Chromosome 17q25 genes, *RHBDF2* and *CYGB*, in ovarian cancer

PAULINA M. WOJNAROWICZ[1], DIANE M. PROVENCHER[2-4], ANNE-MARIE MES-MASSON[2,5] and PATRICIA N. TONIN[1,6,7]

[1]Department of Human Genetics, McGill University; [2]Research Centre of the University of Montreal Hospital Centre (CRCHUM)/Montreal Cancer Institute; [3]Division of Gynecologic Oncology, University of Montreal; [4]Department of Obstetrics and Gynecology, University of Montreal; [5]Department of Medicine, University of Montreal; [6]The Research Institute of the McGill University Health Centre; [7]Department of Medicine, McGill University, Montreal, Quebec, Canada

Received November 15, 2011; Accepted December 29, 2011

DOI: 10.3892/ijo.2012.1371

Abstract. It has been proposed that the frequent loss of heterozygosity (LOH) of an entire chromosome 17 contig in epithelial ovarian cancers (EOC) is the consequence of the inactivation of multiple tumour suppressor genes on this chromosome. We report the characterization of a 453 Kb 17q25 locus shown previously to exhibit a high frequency of LOH in EOC samples. LOH analysis further defined the minimal region of deletion to a 65 Kb interval flanked by *D17S2239* and *D17S2244*, which contains *RHBDF2, CYGB* and *PRCD* as tumour suppressor gene candidates. Tissue specific expression excluded *PRCD* as a candidate. *RHBDF2* was expressed at low levels in the majority of benign and low malignant potential (LMP) tumours, and in a subset of malignant ovarian tumour samples, as compared with primary cultures of normal ovarian surface epithelial cell (NOSE) samples. *CYGB* was expressed at low levels in the majority of LMP and malignant samples compared with benign and NOSE samples. In contrast to *CYGB* expression, *RHBDF2* was expressed at low or undetectable levels in EOC cell lines exhibiting tumourigenic characteristics and up-regulated in a genetically modified EOC cell line rendered non-tumourigenic. DNA sequence analysis identified variants but no apparent deleterious mutations in either gene. Methylation-specific PCR analysis suggested that promoter methylation of *CYGB* but not *RHBDF2* occurred in 6 of 31 malignant samples. The results combined suggest that *RHBDF2* and *CYGB* may play distinctive roles in ovarian cancer and could be added to the growing roster of chromosome 17 genes implicated in this disease.

Correspondence to: Dr Patricia N. Tonin, Medical Genetics, Room L10-120, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec H3G 1A4, Canada
E-mail: patricia.tonin@mcgill.ca

Key words: ovarian cancer, chromosome 17, tumour suppressor, *RHBDF2*, *CYGB*

Introduction

It is well established that loss of chromosome 17 is a common occurrence in epithelial ovarian cancers (EOCs), as suggested by karyotype and loss of heterozygosity (LOH) studies (1,2). This observation, together with complementation studies involving the transfer of chromosome 17 which resulted in reduced tumourigenicity of an EOC cell line (3), suggest that this chromosome harbours tumour suppressor genes (TSGs). Chromosome 17 contains a number of very well characterized TSGs. The 17p13.1 region harbours *TP53*, a TSG that is the most frequently mutated gene in EOC samples, particularly in serous ovarian carcinoma (4,5), which is the most common histopathological subtype reported for EOC. The 17q21.31 region harbours *BRCA1*, a TSG that when inherited in a mutated form in an autosomal dominant fashion significantly increases the risk for developing breast and ovarian cancers (6-8). Although *BRCA1* is very rarely mutated in sporadic disease, the low level of *BRCA1* expression reported in EOC samples has been attributed to alteration of its promoter by CpG methylation (9,10). In addition to these genes, *DPH1* (17p13.3), *HIC1* (17p13.3), *NF1* (17q11.2), and *RARA* (17q21.1), have also been proposed to function as TSGs in EOC (11-14). Unique loci exhibiting a high frequency of LOH have also been identified, suggesting the possibility of new TSGs in ovarian cancer (15-22). It is thus likely that several genes act in concert in suppressing tumourigenicity and thus account for the unusually high frequency of LOH that often involves an entire chromosome 17 contig in ovarian cancer.

Using LOH analyses we reported a 453 kilobase (Kb) minimal region of deletion (MRD) at 17q25.1-q25.2 that is defined by microsatellite markers *D17S1817* and *D17S751* (22). This genomic region contained nine known or strongly predicted genes where rhomboid 5 homolog 2 *(Drosophila)* (*RHBDF2*, formerly *FLJ22341*) and cytoglobin *(CYGB)* emerged as strong TSG candidates based on decreased gene expression in malignant ovarian cancers (22). Of note, our candidate interval overlaps the familial tylosis with esophageal cancer locus (23). Although mutation analyses of *RHBDF2*

and *CYGB* did not identify any disease associated mutations, decreased expression of *CYGB* was observed in familial tylosis with esophageal cancer samples and cell lines derived from sporadic squamous cell esophageal carcinomas (23-26). Further characterization of *CYGB* showed alterations in promoter CpG methylation in sporadic, but not familial tylosis with esophageal cancer (26). Altered promoter methylation, which in some cases correlated with decreased expression of *CYGB*, has also been observed in oral squamous cell carcinomas and non-small cell lung carcinomas (NSCLC) (27-30). Thus, *CYGB* appears to be emerging as a strong TSG candidate. *RHBDF2* has not been investigated in cancers to the same extent, and thus its role in cancer is unknown (22-24).

In the present study we have further characterized the 17q25.1-25.2 MRD and excluded all but three of the nine TSG candidates mapping to this region in ovarian cancer. These three genes are *RHBDF2* and *CYGB*, our top TSG candidates from our initial study of the chromosome 17q25 region in ovarian cancer (22), and *PRCD* (progressive rod-cone degeneration), previously known as *AK054729*, an expressed sequence tag of unknown function (22). We have investigated gene expression in an expanded panel of serous histopathological subtype ovarian cancer samples and various EOC cell lines exhibiting differences in tumourigenic potential, as well as extended our analysis to include benign and low malignancy potential (LMP) or borderline ovarian tumours of the serous subtype. We have also investigated gene expression of an EOC cell line rendered non-tumourigenic as a consequence of chromosome transfer from our group (31). The genetically modified EOC cell line was investigated to determine if the observed reprogramming (alteration in the transcriptome) of key genes involved in ovarian cancer (31,32) that has occurred as a consequence of tumour suppression also included 17q25 candidates identified in our LOH analyses. To begin to investigate mechanisms of altered gene expression, we have performed DNA sequencing and promoter methylation assays.

Materials and methods

Ovarian tumour samples. Tumour samples and peripheral blood lymphocytes were collected with informed consent from participants undergoing surgeries performed at the Research Centre of the University of Montreal Hospital Centre (CRCHUM)-Notre Dame Hospital site or from surgeries performed at the McGill University Health Centre - Montreal General Hospital as described (21,33). Clinical features such as disease stage, and tumour characteristics such as grade and histopathological subtype, were assigned by a gynecologic-oncologist and/or gynecologic-pathologist according to the criteria established by the International Federation of Gynecology and Obstetrics as reported in previous studies for some cases (21,22,34) and summarized in Table I.

Primary cultures of NOSE samples. Primary cultures were derived from normal ovarian surface epithelial (NOSE) cells from the ovaries of eight participants with no prior history of ovarian cancer, following prophylactic oophorectomy at the CRCHUM Notre Dame Hospital site, and were established as described previously (33-35). Cells were cultured in OSE Medium supplemented with 2.5 μg/ml amphotericin B, 50 μg/ml gentamicin and 15% fetal bovine serum (FBS) as previously described (34).

EOC cell lines. EOC cell lines were derived from a stage IIIc/grade 1-2 papillary serous adenocarcinoma (TOV-81D), a stage III/grade 3 clear cell carcinoma (TOV-21G), a stage IIIc/grade 3 endometrioid carcinoma (TOV-112D), the ascites fluid of a stage IIIc/grade 3 adenocarcinoma (OV-90), a stage IIIc/grade 3 serous carcinoma (TOV-2223G), and both the tumour and the ascites fluid of a stage IIIc/grade 3 serous tumour (TOV-1946 and OV-1946), all from chemotherapy naïve patients, as described (36,37). The non-tumourigenic chromosome 3 transfer radiation hybrids (RH), RH-5, RH-6, and RH-10 cell lines, were derived using a neomycin clone of OV-90 (OV-90 neo^r), and the B78MC166 mouse cell line containing human chromosome 3 as described previously (31). The cell lines were cultured in OSE Medium supplemented with 2.5 μg/ml amphotericin B, 50 μg/ml gentamicin and 10% FBS as described previously (36,37).

Nucleic acid extraction. DNA was extracted from clinical specimens, peripheral blood lymphocytes, and EOC cell lines, as described previously (31,33). Total RNA was extracted with TRIzol™ reagent (Gibco/BRL, Life Technologies Inc., Grand Island, NY) from the primary cultures of NOSE samples, and the EOC cell lines, grown to 80% confluency in 100 mm Petri dishes, and from frozen ovarian tumour samples as described previously (31,38). RNA quality was assessed by gel electrophoresis and 2100 Bioanalyzer analysis using the Agilent RNA 6000 Nano kit.

LOH analysis. The polymorphic microsatellite repeat markers, *D17S2238, D17S2239, D17S2244, D17S2245* and *D17S2246*, were used in LOH analysis. The genomic location of the markers was based on the March 2006 human reference sequence (NCBI Build 36.1/hg18) assembly (39). LOH was performed using a PCR-based assay essentially as previously described (22,40) using primer sets and annealing temperatures described by Langan *et al* (23). LOH (or allelic imbalance) was scored based on the absence or difference in the relative intensity of alleles in tumour DNA compared with the DNA extracted from patient-matched peripheral lymphocytes.

Gene expression analyses. A semi-quantitative RT-PCR assay of cDNA was used to assess gene expression essentially as described (22,31,41). About 200 ng of a 1:10 dilution of the reverse transcribed cDNAs were used in PCR assays with gene-specific primers (Table II). Primers were designed using Primer3 software (42) based on the genomic structures of *RHBDF2* and *PRCD* available from the March 2006 human reference sequence (NCBI Build 36.1/hg18) assembly (39), and alignment of reference sequences *NM_024599* and *NM_001077620*, of each gene, respectively. The primers for *CYGB* were designed as described previously (22). The RT-PCR-based assays were performed essentially as previously described (43). The PCR conditions were 3 min at 95˚C, 30 cycles of 94˚C for 30 sec, annealing temperatures for 30 sec and 72˚C for 30 sec. Products were electrophorosed on a 1% agarose gel, visualized by ethidium bromide staining and RNA quality was assessed by *18S* expression (25 PCR cycles). RT-PCR analysis was also performed on cDNA prepared from commercially available

Table I. Ovarian tumour samples used in analyses.

