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Rare Germline Mutations in *PALB2* and Breast Cancer Risk: A Population-Based Study

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Abstract

Germline mutations in the *PALB2* gene are associated with an increased risk of developing breast but little is known about the frequencies of rare variants in *PALB2* and the nature of the variants that influence risk. We selected participants recruited to the Women's Environment, Cancer, and Radiation Epidemiology (WECARE) Study and screened lymphocyte DNA from cases with contralateral breast cancer (n = 559) and matched controls with unilateral breast cancer (n = 565) for *PALB2* mutations. Five pathogenic *PALB2* mutations were identified among the cases (0.9%) versus none among the controls (p=0.04). The first degree female relatives of these five carriers demonstrated significantly higher incidence of breast cancer than relatives of non-carrier cases, indicating that pathogenic *PALB2* mutations confer an estimated 5.3 fold increase in risk (95% CI: 1.8–13.2). The frequency of rare (<1% MAF) missense mutations was similar in both groups (23 versus 21). Our findings confirm in a population-based study setting of women with breast cancer the strong risk associated with truncating mutations in *PALB2* that has been reported in family studies. Conversely, there is no evidence from this study that rare *PALB2* missense mutations strongly influence breast cancer risk.

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Keywords

PALB2; breast cancer; case-control; contralateral

Introduction

PALB2, originally identified as a BRCA2-interacting protein, is crucial for key BRCA2 genome caretaker functions (Xia, et al., 2006) and more recently has also been shown to interact with BRCA1 (Sy, et al., 2009a; Sy, et al., 2009b; Zhang, et al., 2009). Germline mutations in *PALB2* (MIM# 610355), are associated with increased risk of breast (Rahman, et al., 2007) and mutations have been identified in approximately 1% of hereditary breast cancer (HBC) families throughout the world, summarized in (Tischkowitz and Xia, 2010). The first study showing an association with germline mutations in *PALB2* and breast cancer estimated the relative risk to be 2.3 (95% confidence interval (C.I.) 1.4–3.9) (Rahman, et al., 2007), but subsequent studies have suggested that the risk could be higher (Erkko, et al., 2008; Southey, et al., 2010). In this investigation we examine the breast cancer risk conferred by *PALB2* mutations in a population-based case-control study of high risk women, the WECARE Study (Bernstein, et al., 2004). In this study, mutation frequencies in incident cases of contralateral breast cancer (CBC) are compared with the population from which CBC cases emerge, namely survivors of unilateral breast cancer (UBC). This study design is especially advantageous for studying rare genetic mutations, since the frequencies of true risk variants are higher in both cases and controls as both have breast cancer (Begg and Berwick, 1997). Other HBC genes such as *BRCA1/BRCA2* and the *CHEK2**1100delC variant have been studied in this way and have demonstrated increased risk of contralateral breast cancer (CBC) (Fletcher, et al., 2009; Malone, et al., 2010). We screened the coding regions and adjacent intronic areas of the *PALB2* gene in lymphocyte DNA from *BRCA1/BRCA2*-negative women participating in the WECARE study to determine the mutation frequency in CBC cases compared to UBC controls.

Methods

Study Population

The Women's Environment, Cancer, and Radiation Epidemiology Study is a population-based, nested case-control study of CBC, which has been described previously (Bernstein, et al., 2004). All WECARE Study participants were ascertained through one of five population-based cancer registries covering the country of Denmark along with the State of Iowa, Los Angeles County and the Orange County-San Diego regions of California, and three western Washington counties in the United States (the US registries participate in the Surveillance, Epidemiology, and End Results [SEER] registry system). All participants were diagnosed before age 55 years between 1985 and 2000 with a first primary invasive breast cancer that had not spread beyond regional lymph nodes. Cases were diagnosed with a second primary invasive or *in situ* CBC at least one year after first primary diagnosis in the period 1986 to 2001; controls were women with unilateral breast cancer (UBC) who did not develop a second primary breast cancer during the study period (until 2001). For this investigation we selected cases and controls that had been previously screened for pathogenic *BRCA1* and *BRCA2* mutations by denaturing high-performance liquid chromatography (Malone, et al., 2010) and excluded individuals with detectable pathogenic mutations in these genes according to the following criteria: 1) exon sequence changes predicted to truncate protein production; 2) splice site mutations located within two base pairs classified according to the of an intron/exon boundary or shown to cause aberrant splicing; 3) missense changes with known pathogenic functional effects. Our final analytic

group consisted of 559 CBC cases and 565 UBC matched controls and the characteristics of the study participants are given in Table 1.

