoma	1	1
invasive ductal carcinoma	158	178
mixed: invasive ductal carcinoma and DCIS	55	59
medullary carcinoma	5	12
MP		
premenopausal	81	91
postmenopausal	137	157
unknown	1	2

HER2		
neg	189	218
pos	28	32
unknown	2	0
ER		
neg	42	51
pos	177	198
unknown	0	1
PR		
neg	53	53
pos	166	195
unknown	0	2
HER2-ER-PR		
neg (Triple negative tumours)	27	31
pos	192	218
unknown	0	1

B

	All analyzed ovarian cancer patients	
	Lower Inn Valley (Schwaz, Kufstein) (n = 59)	**Remaining Tyrol** (n = 125)
Patient age at diagnosis (years)		
Median	59.1	66.7
Range	39.5 - 84.1	(30.8 - 90.2)
Sample age (years)		
Median	8.1	8.1
Range	1.4 - 13.9	1.4 - 13.9
FIGO		
I	10	19
II	2	8
III	40	74
IV	7	23
unknown	0	1
Tumour grade		
I	9	13
II	23	48
III	25	54
unknown	2	10
Histology		
serous cystadeno CA	49	101
endometroid CA	9	22
clear cell	1	2
Remaining tumour		
no tumour left	30	70
tumour rest < 2c	13	14
tumour rest > 2c	13	33
unknown	3	8
Metastases at diagnosis		
no	52	102
yes	7	23

Supplementary Table S2. Primer sequences.

(A) For Microsatellite markers D17S855, D17S1322, and D17S1323; forward primers labelled with fluorescent dye FAM.

(B) B: To generate small amplicons from FFPE-tissue-extracted DNA that contain the BRCA1 mutation c.4183C>T as well as the four SNP variants defining the B-allele (underlined: M13 Sequence).

A		**Primer Name**	**Primer Sequence**	**f/r**
	D17S855	D17S855_f_fam	CACACAGACTTGTCCTACTGCC	fwd
		D17S855_r	CCACTTTCTAAAAGGCCATCC	rev
	D17S1322	D17S1322_f_fam	GAAGCAGGAATGGAACCAG	fwd
		D17S1322_r	CAGGCTAGAGTGCATGGAGT	rev
	D17S1323	D17S1323_f_fam	TCTGTTCCCGATAGGAGATGG	fwd
		D17S1323_r	CACTCATTGCAAAGTTCCTTCA	rev

B		**Primer Name**	**Primer Sequence**	**f/r**
	***BRCA1* mutation c.4183C>T**	BRCA1-Mut-fw	GTAAAACGACGGCCAGTAGGGCTATCCTCTCAGAGTGACA	fwd
		BRCA1-Mut-rv	CAGGAAACAGCTATGACGAATGCAAAGGACACCACACA	rev
	SNP *BRCA1* c.2082T	BRCA1-SNP1-fw	GTAAAACGACGGCCAGTGAGTAACAAGCCAAATGAACA	fwd
		BRCA1-SNP1-rv	CAGGAAACAGCTATGACGAACCAGGTGCATTTGTTAACT	rev
	SNP *BRCA1* c.2311C	BRCA1-SNP2-fw	GTAAAACGACGGCCAGTGCAAACTGAAAGATCTGTAGAG	fwd
		BRCA1-SNP2-rv	CAGGAAACAGCTATGACACTTCCAGTAACGAGATACTTT	rev
	SNP *BRCA1* c.2612T	BRCA1-SNP3-fw	GTAAAACGACGGCCAGTTTTGCAGAATACATTCAAGGTT	fwd
		BRCA1-SNP3-rv	CAGGAAACAGCTATGACGGCAGAGAATGTTGCACATT	rev
	SNP *BRCA1* c.3113G	BRCA1-SNP4-fw	GTAAAACGACGGCCAGTTGAGCACAATTAGCCGTAATA	fwd
		BRCA1-SNP4-rv	CAGGAAACAGCTATGACTTCATTAGTACTGGAACCTACTTC	rev

Supplementary Table S3: Mutation spectrum in Western Austria. (A) BRCA1 mutations, (B) BRCA2 mutations.