Sample		Pathology	Age	Stage	Histopathology	LOH analysis	Gene expression analysis	DNA sequencing analysis	Methylation analysis	Ref.
TOV944	GT	TOV	67	IIIc	Serous papillary	+		+	+	22
TOV947	D	TOV	53	Ia	Serous papillary	+		+	+	22
TOV921	GT	TOV	55	IIIc	Endometrioid	+		+	+	22
TOV962	T	TOV	55	IIIc	Endometrioid	+		+	+	22
TOV858	GT	TOV	59	IIIc	Endometrioid	+		+	+	22
TOV863	DT	TOV	72	IIIc	Endometrioid	+		+		22
TOV760	T	TOV	73	IIIc	Clear cell	+		+	+	22
TOV903	D	TOV	48	IIIc	Serous papillary	+		+	+	22
1167	T	TOV	54	II	Endometrioid	+		+	+	22
4093	T	TOV	85	IIIc	Mucinous	+		+	+	22
TOV391	GT	TOV	53	IIIc	Clear cell	+		+		22
OV272		TOV	76	IIIc	Serous papillary	+		+		22
TOV676	GT	TOV	78	Ic	Endometrioid	+		+		22
TOV918	T	TOV	54	Ia	Serous papillary	+		+		22
TOV701	T	TOV	34	IIIc	Serous papillary	+		+	+	22
OV747		TOV	60	III	Serous carcinoma	+		+	+	22
2559	T	LMP	64	Ia	Mucinous	+		+	+	22
TOV696	GT	LMP	41	IIIc	Serous	+		+	+	22
TOV925	T	TOV	70	IIIc	Serous				+	
TOV3294	DT	TOV	38	IIIc	Serous				+	
TOV1232	DT	TOV	55	IIIc	Serous				+	
TOV1107	EPT	TOV	78	IIIc	Serous				+	
TOV1835	GT	TOV	68	IIIc	Serous				+	
TOV4057	DT	TOV	40	IIIc	Serous				+	
TOV1223	GT	TOV	69	IIc	Serous				+	
TOV3291	GT	TOV	59	IIIc	Serous				+	
TOV4249	DT	TOV	70	Ia	Serous				+	
TOV1066	EPT	TOV	60	IIIc	Serous				+	
TOV702	T	TOV	56	IIIc	Serous				+	
TOV800	EPT	TOV	76	III	Serous		+		+	34
TOV881	MT	TOV	52	IIIc	Serous		+		+	34
TOV908	DT	TOV	53	IIIc	Serous		+		+	34
TOV974	EPT	TOV	57	IIIc	Serous		+		+	34
TOV1007	EPT	TOV	65	IIIc	Serous		+			34
TOV1054	GT	TOV	76	II	Serous		+			34
TOV1095	DT	TOV	54	III	Serous		+		+	34
TOV1108	DT	TOV	57	IIIc	Serous		+		+	34
TOV1127	GT	TOV	49	III	Serous		+		+	34
TOV1142	DT	TOV	48	IIIb	Serous		+		+	34
TOV1148	GT	TOV	43	III	Serous		+			34
TOV1150	GT	TOV	50	IIc	Serous		+		+	34
BOV2023	DT	BOV	70	-	Serous cyst		+			32
BOV1325	GT	BOV	59	-	Serous cyst		+			32
BOV2270	DT	BOV	64	-	Serous cyst		+			
BOV2312	DT	BOV	45	-	Serous cyst		+			32
BOV2331	GT	BOV	57	-	Serous cyst		+		+	32
BOV2418	GT	BOV	71	-	Serous cyst		+		+	32
BOV1207	DT	BOV	51	-	Serous		+		+	32
BOV1695	GT	BOV	41	-	Serous cyst		+			
BOV1296	DT	BOV	71	-	Serous cyst				+	
BOV2328	DT	BOV	52	-	Serous cyst				+	
BOV2506	DT	BOV	67	-	Serous cyst				+	
TOV838	GT	LMP	38	Ib	Serous adeno		+		+	
TOV978	DT	LMP	34	Ia	Serous cyst		+		+	
TOV1010	GT	LMP	31	Ib	Serous cyst		+		+	
TOV1101	GT	LMP	52	IIIc	Serous cyst		+		+	
TOV1228	GT	LMP	42	IIIc	Serous cyst		+		+	
TOV1267	DT	LMP	44	IIIa	Serous cyst		+		+	
TOV1774	GT	LMP	50	IIIa	Serous		+			
TOV2173	T	LMP	49	Ia	Serous cyst		+		+	

T, tumour; D, tumour on right ovary; M, metastasis; DT, tumour on right ovary; GT, tumour on left ovary; EPT, tumour on omentum; TOV, malignant; BOV, benign; LMP, low malignant potential; Serous cyst, Serous cystadenoma; Serous adeno, Serous adenofibroma.

Table II. Primer sequences and annealing temperatures used in sequencing, RT-PCR, MSP, RFLP and SSCP analyses.

Gene	Exon; cDNA; genomic	Assay	Forward primer (5'>3')	Reverse primer (5'>3')	Size (bp)	Annealing temp. (°C)
RHBDF2	3	DNA sequencing	CTCTCCCGGTCTCCATTC	TCTGTTACCTACGGCAGAAC	239	54
RHBDF2	4	DNA sequencing	GTTTGTTCTCAGGGTTTTCA	GAGAATCCACAGGTTGCTG	277	62
RHBDF2	5-6	DNA sequencing	CTTCTGCTCATCTGGTCACT	ATCCCAGCACATCCACTGT	780	55
RHBDF2	7	DNA sequencing	GGAAGCTGGTGTCTCATTT	ACCCTACCCAACAGAAAGAT	510	55
RHBDF2	8-9	DNA sequencing	TGTTGGGTAGGGTGAAGAC	CCTTCGAGCTTGTCACTACA	808	55
RHBDF2	10-11	DNA sequencing	GTTCCTCTGGTTTGAGATGA	GATGCAGGGTGAGAACTTG	790	55
RHBDF2	12	DNA sequencing	GAAGTACATCCAGCAGGAGA	GACAAAAGTGGCCAAAGTC	651	56
RHBDF2	13	DNA sequencing	GTGACCACGTTCCCTCCT	TGGAGACAGGGTTCAGATT	391	55
RHBDF2	14-15	DNA sequencing	CATGGACAAGTCTGATCTGG	GGTGGTGATCTCACAGCTAA	784	56
RHBDF2	16-17	DNA sequencing	CAGAGTCGAAGGTTCTGGAT	GATGAAGATGATGGCGATAC	722	55
RHBDF2	18	DNA sequencing	GGTCCCAGATCAGTTCTACA	CATCACTGTTCCCTCTCACT	556	55
RHBDF2	19	DNA sequencing	CTGATCGATGGGATGACC	GCCTTAACCAACCATCTCA	687	60
CYGB	1	DNA sequencing	AGCTGCTCATGGAGAAAGT	GACACCTACCTCACCAGGAT	160	60
CYGB	2	DNA sequencing	ATGCTGAGGTCTACTGATGG	CTCTCAGGACAAGGGTTGC	567	60
CYGB	3	DNA sequencing	AGACTGTTGCGTTAGGAATG	TTTGGGAGGATTTTTGCT	514	55
CYGB	4	DNA sequencing	TAGCAGGACGGAGACTAGAA	GGGCTGGTTTATTCCCTAA	529	60
RHBDF2		RT-PCR	GCCCGATGACATCACTAA	TGAAAGACCACAGACACGA	330	55
CYGB		RT-PCR	CAGTTCAAGCACATGGAGGA	GTGGGAAGTCACTGGCAAAT	250	55
PRCD		RT-PCR	AAGGAAAAAGCCTGACACTT	GGGGGTTTTTGAAGACTAATA	219	55
CYGB		Methylation-methylated	TTGATTTAAAGTTTAATATTTTCGA	AAAACCCAACTAAATCCACGAC	111	60
CYGB		Methylation-unmethylated	AATTGATTTAAAGTTTAATATTTTTGA	AAAAAACCCAACTAAATCCACAAC	115	55
RHBDF2		Methylation-methylated	TTTGAGTTAGTTCGGGTTTTTAGTC	GAACCTACCCTAAAACGATAATACG	115	60
RHBDF2		Methylation-unmethylated	TGAGTTAGTTTGGGTTTTTAGTTGA	CCAAACCTACCCTAAAACAATAATACA	115	55
RHBDF2	4	RFLP	GTTTGTTCTCAGGGTTTTCA	GAGAATCCACAGGTTGCTG	277	62
RHBDF2	15	SSCP	CAGGAGCAACCACACAGG	AGCAAAGGACAGTTATTGGAG	210	59

human normal ovary, testes, and placenta RNAs (Stratagene, La Jolla, CA) and human retina total RNA (Clontech, Mountain View, CA). Primer sequences and annealing temperatures are shown in Table II, primer sequences for *18S* were reported previously (22).

Affymetrix GeneChip U133 Plus 2 analysis of *RHBDF2* and *CYGB* was extracted from the transcriptome analyses performed for OV-90neo^r and the non-tumourigenic chromosome 3 transfer radiation hybrids, RH-5, RH-6, and RH-10 cell lines, described previously (31).

Genomic sequencing analyses. Sequencing analysis was performed on PCR products representing the protein coding exons and flanking splice sites of *RHBDF2* and *CYGB* derived from genomic DNA extracted from the EOC cell lines and ovarian tumour samples. Primers designed to analyze *RHBDF2* and *CYGB* were initially based on their respective reference sequences *NM_024599*, and *NM_134268*, as aligned to the genomic structure of each gene according to the March 2006 (NCBI36/hg18) assembly. Although the location of each gene has changed the annotation has been maintained in the most recent [February 2009 (GRCh37/hg19)] assembly of the human genome (44,45). The primers were designed using Primer3 software (42). The PCR assays were carried out as previously described (43). Primer sequences and PCR annealing temperatures are found in Table II. The PCR products were sequenced in both directions using the 3730XL capillary DNA Analyzer system platform from Applied Biosystems at the McGill University and Genome Quebec Innovation Center, Montreal, Canada as previously described (43). Sequence chromatograms from both directions were aligned and compared with the *RHBDF2* and *CYGB* reference sequences. Sequence variants were compared with those reported in the Single Nucleotide Polymorphism (dbSNP) database (www.ncbi.nlm.nih.gov/projects/SNP/) (46). The variants resulting in amino acid substitutions were examined by PolyPhen (genetics.bwh.harvard.edu/pph) (47), Panther (www.pantherdb.org/tools/csnpScoreForm.jsp) (48), SNPeffect (snpeffect.vib.be) (49), F-SNP (compbio.cs.queensu.ca/F-SNP/) (50) and SIFT dbSNP (sift.jcvi.org/www/SIFT_dbSNP.html) (51), and aligned to orthologous genes from various species using the UCSC Genome Bioinformatics Site (genome.ucsc.edu) in order to predict the possible impact on the structure and function of the encoded protein (45).

Allele frequencies of sequence variants. DNA sequence analysis of the EOC cell lines identified the *RHBDF2* variants *2064A>G* and *492G>T*. Allele frequencies of these variants were determined by comparing genotypes of peripheral blood lymphocytes from 111 healthy women and 104 ovarian cancer cases. The samples were collected with informed consent from the CHUM at Notre Dame Hospital.

PCR-based assays were used to detect the presence of the variants using primers and annealing temperatures described in Table II. The *2064A>G* variant, located in exon 15, was detected by single-strand conformation polymorphism (SSCP) analysis using previously described conditions (40). Briefly, the reaction products were diluted 2:3 with stop buffer and heated at 95˚C for 15 min before loading on a 30% acrylamide non-denaturing gel. The products were electrophoresed at 20 W for 15 min followed by 3 W for 18 h at room temperature. Gels were dried and autoradiographed. Variants were detected as a band shift and compared with patterns from a variant-positive EOC cell line. The *492G>T* variant, located in exon four, was detected using a PCR-based restriction fragment length polymorphism (RFLP) assay. The variant alters the sequence that would normally be recognized and cleaved by the *HpyCH4V* restriction endonuclease. PCR products were digested overnight at 37˚C and resolved by agarose (1%) gel electrophoresis. Allele and genotype frequency distributions were compared by Pearson's Chi-square test (Statistical Product and Service Solution Package, SPSS, Chicago, IL).