A

BRCA1	**Exon**	**Mutation name**	**Protein code**	**No. of patients in BIC database**	**Prevalence (%) at MUI**
1	11	c.676delT	p.(Cys226ValfsTer8)	16	1
2	11	c.1687C>T	p.(Gln563Ter)	94	3
3	11	c.1874_1877dup	p.(Val627SerfsTer4)	14	1
4	11	c.1961delA	p.(Lys654SerfsTer47)	39	1
5	11	c.2197_2201delGAGAA	p.(Glu733ThrfsTer5)	5	1
6	11	c.3018_3021delTTCA	p.(His1006GlnfsTer17)	7	7 (15,2%)
7	11	c.3398T>G	p.(Leu1133Ter)	0	2
8	11	c.3481_3491delGAAGATACTAG	p.(Glu1161PhefsTer3)	64	1
9	**11**	**c.3511A>T**	**p.(Lys1171Ter)**	**novel**	**2**
10	11	c.3756_3759delGTCT	p.(Ser1253ArgfsTer10)	124	3
11	12	c.4113delG	p.(Cys1372ValfsTer21)	2	1
12	12	c.4183C>T	p.(Gln1395Ter)	28	14 (30,4%)
13	17	c.4998C>A	p.(Tyr1666Ter)	0	1
14	17	c.5057A>G	p.(His1686Arg) [$]	0	1
15	18	c.5096G>A	p.(Arg1699Gln)*	11	1
16	18	c.5123C>A	p.(Ala1708Glu)	46	1
17	**19**	**c.5167delAinsTTT**	**p.(Ile1723PhefsTer8)**	**novel**	**1**
18	20	c.5194-?_5277+?del (exon 20 del)	p.His1732_Lys1759del (r.5194_5277del84)	1	1
19	20	c.5251C>T	p.(Arg1751Ter)	44	1
20	20	c.5266dupC	p.(Gln1756ProfsTer74)	>1000	1
21	22	c.5346G>A	p.(Trp1782Ter)	13	1
					total 46

B

BRCA2	**Exon/ Intron**	**Mutation name**	**Protein/transcript code**	**No. of patients in BIC database**	**Prevalence (%) at MUI**
1	**9**	**c.712G>T**	**p.(Glu238Ter)**	**novel**	**1**
2	10	c.1813dupA	p.(Ile605AsnfsTer11)	75	1
3	11	c.2808_2811delACAA	p.(Ala938ProfsTer21)	109	1
4	11	c.3172A>T	p.(Lys1058Ter)	0	1
5	11	c.3283C>T	p.(Gln1095Ter)	0	1
6	11	c.3545_3546delTT	p.(Phe1182Ter)	14	1
7	11	c.3847_3848delGT	p.(Val1283LysfsTer2)	64	1
8	11	c.3915delT	p.(Phe1305LeufsTer30)	0	1
9	11	c.5303_5304delTT	p.(Leu1768ArgfsTer5)	5	1
10	**11**	**c.5390delC**	**p.(Ala1797GlufsTer8)**	**novel**	**1**
11	11	c.5645C>A	p.(Ser1882Ter)	27	1
12	11	c.5909C>A	p.(Ser1970Ter)	11	1
13	**11**	**c.5952dupA**	**p.(Ser1985IlefsTer18)**	**novel**	**1**
14	11	c.6202dupA	p.(Ile2068AsnfsTer10)	1	1
15	11	c.6405_6409delCTTAA	p.(Asn2135LysfsTer3)	13	1
16	**14**	**c.7266T>A**	**p.(Cys2422Ter)**	**novel**	**1**
17	17	c.7846delT	p.(Ser2616LeufsTer32)	0	1
18	17	c.7878G>C	p.(Trp2626Cys) [#]	13	1
19	19	c.8363G>A	p.(Trp2788Ter)	0	1
20	19	c.8486A>G	r.8332_8487del156 [§]	2	1
21	21	c.8755-1G>A	?	6	2
22	23	c.9117+1G>A	p.(Val2985GlyfsTer4)	1	1
23	25	c.9382C>T	p.(Arg3128Ter)	50	1
					total 24

Abbreviations:
BIC database, Breast Cancer Information Core mutation database; MUI, Medical University Innsbruck. Novel mutations are printed in bold.