DNA methylation analysis. Hypermethylation of the putative promoter regions of *RHBDF2* and *CYGB* were examined using methylation specific PCR assays following bisulfite conversion of cytosine residues. The bisulfite conversion reactions were performed using the Imprint™ DNA Modification kit (Sigma-Aldrich Canada Ltd., Oakville, Ontario) with 1 μg of DNA from the EOC cell lines and ovarian tumour samples. The methylation specific primers for *CYGB* were designed using the MethPrimer program (52) to overlap genomic regions previously investigated for methylation (27). The primers for *RHBDF2* were also designed using MethPrimer, based on a putative CpG island that maps 123 Kb upstream of the gene, and overlaps the first exon (39). The PCR reactions were carried out as previously described (43). In the absence of *CYGB* and *RHBDF2* methylated control, *CDKN2A* was investigated to assess bisulfite conversion in all assays as it has been shown methylated in the TOV-112D EOC cell line (13). Primer sequences and annealing temperatures can be found in Table II.

Results

Defining the 17q25 TSG locus. A previous LOH analysis identified a 453 Kb minimal region of deletion defined by the microsatellite markers *D17S1817* and *D17S751* which contained nine known or strongly predicted genes and two ESTs (22). Recent annotations of the human genome have further defined this interval with the addition of genes predicted to encode small nucleolar RNAs (Fig. 1A). To further define this region of deletion and select TSG candidates for molecular genetic analyses, LOH analysis was performed on the subset of 16 malignant ovarian cancer samples and two LMP samples previously shown to contain interstitial deletions (22). Since our report, microsatellite markers *D17S2238, D17S2239, D17S2244, D17S2245* and *D17S2246* were identified, but upon further testing only *D17S2239* and *D17S2244* were found to be informative for LOH analyses (Fig. 1B). The LOH results are summarized along with previously published reports for the analysis of markers *D17S1817, D17S801* and *D17S751* (reproduced in Fig. 1B). Taken together, these data further refined the MRD to an estimated 65.08 Kb interval flanked by the markers *D17S2239* and *D17S2244* based on the February 2009 human reference sequence (GRCh37) (45). The MRD was observed in malignant tumours of the serous, endometrioid, clear cell and mucinous EOC histopathological subtypes. Notably, ovarian cancer samples 1167T and OV747 exhibited LOH only for *D17S801*. This newly refined candidate TSG-containing locus contains the entirety of *CYGB* and *PRCD*, as well as the first exon of *RHBDF2*.

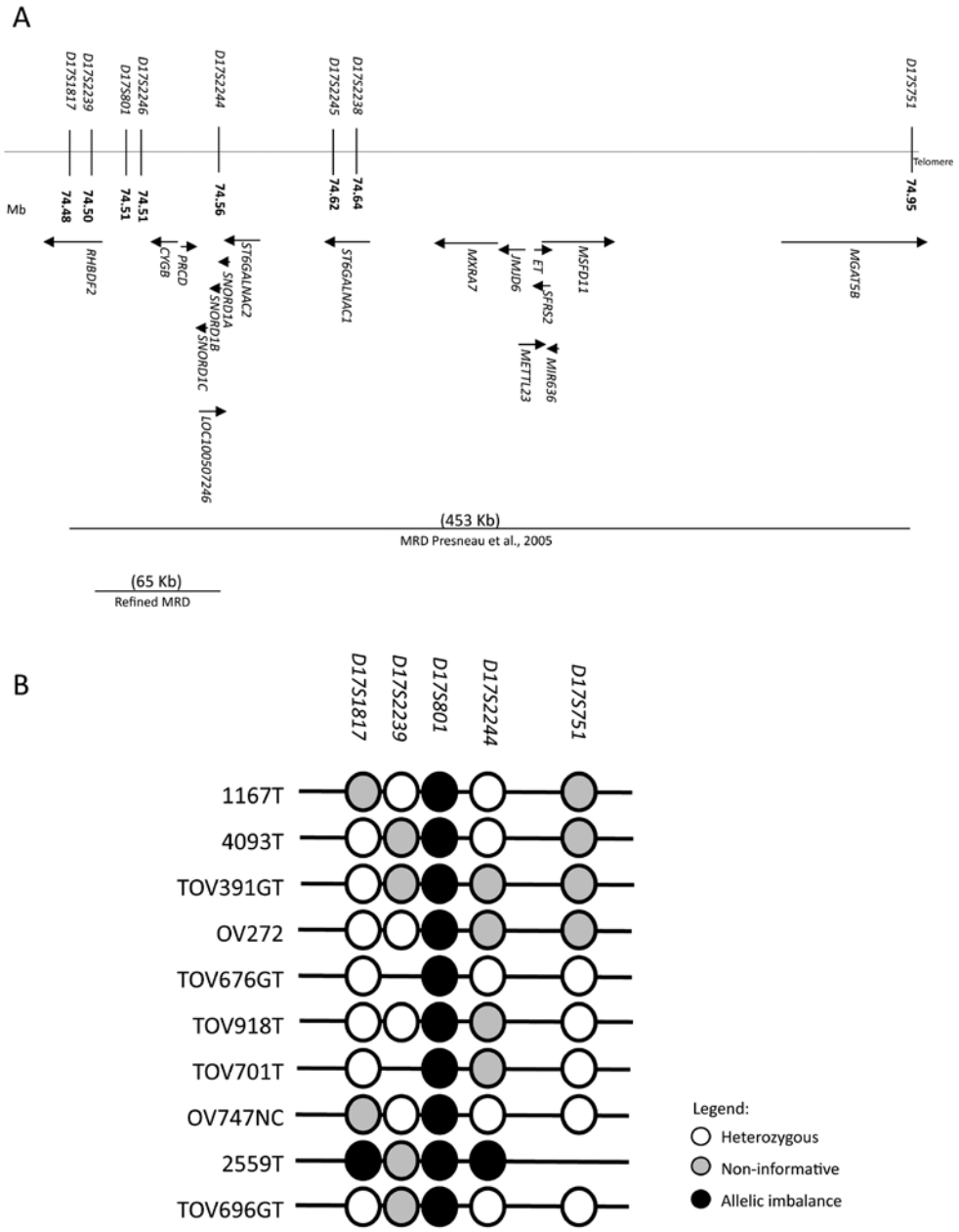

Figure 1. Genomic organization of the 17q25 MRDs defined by LOH analysis. (A) The genomic organization of the 453 Kb MRD defined by polymorphic genetic markers *D17S751* and *D17S1817*, reported in a previous LOH analysis (22), is shown using a recent human genome assembly (hg18) annotation. The genetic map contains genes (RefSeq designation) and expressed sequences aligned (arrows indicate orientation) relative to the location of polymorphic genetic markers (with chromosomal position in Mb). The position of the 65 Kb MRD defined by genetic markers *D17S2244* and *D17S2239* as refined by LOH analyses using additional polymorphic markers is shown. (B) LOH results of the EOC samples used to further define the MRD locus. The refined 65 Kb locus is inferred by the retention of heterozygosity of alleles for *D17S2244* and/or *D17S2239*, in EOC samples harbouring LOH of *D17S801*.

Gene expression analyses. The LOH analysis identified *RHBDF2, CYGB* and *PRCD* as top TSG candidates, as *ST6GALNAC2, ST6GALNAC1, MXRA7, JMJD6, METTL23, SFRS2, ET, MFSD11* and *MGAT5B* fell outside of the newly defined minimal region of deletion (Fig. 1A). We previously showed low or absent expression of *RHBDF2* and variable expression of *CYGB* in malignant ovarian cancer samples exhibiting LOH of the 17q25 locus including *D17S801* relative to primary cultures of NOSE (22). To further characterize the gene expression of our candidates, and as *PRCD* was not previously investigated, we have extended this analysis to include a larger panel of NOSE samples and malignant ovarian cancer samples and also included an investigation of benign and LMP ovarian tumours. All ovarian tumour samples investigated were histopathologically classified as serous, which represents the most common subtype of epithelial ovarian cancers. We performed semi-quantitative RT-PCR analyses as our studies have consistently shown a very good concordance of gene expression when compared with more quantitative methods such as gene expression microarray analyses or quantitative RT-PCR in comparing primary cultures of NOSE samples, benign and LMP tumours, and malignant ovarian cancer samples and our EOC cell lines, where overt differences in gene expression were detectable in these ovarian cancer model systems of study (22,32,34,41,53,54).

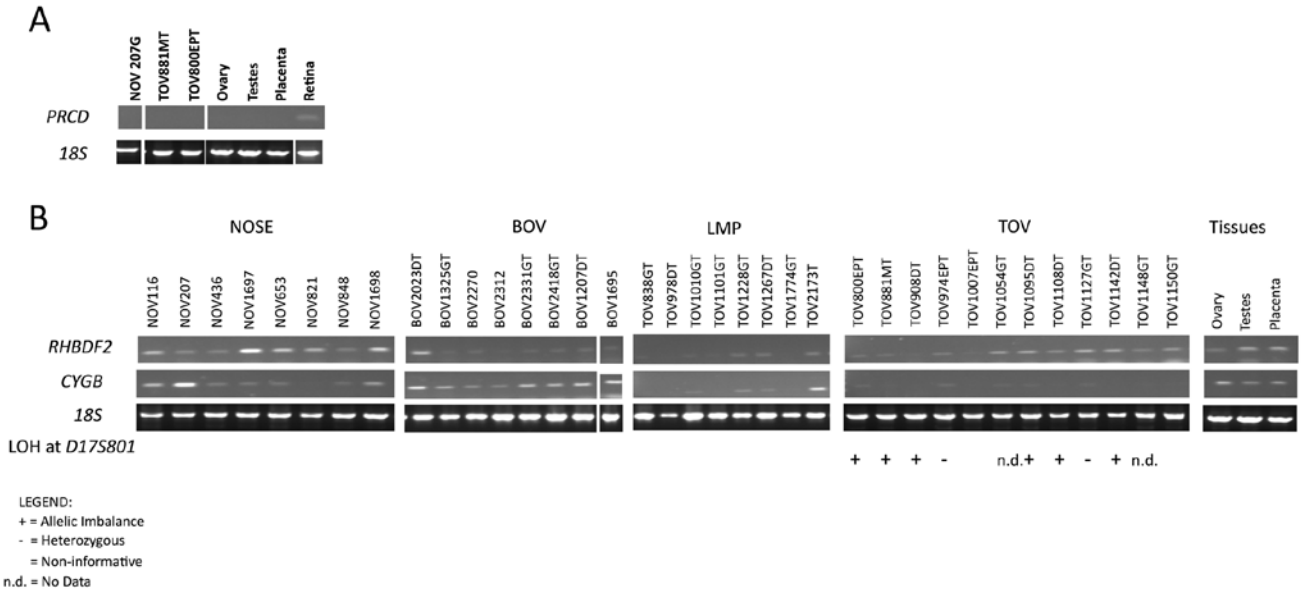

Figure 2. RT-PCR expression analyses of *RHBDF2, CYGB* and *PRCD*. (A) RT-PCR assays of *PRCD* shown for primary culture of normal ovarian surface epithelial cells sample *NOV207G*, malignant serous ovarian carcinoma samples (TOV881MT and TOV800EPT), and commercially available RNA from ovary, testes, placenta and retina. Expression assays of *18S* RNA are shown for RNA quality. (B) Expression assays of *RHBDF2* and *CYGB* shown for primary cultures of normal ovarian surface epithelial (NOSE) cells, benign serous subtype ovarian tumours (BOV), low malignancy potential (LMP) serous subtype tumours, malignant serous ovarian subtype carcinoma samples (TOV), and commercially available normal ovary, testes, and placenta RNAs. Expression assays of *18S* RNA are shown for RNA quality. LOH status for the polymorphic genetic marker *D17S801* is shown for the TOV samples as reported previously (34).