[$]The *BRCA1* variant c.5057A>G, p.(His1686Arg) not recorded in the BIC database affects a highly conserved amino acid, segregates with premenopausal breast cancer in our patient family and was retained in tumour tissue from one affected individual that showed loss of heterozygosity (LOH) of the wild-type allele. Pathogenicity of this variant is also supported by its inability to functionally complement Brca1-deficient mouse embryonic stem cells.[9] * A full-length cDNA-based complementation assay shows that *BRCA1* variant c.5096G>A, p.(Arg1699Gln), did not restore homologous recombination activity and confers sensitivity to PARP inhibition in Brca1-deficient mouse embryonic stem cells.[9] Furthermore, family history and segregation analyses in a pooled family study strongly supported the clinical relevance of this variant and show that it is associated with an intermediate breast and ovarian cancer risk.[10] [#]The *BRCA2* variant c.7878G>C, p. (Trp2626Cys), is predicted to be deleterious with a likelihood ratio of 1:48.[11] It caused loss of function of the protein in in vitro studies.[12] [§]The *BRCA2* variant c.8486A>G led to skipping of exon 19 in transcripts of our own index patient as well as in another patient.[13]

SUPPLEMENTARY REFERENCES

1 Wockel A and Kreienberg R: First Revision of the German S3 Guideline 'Diagnosis, Therapy, and Follow-Up of Breast Cancer'. *Breast Care (Basel)* 2008; **3:** 82-86.

2 TAKO, Tiroler Arbeitskreis für Onkologie: Mammakarzinom. Empfehlungen zu Diagnostik, Therapie und Nachsorgeuntersuchungen in Tirol. 2010.

3 Evans DG, Young K, Bulman M, Shenton A, Wallace A, Lalloo F: Probability of BRCA1/2 mutation varies with ovarian histology: results from screening 442 ovarian cancer families. *Clin Genet* 2008; **73:** 338-345.

4 Frosk P, Burgess S, Dyck T, Jobse R, Spriggs EL: The use of ancestral haplotypes in the molecular diagnosis of familial breast cancer. *Genet Test* 2007; **11:** 208-215.

5 Oberaigner W and Siebert U: Are survival rates for Tyrol published in the Eurocare studies biased? *Acta Oncol* 2009; **48:** 984-991.

6 Forman D, Bray F, Brewster DH *et al* (eds.): Cancer Incidence in Five Continents, Vol. X (electronic version). Lyon, 2013. Available: http://ci5.iarc.fr.

7 Sasieni P: On the expected number of cancer deaths during follow-up of an initially cancer-free cohort. *Epidemiology* 2003; **14:** 108-110.

8 Jensen OM, Parkin DM, Maclennan R, Muir CS, Skeet RG (eds.): Cancer Registration: Principles and Methods. Lyon, 1991.

9 Bouwman P, van der Gulden H, van der Heijden I *et al*: A high-throughput functional complementation assay for classification of BRCA1 missense variants. *Cancer Discov* 2013; **3:** 1142-1155.

10 Spurdle AB, Whiley PJ, Thompson B *et al*: BRCA1 R1699Q variant displaying ambiguous functional abrogation confers intermediate breast and ovarian cancer risk. *J Med Genet* 2012; **49:** 525-532.

11 Easton DF, Deffenbaugh AM, Pruss D *et al*: A systematic genetic assessment of 1,433 sequence variants of unknown clinical significance in the BRCA1 and BRCA2 breast cancer-predisposition genes. *Am J Hum Genet* 2007; **81:** 873-883.

12 Biswas K, Das R, Alter BP *et al*: A comprehensive functional characterization of BRCA2 variants associated with Fanconi anemia using mouse ES cell-based assay. *Blood* 2011; **118:** 2430-2442.

13 Houdayer C, Caux-Moncoutier V, Krieger S *et al*: Guidelines for Splicing Analysis in Molecular Diagnosis Derived from a Set of 327 Combined In Silico/In Vitro Studies on BRCA1 and BRCA2 Variants. *Human Mutation* 2012; **33:** 1228-1238.