PRCD expression by RT-PCR analysis was not detectable in NOSE samples, malignant ovarian tumour samples or from ovary, testes or placental tissues (Fig. 2A). However, low-level expression was detectable in retina, which is consistent with independent reports showing *PRCD* expression in retina and retinal pigment epithelium/choroids (55). In contrast, *RHBDF2* and *CYGB* expression was detectable by RT-PCR in NOSE, benign and LMP tumour samples, and malignant ovarian cancer samples (Fig. 2B). *RHBDF2* expression was detectable in all NOSE samples. In contrast, *RHBDF2* was expressed at low or undetectable levels in the majority of benign and LMP samples, and a subset of malignant samples. The low or undetectable levels of *RHBDF2* expression in a subset of malignant ovarian cancer samples (particularly TOV800EPT, TOV881MT, TOV908DT, TOV974EPT, and TOV1007EPT) is consistent in part with our previous study where expression was not detectable in malignant ovarian cancer samples of different histopathological subtypes (endometrioid, clear cell, mucinous and serous) where all exhibited LOH of the *D17S801* locus (Fig. 1B) (22).

Of note, some of the malignant ovarian cancer samples exhibiting low levels of *RHBDF2* expression, such as TOV800EPT, TOV881MT and TOV908DT, also harbor LOH (or allelic imbalance) of the *D17S801* locus as determined in an independent study from our group (34) (Fig. 2B). Although *CYGB* expression was also detectable in all NOSE samples, expression was clearly detectable in all benign ovarian tumour samples (Fig. 2B). *CYGB* expression was low or undetectable in the majority of LMP samples and in all malignant ovarian cancer samples (Fig. 2B). As the same batch of cDNAs were used in the semi-quantitative RT-PCR analyses, the expression profiles likely reflect differential expression of *RHBDF2* and *CYGB* in ovarian tumour samples as has been observed in our previous studies with other genes (22,32,34,41,53,54). *RHBDF2* and *CYGB* expression was also detectable in whole ovary, testes and placenta. Given the apparent tissue specific expression of *PRCD* and absence of ectopic expression in NOSE and ovarian tumour samples (Fig. 2A), we excluded this gene from further investigation as a TSG candidate.

Gene expression analysis of *RHBDF2* and *CYGB* was also investigated by semi-quantitative RT-PCR in EOC cell lines established as long-term passages from chemotherapy naïve ovarian cancer samples in our research group (36,37). The cell lines differ in their *in vivo* tumourigenic potential, where TOV-81D and TOV-2223G are incapable of forming tumours as xenografts in immunocompromised mice in contrast to OV-90, TOV-21G, TOV-112D, TOV-1946, and OV-1946 (36,37). However, in contrast to TOV-81D, TOV-2223G retains some *in vitro* features of tumourigenic cells, such as the ability to invade matrigel and form colonies in soft agarose (37). By semi-quantitative RT-PCR or gene expression microarray analyses, TOV-81D has also been shown to retain gene expression profiles that resembled NOSE samples (22,32,34,41,53,54). Notably, TOV-81D exhibited the highest level of expression of *RHBDF2* whereas low or undetectable levels of expression were observed in all of the other EOC cell lines (Fig. 3A). Although *CYGB* expression was also robust in the TOV-81D cell line, expression was also clearly detectable in OV-90, TOV-21G and TOV-2223G. As these EOC cell lines differ in their allelic content of the *D17S801* locus (Fig. 3A), it is possible that differences in gene expression in some of the EOC cell lines could be due to dosage effects.

Expression analysis was also performed on the OV-90 derivative cell lines, RH-5, RH-6 and RH-10, rendered non-tumourigenic as a consequence of chromosome 3 fragment transfer (31). These cell lines were derived in the context of

Table III. Sequence variants in *RHBDF2* and *CYGB* indentified in seven EOC cell lines.

Gene	Variant identified	Genomic region	Reference allele-strand	OV90	TOV112D	TOV21G	TOV81D	TOV1946	OV1946	TOV2223	Codon change	Amino acid substitution	Function	SNP	CEU population diversity
RHBDF2	*280G>A*	Exon 3	G	G,A	A	G	G	G	G	G			UTR	*rs12943385*	G: 0.908, A: 0.092
	492G>T	Exon 4	G	G	G	G,T	T	G	G	T	GCA>TCA	A67S	Missense	*rs3809694*	
	916C>T	Exon 6	C	T	T	T	T	T	T	T	CCG>CTG	P208L	Missense	*rs3744045*	T: 1.000, C: 0.000
	1181+145_1181+146insC	Intron 7	-	insC	insC	insC,-	-	insC	insC	-			Intronic	*rs35408486*	
	1300+50G>A	Intron 8	G	G	G	G	A,G	G	G	G			Intronic	*rs61267327*	G: 0.950, A: 0.050
	1496-76C>T	Intron 9	C	T	T	C,T	C	T	T	C			Intronic	*rs12951122*	T: 1.000, C: 0.000
	2064A>G	Exon 15	A	A	A	A	A,G	A	A	A	ATG>GTG	M591V	Missense	*rs73998915*	
CYGB	*302-72insC*	Intron 1	-	-	-	-	insC,-	-	-	insC			Intronic	*rs3217541*	
	697-79C>T	Intron 3		C	C	C	C	T	T	C			Intronic		
	739A>C	Exon 4	A	A,C	C	A,C	A,C	C	C	A			UTR	*rs8077736*	A: 0.639, C: 0.361
	740A>C	Exon 4	A	A,C	C	A,C	A,C	C	C	A			UTR	*rs4238995*	

Both genes are on the minus strand.

research involving the identification of chromosome 3p TSGs (31,54). Affymetrix gene expression analyses showed an altered transcriptome in comparison to the parental OV-90 cell line, wherein some genes were reprogrammed and reflected the expression profile observed in NOSE samples as compared with malignant ovarian cancer samples (31,32). Affymetrix gene expression analysis showed a consistent up-regulation of *RHBDF2* expression in the three independently derived non-tumourigenic OV-90 hybrid cell lines RH-5, RH-6 and RH-10 but no detectable difference in *CYGB* expression as compared with the tumourigenic parental cell line OV-90 (31) (unpublished data). These observations are consistent with RT-PCR results (Fig. 3B).

Sequence analysis of RHBDF2 and CYGB. A classical mechanism of inactivation of tumour suppressor genes involves the loss of one parental allele possibly through chromosome nondisjunction and somatic mutation of the remaining parental allele. We investigated the possibility of somatic inactivation by sequencing the genomic DNA regions that represent the protein coding exons and splice junction sites of *RHBDF2* and *CYGB*. We began our analysis by investigating the EOC cell lines as some of these cell lines exhibited allelic imbalance or loss of heterozygosity of the 17q25 locus containing the gene candidates. Relative to the reference sequences of each candidate gene, sequence variants were identified in both genes, in all EOC cell lines (Table III). In sequencing *RHBDF2*, we identified three sequence variants that confer an amino acid substitution, one variant in the gene's 5'UTR, and four variants in intronic regions. The variants *492G>T, 916C>T* and *2064A>G* result in the amino acid substitutions A67S, P208L, and M591V, respectively. Sequencing *CYGB* resulted in the identification of two intronic variants, and two adjacent variants that are situated in the 3'UTR. With one exception, all variants identified have been reported in the dbSNP database (www.ncbi.nlm.nih.gov/projects/SNP) (46) (Table III). The *CYGB 697-79C>T* variant identified in both TOV-1946 and OV-1946, which are cell lines derived from the malignant tumour and ascites sampled from the same patient (37), has not been reported previously. None of the identified intronic variants are close to splice sites, or create novel consensus GTAG splice site sequences.

We then extended our sequence analysis to the ovarian tumour samples that exhibited LOH of the *D17S801* locus (22), of which all but one was available for this analysis (Table IV). Eight of the 11 sequence variants identified in the EOC cell lines were also identified in the tumour samples, and no other sequence variants unique to these tumour samples were observed.

Frequencies for northern and western European populations (CEU) are available for 5 of the 11 reported SNPs (Table III). Data for this population was reviewed as our cancer samples and derived cell lines were ascertained from the French Canadian population of Quebec, a population sharing common European ancestry (56). Notably, the CEU population frequency of *RHBDF2 916C>T* variant, conferring a P208L amino acid change, is 100% for the T allele, which is consistent with our results for all EOC cell lines, suggesting that this is the most common allele in the population. Population frequencies for the *RHBDF2* variants *492G>T* and *2064A>G*, that are both predicted to result in amino acid substitutions, are not reported

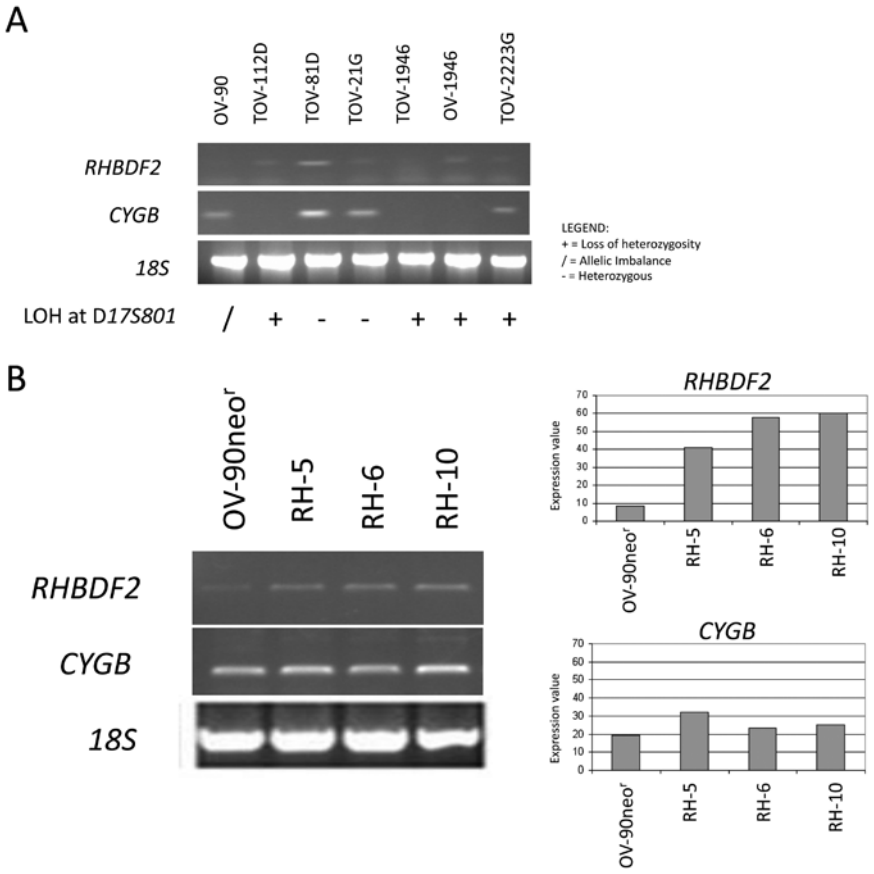

Figure 3. Expression analyses in EOC cell lines. (A) RT-PCR assays of *RHBDF2* and *CYGB* in the EOC cell lines, OV-90, TOV-112D, TOV-81D, TOV-21G, TOV-1946, OV-1946, and TOV-2223G. Expression assays of *18S* RNA are shown for RNA quality. The LOH status of the *D17S801* locus is shown for each EOC cell line. (B) Left panel: RT-PCR assays of *RHBDF2* and *CYGB* shown for the non-tumourigenic hybrids (RH-5, RH-6 and RH-10) generated by the transfer of chromosome 3 fragments into a neomycin-resistance derivative of OV-90 (OV-90neo^r), a tumourigenic EOC cell line. Expression assays of *18S* RNA are shown for RNA quality. Right panel: Affymetrix GeneChip U133 Plus2 microarray expression data shown for probe sets *219202_at* and *1570410_at*, representing *RHBDF2* and *CYGB*, respectively were extracted from previously performed transcriptome assays (31) of OV-90neo^r, RH-5, RH-6, and RH-10.

in dbSNP. Consequently, we determined the genotype and allele frequencies in peripheral blood lymphocyte DNA from healthy women and women affected with ovarian cancer representative of our population. There were no significant differences in the genotype and allele frequencies between unaffected and affected women for either variant (p=0.06-0.76) (Table V).

The *RHBDF2* variant *492G>T* (in exon 4) is predicted to result in the substitution of a nonpolar alanine residue with the polar serine residue at amino acid position 67, whereas the variant *2064A>G* (in exon 15) results in the substitution of a methionine with a valine residue which are both basic amino acids. The predicted structure of *RHBDF2* is that of a seven helical transmembrane protein with an extended intracellular N terminus and a highly conserved loop region, termed the iRhom homology domain (IRHD), that is found between the first and second transmembrane domains (57). According to this structure, the A67S substitution would affect an amino acid located in the intracellular N terminus domain of the protein, whereas the M591V substitution would affect the amino acid sequence of the highly conserved IRHD. In applying various computational tools that predict the functional effects of sequence variants, neither substitution was predicted to have a biological impact on the encoded protein (see Materials and methods). As evolutionarily conserved amino acids may be indicative of functionally important residues, we also investigated the conservation of amino acid sequences encoded by *492G>T* (A67S) and *2064A>G* (M591V) using the UCSC genome browser vertebrate multiz alignment and conservation (44 species) track (45). Both variants and their flanking amino acid sequences appear to be conserved in primates (Fig. 4). The A67S variant, and flanking amino acid sequences, appear not to be well conserved beyond primates (Fig. 4). The M591V variant, and flanking amino acids also appear to be conserved in other placentals and vertebrates including the rabbit, the dolphin and the chicken (Fig. 4).

Methylation analyses of CYGB and RHBDF2. Loss of gene function in cancer cells can also be achieved by affecting the regulation of gene expression by methylation of CpG regions in the promoter regions of tumour suppressor genes. Although previous studies have shown methylation of *CYGB* in other cancer types (26-28), methylation of *CYGB* promoter regions has not been investigated in the context of ovarian cancer. Moreover, to our knowledge, promoter methylation of *RHBDF2* has not been investigated in any cancer context. We applied methylation-specific PCR analysis to investigate the possibility of altered CpG methylation in the inferred promoter regions of *CYGB* and *RHBDF2*. As we did not have any samples exhibiting methylation of these genes, we also included a

Table IV. Sequence variants in *RHBDF2* and *CYGB* identified in EOC tumour samples.

	RHBDF2					*CYGB*		
Sample	*280G>A* exon 3 5'UTR	*492G>T* exon 4 A67S	*916C>T* exon 6 P208L	*1181+145_1181+146insC* intron 7 intronic	*1496-76C>T* intron 9 intronic	*302-72insC* intron 1 intronic	*739A>C* exon 4 3'UTR	*740A>C* exon 4 3'UTR
Malignant								
TOV944GT	G	G	T	insC	T	-	A	A
TOV947D	G	TG	T	insC	T	-	A	A
TOV921GT	G	T	T	(insC) (insC-)	T	-	A	A
TOV962T	G	TG	T	insC	T	(insC) (insC-) AI	AC	AC
TOV858GT	G	G	T	insC	T	-	A	A
TOV863DT	AG	T	T	insC	T	-	C	C
TOV760T	G	TG	T	insC	T	-	A	A
TOV903D	G	T	T	(insC) (insC-)	T	-	A	A
1167T	AG	TG	T	insC	T	(insC) (insC-)	A	A
4093T	G	T	T	insC	T	-	A	A
TOV391GT	G	TG	T	insC	T	(insC) (insC-)	A	A
OV272	AG	TG	T	NA	NA	NA	AC	AC
TOV676GT	AG	TG	T	insC	T	(insC) (insC-)	AC	AC
TOV918T	AG	TG	T	insC	T	(insC) (insC-)	A	A
TOV701T	AG	T	T	(insC) (insC-)	T	-	A	A
OV747	AG	G	T	insC	T	-	A	A
LMP								
2559T	G	TG	T	insC	T	(insC) (insC-) AI	AC	AC
TOV696GT	G	T	T	insC	T	-	AC	AC

NA, DNA no longer available to perform assay; LMP, low malignant potential; T, tumour; D/DT, tumour on right ovary; GT, tumour on left ovary.

Table V. Allele frequency analysis of *RHBDF2* variants *492G>T* and *2064A>G*.

A, *492G>T*

		Genotype (amino acid) (%)				Allele frequency (%)	
Group	Total (individuals)	GG (Ala/Ala)	GT (Ala/Ser)	TT (Ser/Ser)	Total (chromosomes)	G	T
Affected (blood)	104	19 (18.27)	50 (48.08)	35 (33.65)	208	88 (42.31)	120 (57.69)
Unaffected	73	22 (30.14)	28 (38.36)	23 (31.51)	146	72 (49.32)	74 (50.68)
Total	177	41	78	58	354	160	194

B, *2064A>G*

		Genotype (amino acid) (%)				Allele frequency (%)	
Group	Total (individuals)	AA (Met/Met)	AG (Met/Val)	GG (Val/Val)	Total (chromosomes)	A	G
Affected (blood)	99	95 (95.96)	4 (4.04)	0 (0)	198	194 (97.98)	4 (2.02)
Unaffected	111	103 (92.79)	8 (7.21)	0 (0)	222	214 (96.40)	8 (3.60)
Total	210	198	12	0	420	408	12

methylation-specific PCR assay for *CDKN2A*, a gene that was previously shown methylated in the TOV-112D EOC cell line by an independent group (13). The results for the EOC cell lines are shown in Fig. 5A. Although there is clear evidence of methylation of *CDKN2A* in TOV-112D, there is no evidence of methylation of *RHBDF2* in any of the EOC cell lines.

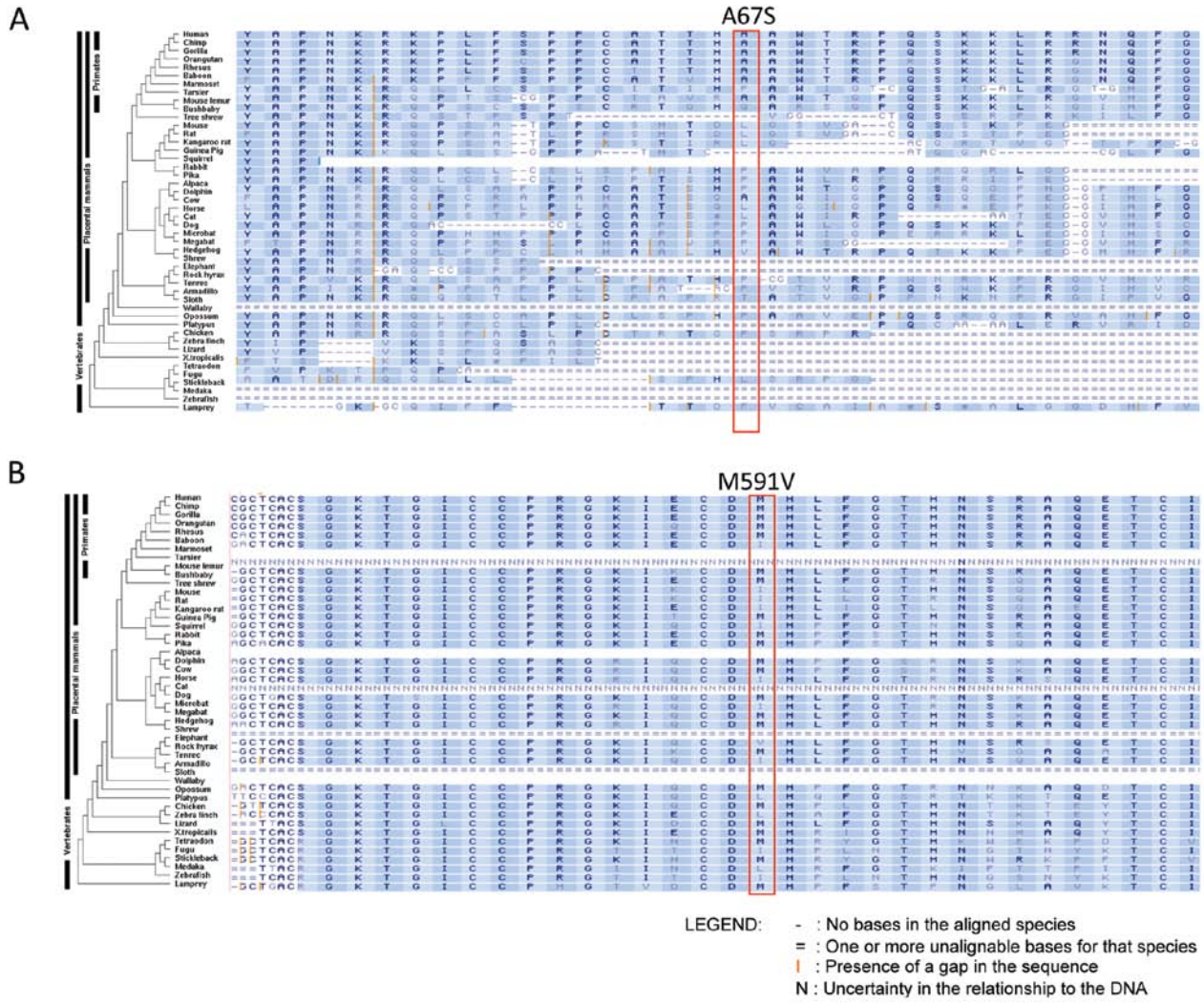

Figure 4. Amino acid conservation of *RHBDF2* missense variants *492G>T* and *2064A>G*. Pair-wise amino acid alignments of up to 44 vertebrates species containing the A67S (A) and M591V (B) amino acid missense variants encoded by *RHBDF2 492G>T* and *2064A>G* sequence variants, respectively and flanking amino acid sequences. The alignments were generated using the UCSC genome browser Vertebrate Multiz Alignment & Conservation conservation track (45).

The methylation specific PCR results show the presence of a doublet, which could perhaps be attributed to the high GC content of the amplified region. Methylation-specific PCR assays however, did provide evidence of methylation in the OV-1946 EOC cell line (Fig. 5A). These results are interesting given that both OV-1946 and TOV-1946 cell lines exhibited undetectable levels of *CYGB* expression (Fig. 3A). They were derived from malignant ovarian ascites (OV-1946) and tumour (TOV-1946) samples from the same ovarian cancer patient (37). Both cell lines exhibited LOH of the 17q25 locus (Fig. 3A), and likely share the same alleles as suggested by DNA sequencing analysis (Table III). Moreover, these cell lines have been shown to exhibit similarities in their molecular genetic features (such as sharing a common somatic *TP53* mutation), growth characteristics and tumourigenic potential (37). Although we cannot exclude the possibility of tissue culture effects, these observations combined suggest that alteration in methylation status of OV-1946 was unlikely to occur as an early event in the evolution of ovarian cancer disease in the patient from which these EOC cell lines were derived.

We then investigated the malignant ovarian cancer samples, LMP and benign ovarian tumours that were examined by gene expression analysis, to determine if the low or undetectable levels of expression observed could be due to alteration of promoter methylation. Genomic DNA was available for a subset of malignant ovarian cancer samples investigated by semi-quantitative RT-PCR analyses in the present study (Figs. 5B, and 2B). In addition, genomic DNA was also available for a subset of malignant ovarian cancer samples examined for gene expression in our previous analysis of 17q25 candidates, which were shown to exhibit LOH overlapping the 17q25 locus (Fig. 5B). In an earlier study, we reported no detectable expression of *RHBDF2* in these samples, although *CYGB* expression was detected in TOV944GT, TOV760T and TOV903D (22). Although there was no evidence of promoter methylation for the benign and LMP tumour samples (Fig. 5C), six malignant ovarian cancer samples TOV881MT, TOV974EPT, TOV1095DT, TOV1108DT, TOV1127GT and TOV1142DT exhibited evidence of promoter methylation of *CYGB* (Fig. 5B). These results are interesting given the low or undetectable levels of *CYGB* expression observed

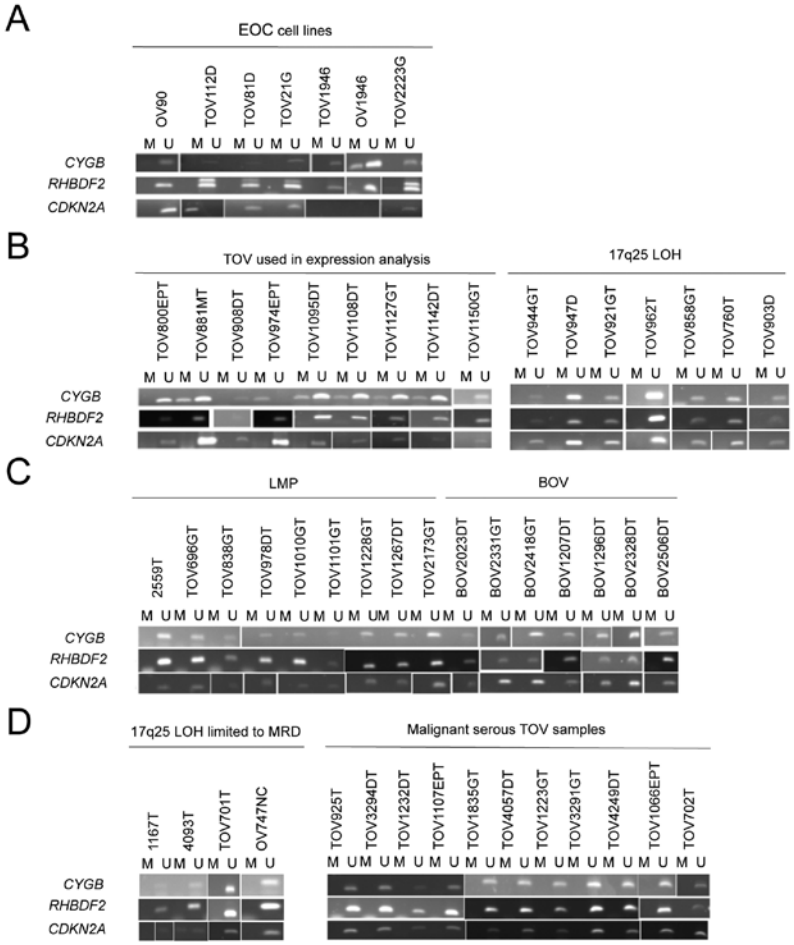

Figure 5. Methylation specific PCR analysis of *CYGB* and *RHBDF2*. Methylation specific PCR assays of *RHBDF2* and *CYGB* were performed on bisulfite-treated DNA with primers designed to detect methylated (M) or unmethylated (U) sequences. Methylation specific analysis was also performed for *CDKN2A*, shown previously to exhibit methylation in the TOV-112D EOC cell line, to control for bisulfite conversion. Shown are results from EOC cell lines (A); the malignant ovarian serous cancers also examined for gene expression in the present study and the previous studies where samples TOV944GT, TOV947D, and TOV903D represent serous histopathological subtype cases (B); the LMP and benign ovarian tumours examined for gene expression in the present study (C); and the samples shown to exhibit interstitial loss of 17q25 locus where samples TOV701T and TOV747NC (not cultured) represent serous histopathological subtype cases, and a new set of malignant ovarian cancers of the serous histopathological subtype (D).

in all samples analyzed in the present study (Fig. 2B). All of the samples exhibiting evidence of methylation silencing are of the serous histopathological subtype of ovarian cancer (Table I). Given the unexpectedly high frequency of methylation results in the analysis of *CYGB*, we extended our analysis of *CYGB* (as well as *RHBDF2* and *CDKN2A*) to the malignant ovarian cancer samples exhibiting interstitial loss of the 17q25 locus that was limited to the minimal region of deletion (Figs. 5D, and 1B) and a new panel of malignant ovarian serous subtype cancer samples (Fig. 5D). We observed no other examples of *CYGB* methylation in these samples. Thus we observed *CYGB* promoter methylation in 6 of 31 malignant ovarian cancer samples, where 6 of 25 (24%) samples examined share in common the serous histopathological subtype classification.

Discussion

LOH mapping further refined a 65 Kb overlapping region of deletion containing three known protein-encoding genes, *RHBDF2, CYGB* and *PRCD* to pursue as TSG candidates in the context of ovarian cancer. The absence of *PRCD* expression in normal and tumour ovarian tissues combined with reported tissue-specific expression in retinal cells (55) suggests that this gene is unlikely to play a role in ovarian cancer. In contrast, the differential expression of both *RHBDF2* and *CYGB* in malignant ovarian cancer samples and EOC cell lines suggests a possible role in this disease. Low or undetectable levels of *RHBDF2* expression were apparent in a subset of malignant ovarian cancer samples relative to NOSE samples. Whereas low or undetectable expression of *CYGB* was more clearly apparent across all samples analyzed. Although these results are consistent with our previous studies (22), low or undetectable levels of expression of *RHBDF2* were also apparent in other histopathological subtypes than that observed with *CYGB* expression. This suggests a role for *RHBDF2* in a subset of malignant ovarian cancer samples and perhaps a more extensive role for *CYGB*, particularly in the serous histopathological subtype.

An interesting observation is the decreased or absent expression of *RHBDF2* in EOC cell lines that exhibit various *in vivo* and *in vitro* features associated with tumourigenic potential. The apparent up-regulation of *RHBDF2* in the OV-90 EOC

cell lines rendered non-tumourigenic as a result of transfer of a normal copy of chromosome 3 also suggests an association of gene expression with tumourigenicity (31). Thus, *RHBDF2* may be one of a number of genes transcriptionally reprogrammed as a consequence of tumour suppression (32). *RHBDF2* under-expression could not be attributed to somatic mutations or alteration of promoter methylation status, mechanisms that have been shown to inactivate or alter TSG function (58). Thus alternative epigenetic mechanisms deregulating *RHBDF2* expression are involved, and this is also inferred by our transcriptome analyses of the genetically modified OV-90 cell line (31,32). In contrast to *RHBDF2*, differential expression of *CYGB* in the EOC cell lines was observed and there was no alteration in gene expression profile in the genetically modified OV-90 derived EOC cell lines. However, it is interesting to note the undetectable levels of *CYGB* expression in TOV-1946 and OV-1946 EOC cell lines relative to TOV-81D and TOV-2223G, given that these cell lines were all derived from ovarian cancers of the serous histopathological subtype, and that the former exhibit both *in vitro* and *in vivo* characteristics associated with tumourigenic potential (36,37). It is possible that the *CYGB* expression profile is more characteristic of malignant ovarian carcinomas of the serous histopathological subtype as has been observed for other genes differentially expressed in serous ovarian cancer (32). Although dysregulation of *CYGB* expression could not be attributed to somatic mutations, alteration of promoter methylation status was observed in some malignant ovarian cancer cases.

An interesting observation is that methylation was observed in malignant samples of the serous histopathological subtype. Although alteration of promoter methylation is suggestive of a mechanism of dysregulating *CYGB* expression, not all samples exhibiting undetectable levels of *CYGB* expression exhibited evidence of promoter methylation by the methylation-specific PCR-based assay. As independent studies of *CYGB* applied a pyrosequencing-based methylation analysis of cancer samples (a methodology not feasible in the present study) (26-28,30), which investigates quantitatively the degree of methylation at CpG positions in close proximity to the promoter, it is possible that methylation of *CYGB* is more extensive than detected in our analysis or alternatively not associated with alteration in gene expression. Future studies examining a large number of malignant ovarian carcinomas would be required to determine the association of *CYGB* expression and methylation status, and its extent in malignant ovarian cancer, particularly in the serous histopathological subtype of this disease.

The refined TSG locus resides in a region containing high GC and *Alu* repeat content, the latter of which has been associated with regions prone to deletions or rearrangements (59, 60). Another interesting structural feature of our candidate region is that it contains a 1.5 Kb inverted, duplicated sequence, that spans 13 Kb and includes the polymorphic microsatellite marker *D17S801* (22) We have proposed that this structure may contribute to the instability of the genomic region and thus account for the high frequency of LOH of *D17S801* in ovarian cancer specimens (22). Although our refined region also contains *SNORD1A, SNORD1B*, and *SNORD1C*, which encode small nucleolar mRNAs that are predicted to guide the 2'O-ribose methylation of 28S rRNA G4362 (61), and *LOC100507246* (62), the *SNORD1* genes have been provisionally mapped and *LOC100507246* is a recently annotated hypothetical gene for a non-coding RNA of unknown function, neither of which have been implicated in cancer.

An interesting observation was the differential expression pattern of *RHBDF2* and *CYGB* in benign versus LMP serous subtype ovarian tumours. Low or undetectable levels of *RHBDF2* expression were observed in the majority of benign and LMP ovarian tumours relative to the NOSE samples. *CYGB* was expressed in all benign samples but showed low or undetectable gene expression levels in the majority of LMP tumours. There is mounting evidence that the serous histopathological subtype of ovarian cancer develops along multiple pathways. In one pathway, tumours develop in a stepwise manner, from a benign lesion to an LMP tumour and finally to a low-grade malignant serous subtype tumour (63). In an alternate pathway, high-grade serous tumours arise without a recognizable precursor or intermediate lesion (63). Comparative genomic hybridization analyses have identified chromosome 17 loss in both LMP and stage I invasive tumours, suggesting chromosome 17 loss to be an early event in the development of serous ovarian cancers or characterizes those LMP tumours that are likely to become invasive (64). Noteworthy, of the 11 LMP tumour samples investigated in our initial LOH analysis of chromosome 17 (21), only one sample exhibited LOH and this sample exhibited loss of the 17q25.1 region. The frequency of LOH of the 17q25.1 locus is in keeping with the observations in the present study where only two of nine LMP samples (2559T and TOV696T) exhibited LOH of the 17q25.1 locus (22). LMP cases are often managed by surgical intervention resulting in excellent outcomes, however recurrences have been reported in ~15% of cases often with poor outcomes (65,66). The long-term survival outcome of our LMP cases with LOH of 17q25.1 is not known, however it would be interesting to determine if the presence of LOH in this chromosomal region is associated with recurrence and/or poor outcome of LMP cases.

CYGB encodes cytoglobin; a highly conserved member of the vertebrate globin family (67,68) and its role in cancer has not been elucidated. Although *CYGB* expression has been observed in various tissues, it is mainly expressed in epithelial cells (69). There is evidence to suggest that *CYGB* is involved in response to hypoxia, and/or protecting cells from oxidative stress. The genomic regions that encode the 5'UTR and 3'UTR and the putative promoter region of *CYGB*, contain hypoxia response elements (HRE) and hypoxia-inducible protein binding sites (HIPBS) (70,71). Of note, DNA sequencing analysis of *CYGB* in the present study identified an alteration of adjacent SNPs *739_740 AA > CC* in 3 of the 7 EOC cell lines that do not express *CYGB*. These SNPs, which were observed in a homozygous state, lie between two putative conserved HIPBS motifs that are purported to stabilize the mRNAs of various hypoxia-responsive genes (70,72). The functional consequences of the SNPs are unknown but warrant further investigation given their location within the conserved HIPBS motifs.

In addition, *CYGB* has been shown to be upregulated by the HIF-1 transcription factor (71). Hypoxia followed by reoxygenation causes overproduction of reactive oxygen species (ROS), and there is evidence to suggest that *CYGB* may protect cells against these ROS under conditions of limited oxygen supply by acting as a ROS scavenger (73,74). As cancer cells are exposed to oxidative stress (75,76), it is possible that *CYGB* may func-

tion as a ROS scavenger in a developing tumour, and loss or decreased *CYGB* expression may contribute to tumourigenesis. Recently, it was shown that ROS exposure of the MCF-7 breast cancer cell line resulted in the induction of *CYGB* expression as well as cell cycle arrest and apoptosis (77). It is also interesting to note that in our previous chromosome 17 transcriptome analyses, a number of genes reported to be hypoxia-related were underexpressed in EOC tumours relative to NOSE samples (34).

In a cancer context, *CYGB* has been implicated as a TSG in familial tylosis with esophageal cancer and sporadic squamous cell esophageal carcinoma (23-26), and there is preliminary evidence suggesting tumour suppressor activity of *CYGB* based on the observation of an inverse relationship between *CYGB* expression and colony formation in NSCLC and breast cancer cell lines (29). Recently, a *CYGB* knockout mouse has been generated and it was found that, upon exposure to N,N-diethylnitrosamine, *CYGB*-deficient mice were more susceptible to liver and lung cancer development, further supporting a role for this gene in cancer (78). Although the mechanism of dysregulating *CYGB* expression is unknown, *CYGB* promoter methylation has been observed in some sporadic squamous cell esophageal carcinomas, oral squamous cell carcinomas, head and neck squamous cell carcinomas and NSCLC that also exhibit low levels of *CYGB* expression (26-28,30).

Less is known about the function of *RHBDF2* and its role in cancer. *RHBDF2* (previously called *RHBDL6, RHBDL5,* and *FLJ22341*) encodes rhomboid 5 homolog 2 *(Drosophila)* (also called *iRhom2*) and belongs to the rhomboid family of proteins. *RHBDF2* bears homology to the *Drosophila* gene *rhomboid-1*, which encodes an intermembrane serine protease, with seven transmembrane domains and a cytoplasmic amino terminus. *Rhomboid-1* is located in the Golgi and functions in the cleavage of *Drosophila* epidermal growth factor receptor ligands (79-81). *RHBDF2* (along with *RHBDF1*) is part of the iRhom subfamily, (57). Although protein function has not been elucidated, iRhoms resemble other rhomboids but as they lack a catalytic serine-histidine dyad, shown to possess protease function, they have been referred to as the 'inactive rhomboid subfamily'. iRhoms have extended N termini and an IRHD, which is a highly conserved loop region between the first and second transmembrane domains (57). The function of the IRHD is currently unknown, but it has been suggested that the cysteine residues may form disulfide bridges that could stabilize a globular fold (57). iRhoms are highly conserved in all animal species, and they may play a regulatory role by binding to and sequestering substrates (81). Notably, the *RHBDF2* sequence variant *2064A>G*, which would give rise to the amino acid substitution M591V, is found within the highly conserved IRHD. Although this variant is not predicted to be deleterious according to various computational tools, functional analyses could elucidate its affect on protein activity.

In summary, LOH analysis defined a region of deletion containing *RHBDF2*, *CYGB*, and *PRCD* as candidates to pursue in ovarian cancer. Gene expression analyses of ovarian tumour samples, cell lines and a unique cell line model rendered non-tumourigenic, supports a role for *RHBDF2* and *CYGB* in this disease. However, the differential expression profiles of these genes suggest that their roles may be unique, with *RHBDF2* more closely associated with tumourigenic potential. Mechanisms of gene dysregulation appear not to involve intragenic mutations, which would affect protein function. However, promoter methylation silencing may be responsible for dysregulating *CYGB* expression in some cases. This is the first report of methylation silencing of *CYGB* in ovarian cancer, suggesting that *CYGB* may play a role in diverse cancer types, as previous studies have also shown promoter methylation silencing in esophageal and lung cancers (26-28,30). The observation that methylation occurred in the serous but not other histopathological subtypes of EOC is also interesting in light of subtype specific pathways in EOC (82). It is interesting to note that a recent whole genome sequencing screen of 316 serous ovarian carcinomas identified a case with a missense variant in *CYGB* and another with a frameshift variant in *RHBDF2* (82). Although large-scale genomic sequencing endeavours are likely to under report variants identified, the results are in keeping with our targeted sequencing endeavours indicating that somatic intragenic mutations may be rare in *RHBDF2* and *CYGB* in ovarian cancer. However, the results raise the question if the *RHBDF2* frameshift mutation conferred a selective advantage to the ovarian cancer found to harbour this apparently deleterious mutation. Our results combined suggest that *RHBDF2* and *CYGB* may play distinctive roles in ovarian cancer, could be added to the growing roster of chromosome 17 genes implicated in cancer, and thus warrant further investigation to establish their role in the development of this disease.

Acknowledgements

We would like to thank Suzanna L. Arcand for her expertise. P.M.W. is a recipient of a Doctoral Research Award from the Canadian Institute of Health Research. The Research Institute of the McGill University Health Centre and the Centre de recherche du Centre hospitalier de l'Université de Montréal receives support from the Fonds de recherche du Québec - Santé. Clinical specimens were provided by the Banque de tissus et de données of the Réseau de recherche sur le cancer of the Fonds de recherche du Québec - Santé which is affiliated with the Canadian Tumour Repository Network. This research was supported by grants from the Canadian Institute of Health Research and the Cancer Research Society Strategic Initiative on the Genomics and Proteomics of Metastatic Cancer to P.N.T., D.M.P. and A.-M.M.-M.

References

1. Mitelman F, Johansson B and Mertens F: Mitelman Database of Chromosome Aberrations in Cancer. http://cgap.nci.nih.gov/Chromosomes/Mitelman, 2007.
2. Liu Y and Ganesan TS: Tumour suppressor genes in sporadic epithelial ovarian cancer. Reproduction 123: 341-353, 2002.
3. Cao Q, Abeysinghe H, Chow O, *et al*: Suppression of tumorigenicity in human ovarian carcinoma cell line SKOV-3 by microcell-mediated transfer of chromosome 11. Cancer Genet Cytogenet 129: 131-137, 2001.
4. Salani R, Kurman RJ, Giuntoli R II, *et al*: Assessment of TP53 mutation using purified tissue samples of ovarian serous carcinomas reveals a higher mutation rate than previously reported and does not correlate with drug resistance. Int J Gynecol Cancer 18: 487-491, 2008.
5. Ahmed AA, Etemadmoghadam D, Temple J, *et al*: Driver mutations in TP53 are ubiquitous in high grade serous carcinoma of the ovary. J Pathol 221: 49-56, 2010.
6. Miki Y, Swensen J, Shattuck-Eidens D, *et al*: A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. Science 266: 66-71, 1994.

7. Easton DF, Ford D and Bishop DT: Breast and ovarian cancer incidence in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Am J Hum Genet 56: 265-271, 1995.
8. Ford D, Easton DF, Bishop DT, Narod SA and Goldgar DE: Risks of cancer in BRCA1-mutation carriers. Breast Cancer Linkage Consortium. Lancet 343: 692-695, 1994.
9. Wilcox CB, Baysal BE, Gallion HH, Strange MA and DeLoia JA: High-resolution methylation analysis of the BRCA1 promoter in ovarian tumors. Cancer Genet Cytogenet 159: 114-122, 2005.
10. Baldwin RL, Nemeth E, Tran H, *et al*: BRCA1 promoter region hypermethylation in ovarian carcinoma: a population-based study. Cancer Res 60: 5329-5333, 2000.
11. Sangha N, Wu R, Kuick R, *et al*: Neurofibromin 1 (NF1) defects are common in human ovarian serous carcinomas and co-occur with TP53 mutations. Neoplasia 10: 1362-1372, 2008.
12. Feng Q, Deftereos G, Hawes SE, *et al*: DNA hypermethylation, Her-2/neu overexpression and p53 mutations in ovarian carcinoma. Gynecol Oncol 111: 320-329, 2008.
13. Rathi A, Virmani AK, Schorge JO, *et al*: Methylation profiles of sporadic ovarian tumors and nonmalignant ovaries from high-risk women. Clin Cancer Res 8: 3324-3331, 2002.
14. Pergolizzi R, Appierto V, Crosti M, *et al*: Role of retinoic acid receptor overexpression in sensitivity to fenretinide and tumorigenicity of human ovarian carcinoma cells. Int J Cancer 81: 829-834, 1999.
15. Saretzki G, Hoffmann U, Rohlke P, *et al*: Identification of allelic losses in benign, borderline, and invasive epithelial ovarian tumors and correlation with clinical outcome. Cancer 80: 1241-1249, 1997.
16. Leary JA, Doris CP, Boltz EM, Houghton CR, Kefford RF and Friedlander ML: Investigation of loss of heterozygosity at specific loci on chromosomes 3p, 6q, 11p, 17p and 17q in ovarian cancer. Int J Gynecol Cancer 3: 293-298, 1993.
17. Godwin AK, Vanderveer L, Schultz DC, *et al*: A common region of deletion on chromosome 17q in both sporadic and familial epithelial ovarian tumors distal to BRCA1. Am J Hum Genet 55: 666-677, 1994.
18. Jacobs IJ, Smith SA, Wiseman RW, *et al*: A deletion unit on chromosome 17q in epithelial ovarian tumors distal to the familial breast/ovarian cancer locus. Cancer Res 53: 1218-1221, 1993.
19. Russell SE, McIlhatton MA, Burrows JF, *et al*: Isolation and mapping of a human septin gene to a region on chromosome 17q, commonly deleted in sporadic epithelial ovarian tumors. Cancer Res 60: 4729-4734, 2000.
20. Kalikin LM, Frank TS, Svoboda-Newman SM, Wetzel JC, Cooney KA and Petty EM: A region of interstitial 17q25 allelic loss in ovarian tumors coincides with a defined region of loss in breast tumors. Oncogene 14: 1991-1994, 1997.
21. Dion F, Mes-Masson AM, Seymour RJ, Provencher D and Tonin PN: Allelotyping defines minimal imbalance at chromosomal region 17q25 in non-serous epithelial ovarian cancers. Oncogene 19: 1466-1472, 2000.
22. Presneau N, Dewar K, Forgetta V, Provencher D, Mes-Masson AM and Tonin PN: Loss of heterozygosity and transcriptome analyses of a 1.2 Mb candidate ovarian cancer tumor suppressor locus region at 17q25.1-q25.2. Mol Carcinog 43: 141-154, 2005.
23. Langan JE, Cole CG, Huckle EJ, *et al*: Novel microsatellite markers and single nucleotide polymorphisms refine the tylosis with oesophageal cancer (TOC) minimal region on 17q25 to 42.5 kb: sequencing does not identify the causative gene. Hum Genet 114: 534-540, 2004.
24. Risk JM, Evans KE, Jones J, *et al*: Characterization of a 500 kb region on 17q25 and the exclusion of candidate genes as the familial Tylosis Oesophageal Cancer (TOC) locus. Oncogene 21: 6395-6402, 2002.
25. Shahabi M, Noori Daloii MR, Langan JE, *et al*: An investigation of the tylosis with oesophageal cancer (TOC) locus in Iranian patients with oesophageal squamous cell carcinoma. Int J Oncol 25: 389-395, 2004.
26. McRonald FE, Liloglou T, Xinarianos G, *et al*: Down-regulation of the cytoglobin gene, located on 17q25, in tylosis with oesophageal cancer (TOC): evidence for trans-allele repression. Hum Mol Genet 15: 1271-1277, 2006.
27. Shaw RJ, Liloglou T, Rogers SN, *et al*: Promoter methylation of P16, RARbeta, E-cadherin, cyclin A1 and cytoglobin in oral cancer: quantitative evaluation using pyrosequencing. Br J Cancer 94: 561-568, 2006.
28. Xinarianos G, McRonald FE, Risk JM, *et al*: Frequent genetic and epigenetic abnormalities contribute to the deregulation of cytoglobin in non-small cell lung cancer. Hum Mol Genet 15: 2038-2044, 2006.
29. Shivapurkar N, Stastny V, Okumura N, *et al*: Cytoglobin, the newest member of the globin family, functions as a tumor suppressor gene. Cancer Res 68: 7448-7456, 2008.
30. Shaw RJ, Omar MM, Rokadiya S, *et al*: Cytoglobin is upregulated by tumour hypoxia and silenced by promoter hypermethylation in head and neck cancer. Br J Cancer 101: 139-144, 2009.
31. Cody NA, Ouellet V, Manderson EN, *et al*: Transfer of chromosome 3 fragments suppresses tumorigenicity of an ovarian cancer cell line monoallelic for chromosome 3p. Oncogene 26: 618-632, 2007.
32. Quinn MC, Filali-Mouhim A, Provencher DM, Mes-Masson AM and Tonin PN: Reprogramming of the transcriptome in a novel chromosome 3 transfer tumor suppressor ovarian cancer cell line model affected molecular networks that are characteristic of ovarian cancer. Mol Carcinog 48: 648-661, 2009.
33. Lounis H, Provencher D, Godbout C, Fink D, Milot MJ and Mes-Masson AM: Primary cultures of normal and tumoral human ovarian epithelium: a powerful tool for basic molecular studies. Exp Cell Res 215: 303-309, 1994.
34. Wojnarowicz PM, Breznan A, Arcand SL, *et al*: Construction of a chromosome 17 transcriptome in serous ovarian cancer identifies differentially expressed genes. Int J Gynecol Cancer 18: 963-975, 2008.
35. Kruk PA, Maines-Bandiera SL and Auersperg N: A simplified method to culture human ovarian surface epithelium. Lab Invest 63: 132-136, 1990.
36. Provencher DM, Lounis H, Champoux L, *et al*: Characterization of four novel epithelial ovarian cancer cell lines. In Vitro Cell Dev Biol Anim 36: 357-361, 2000.
37. Ouellet V, Zietarska M, Portelance L, *et al*: Characterization of three new serous epithelial ovarian cancer cell lines. BMC Cancer 8: 152, 2008.
38. Presneau N, Mes-Masson AM, Ge B, Provencher D, Hudson TJ and Tonin PN: Patterns of expression of chromosome 17 genes in primary cultures of normal ovarian surface epithelia and epithelial ovarian cancer cell lines. Oncogene 22: 1568-1579, 2003.
39. Karolchik D, Baertsch R, Diekhans M, *et al*: The UCSC Genome Browser Database. Nucleic Acids Res 31: 51-54, 2003.
40. Arcand SL, Provencher D, Mes-Masson AM and Tonin PN: OGG1 Cys326 variant, allelic imbalance of chromosome band 3p25.3 and TP53 mutations in ovarian cancer. Int J Oncol 27: 1315-1320, 2005.
41. Ouellet V, Provencher DM, Maugard CM, *et al*: Discrimination between serous low malignant potential and invasive epithelial ovarian tumors using molecular profiling. Oncogene 24: 4672-4687, 2005.
42. Rozen S and Skaletsky H: Primer3 on the WWW for general users and for biologist programers. In: Bioinformatics Methods and Protocols: Methods in Molecular Biology. Krawetz S and Misener S (eds). Humana Press, Totowa, NJ, pp365-386, 2000.
43. Arcand SL, Maugard CM, Ghadirian P, *et al*: Germline TP53 mutations in BRCA1 and BRCA2 mutation-negative French Canadian breast cancer families. Breast Cancer Res Treat 108: 399-408, 2008.
44. Kent WJ, Sugnet CW, Furey TS, *et al*: The human genome browser at UCSC. Genome Res 12: 996-1006, 2002.
45. Fujita PA, Rhead B, Zweig AS, *et al*: The UCSC Genome Browser database: update 2011. Nucleic Acids Res 39: D876-D882, 2011.
46. Sherry ST, Ward MH, Kholodov M, *et al*: dbSNP: the NCBI database of genetic variation. Nucleic Acids Res 29: 308-311, 2001.
47. Ramensky V, Bork P and Sunyaev S: Human non-synonymous SNPs: server and survey. Nucleic Acids Res 30: 3894-3900, 2002.
48. Thomas PD, Campbell MJ, Kejariwal A, *et al*: PANTHER: a library of protein families and subfamilies indexed by function. Genome Res 13: 2129-2141, 2003.
49. Reumers J, Schymkowitz J, Ferkinghoff-Borg J, Stricher F, Serrano L and Rousseau F: SNPeffect: a database mapping molecular phenotypic effects of human non-synonymous coding SNPs. Nucleic Acids Res 33: D527-D532, 2005.
50. Lee PH and Shatkay H: F-SNP: computationally predicted functional SNPs for disease association studies. Nucleic Acids Res 36: D820-D824, 2008.
51. Ng PC and Henikoff S: SIFT: Predicting amino acid changes that affect protein function. Nucleic Acids Res 31: 3812-3814, 2003.

52. Li LC and Dahiya R: MethPrimer: designing primers for methylation PCRs. Bioinformatics 18: 1427-1431, 2002.
53. Manderson EN, Birch AH, Shen Z, Mes-Masson AM, Provencher D and Tonin PN: Molecular genetic analysis of a cell adhesion molecule with homology to L1CAM, contactin 6, and contactin 4 candidate chromosome 3p26pter tumor suppressor genes in ovarian cancer. Int J Gynecol Cancer 19: 513-525, 2009.
54. Cody NA, Shen Z, Ripeau JS, *et al*: Characterization of the 3p12.3-pcen region associated with tumor suppression in a novel ovarian cancer cell line model genetically modified by chromosome 3 fragment transfer. Mol Carcinog 48: 1077-1092, 2009.
55. Zangerl B, Goldstein O, Philp AR, *et al*: Identical mutation in a novel retinal gene causes progressive rod-cone degeneration in dogs and retinitis pigmentosa in humans. Genomics 88: 551-563, 2006.
56. Scriver CR: Human genetics: lessons from Quebec populations. Annu Rev Genomics Hum Genet 2: 69-101, 2001.
57. Lemberg MK and Freeman M: Functional and evolutionary implications of enhanced genomic analysis of rhomboid intramembrane proteases. Genome Res 17: 1634-1646, 2007.
58. Liggett WH Jr and Sidransky D: Role of the p16 tumor suppressor gene in cancer. J Clin Oncol 16: 1197-1206, 1998.
59. Smit AF: Interspersed repeats and other mementos of transposable elements in mammalian genomes. Curr Opin Genet Dev 9: 657-663, 1999.
60. Kolomietz E, Meyn MS, Pandita A and Squire JA: The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors. Genes Chromosomes Cancer 35: 97-112, 2002.
61. Zhou H, Zhao J, Yu CH, *et al*: Identification of a novel box C/D snoRNA from mouse nucleolar cDNA library. Gene 327: 99-105, 2004.
62. Strausberg RL, Feingold EA, Grouse LH, *et al*: Generation and initial analysis of more than 15,000 full-length human and mouse cDNA sequences. Proc Natl Acad Sci USA 99: 16899-16903, 2002.
63. Shih IeM and Kurman RJ: Ovarian tumorigenesis: a proposed model based on morphological and molecular genetic analysis. Am J Pathol 164: 1511-1518, 2004.
64. Osterberg L, Akeson M, Levan K, *et al*: Genetic alterations of serous borderline tumors of the ovary compared to stage I serous ovarian carcinomas. Cancer Genet Cytogenet 167: 103-108, 2006.
65. Trimble CL, Kosary C and Trimble EL: Long-term survival and patterns of care in women with ovarian tumors of low malignant potential. Gynecol Oncol 86: 34-37, 2002.
66. Longacre TA, McKenney JK, Tazelaar HD, Kempson RL and Hendrickson MR: Ovarian serous tumors of low malignant potential (borderline tumors): outcome-based study of 276 patients with long-term (> or =5-year) follow-up. Am J Surg Pathol 29: 707-723, 2005.
67. Fordel E, Geuens E, Dewilde S, *et al*: Cytoglobin expression is upregulated in all tissues upon hypoxia: an in vitro and in vivo study by quantitative real-time PCR. Biochem Biophys Res Commun 319: 342-348, 2004.
68. Burmester T, Ebner B, Weich B and Hankeln T: Cytoglobin: a novel globin type ubiquitously expressed in vertebrate tissues. Mol Biol Evol 19: 416-421, 2002.
69. Shigematsu A, Adachi Y, Matsubara J, *et al*: Analyses of expression of cytoglobin by immunohistochemical studies in human tissues. Hemoglobin 32: 287-296, 2008.
70. Wystub S, Ebner B, Fuchs C, Weich B, Burmester T and Hankeln T: Interspecies comparison of neuroglobin, cytoglobin and myoglobin: sequence evolution and candidate regulatory elements. Cytogenet Genome Res 105: 65-78, 2004.
71. Guo X, Philipsen S and Tan-Un KC: Study of the hypoxia-dependent regulation of human CYGB gene. Biochem Biophys Res Commun 364: 145-150, 2007.
72. Czyzyk-Krzeska MF and Beresh JE: Characterization of the hypoxia-inducible protein binding site within the pyrimidine-rich tract in the 3'-untranslated region of the tyrosine hydroxylase mRNA. J Biol Chem 271: 3293-3299, 1996.
73. Fordel E, Thijs L, Martinet W, Schrijvers D, Moens L and Dewilde Y cells: a step closer to the unraveling of neuroglobin and cytoglobin functions. Gene 398: 114-122, 2007.
74. Xu R, Harrison PM, Chen M, *et al*: Cytoglobin overexpression protects against damage-induced fibrosis. Mol Ther 13: 1093-1100, 2006.
75. Valko M, Izakovic M, Mazur M, Rhodes CJ and Telser J: Role of oxygen radicals in DNA damage and cancer incidence. Mol Cell Biochem 266: 37-56, 2004.
76. Klaunig JE and Kamendulis LM: The role of oxidative stress in carcinogenesis. Annu Rev Pharmacol Toxicol 44: 239-267, 2004.
77. Chua PJ, Yip GW and Bay BH: Cell cycle arrest induced by hydrogen peroxide is associated with modulation of oxidative stress related genes in breast cancer cells. Exp Biol Med (Maywood) 234: 1086-1094, 2009.
78. Thuy le TT, Morita T, Yoshida K, *et al*: Promotion of liver and lung tumorigenesis in DEN-treated cytoglobin-deficient mice. Am J Pathol 179: 1050-1060, 2011.
79. Urban S, Lee JR and Freeman M: *Drosophila* rhomboid-1 defines a family of putative intramembrane serine proteases. Cell 107: 173-182, 2001.
80. Urban S, Lee JR and Freeman M: A family of Rhomboid intramembrane proteases activates all *Drosophila* membrane-tethered EGF ligands. EMBO J 21: 4277-4286, 2002.
81. Freeman M: Proteolysis within the membrane: rhomboids revealed. Nat Rev Mol Cell Biol 5: 188-197, 2004.
82. Cancer Genome Atlas Research Network: Integrated genomic analyses of ovarian carcinoma. Nature 474: 609-615, 2011.