Molecular Methods

Genomic DNA was obtained from lymphocytes using standard extraction methods. The 13 coding exons of *PALB2* (NCBI reference sequence NM_024675.3) were screened using high-resolution melt analysis (HRM), a method with similar sensitivity to sequencing (Wittwer, 2009). All work was carried out in a single laboratory using the conditions listed in Table 2. The PCR for HRM was performed in 5ul total volume (2ul mastermix, 0.5ul LCGreen, 0.5ul each of forward and reverse primers (2.5uM stock concentration), 0.5ul of double distilled sterile water, 1ul of DNA (25ng/ul). As indicated in Table 2, Q solution instead of water was used for the exon 1 PCR and 25mM of MgCl₂ was added to the PCRs for exons 5.1, 13.1 and 13.2. The PCR protocol was as follows (for 66°C annealing temperature): 95°C for 2min, 95°C for 45sec, 73°C 45sec Touchdown (-1°C increment for 7 cycles), followed by 95°C for 45sec, 66°C for 45sec for 35 cycles. The PCR products were then analysed by HRM using the LightScanner instrument (Idaho Technologies, Salt Lake City, Utah) according to the manufacturer's protocol. Nucleotide numbering reflects cDNA numbering where +1 corresponds to the A of the ATG translation initiation codon in the reference sequence (NM_024675.3) and the initiation codon is codon 1. Variants were confirmed by Sanger sequencing and were classified as pathogenic using the same criteria as the *BRCA1/BRCA2* study (Malone, et al., 2010) mentioned above. Sequence changes that did not fulfill these criteria for being pathogenic were divided into three categories 1) missense variants, 2) exonic synonymous single nucleotide variants ("synonymous"), and 3) intronic variants. We further examined the effects of missense mutations, classified on the basis of bioinformatic tools that predict functional significance: Sorting Intolerant from Tolerant (SIFT) (Kumar, et al., 2009), Polyphen (Adzhubei, et al., 2010) and Align GVGD (Tavtigian, et al., 2008). SIFT and Polyphen were used under default conditions and with the programs' internally generated alignments. Align-GVGD was used with a curated alignment in which the sequence furthest diverged from human was from the fish *Danio rerio*. This alignment, or updated versions thereof, is available at the Align-GVGD website <http://agvgd.iarc.fr/alignments>.

Statistical methods

First, we evaluated the broad case-control associations with respect to various, possibly overlapping mutation types using individuals who had wild-type *PALB2* as the baseline and estimating the risk of carrying a deleterious, missense synonymous, or intronic mutation. Due to the small sample sizes in some of the analyses presented, we employed Fishers exact test and exact (i.e. permutation-based) methods for constructing confidence intervals using Cytel Studio© software (Cytel Inc., Cambridge, MA, USA). The exact two-sided Cochran-Armitage trend test was employed to investigate the relationship between the case-control status and the bioinformatics tools classifications and the exact Kruskal-Wallis test was performed to test for differences in continuous patient characteristics by mutation type. We estimated the relative risk conferred by pathogenic *PALB2* mutations by comparing the breast cancer incidence in their first degree female relatives with the corresponding incidence in first degree relatives of non-carrier CBC cases using conventional actuarial techniques, and then by transforming the resulting estimate (Saunders and Begg, 2003). We further examined the relative risks of individual rare missense variants (MAF <1%) using a hierarchical modeling approach developed for this purpose (Capanu, et al., 2011). This model included adjustment for eight common polymorphisms (MAF >1%), the presence of a truncation or splicing mutation, the presence of a rare intronic mutation, the presence of a rare synonymous mutation, as well as childbearing history as possible confounders. The effects of individual variants were adjusted on the basis of the average age at diagnosis of

the carriers of the specific variant, family history of breast cancer in first-degree relatives for these carrier probands, as well as the three bioinformatic prediction tools.

Results

We screened the *PALB2* gene in a total of 1124 women with breast cancer and we identified five pathogenic mutations in the cohort of 559 women with CBC versus 0/565 women with UBC ($p=0.04$), Table 3. Of the five mutations, three have been previously reported: 1592delT is a known founder mutation in Finland (Erkko, et al., 2007), while W1038X and Y1183X have been reported by several groups (Table 4). Among the five carriers of these mutations, the median ages of the first and second breast cancers were 46 and 55 years respectively and all probands had at least one first-degree relative affected with breast cancer, although none had an additional young-onset case (under age 50 years) in the family. Nonetheless the breast cancer incidence in these 17 first degree female relatives of *PALB2* carriers was significantly higher than the corresponding incidence in first degree relatives of non-carrier cases, and from this we derived a relative risk estimate for carriers of a pathogenic mutation of 5.3 (95% CI: 1.8–13.2). The majority of the breast cancers in these five women were ER positive, PR positive infiltrating ductal type, with the notable exception of the carrier for the 1592delT mutation where both tumors were ER negative, PR negative, the first being classified as medullary-type (Table 4).

We found no statistically significant difference in the frequency of missense variants between cases and controls (these variants are listed in Supp. Table S1). However, when classified by their predicted degrees of pathogenicity using the three different models, SIFT (Kumar, et al., 2009), Polyphen (Adzhubei, et al., 2010) and Align GVGD (Tavtigian, et al., 2008), for which the criteria was set as GV=0 and GD 65, following the classification at <http://agvgd.iarc.fr/classifiers.php>, there were marginally significant trends for SIFT and align-GVGD, evidenced by the higher relative frequencies of cases in the highest risk categories (Table 3). Further analyses of the individual rare missense mutations using hierarchical modeling revealed no specific mutations as significantly associated with risk (data not shown).

The overall frequency of intronic variants was higher in cases than controls (Table 3), and we did observe a notably increased frequency of rare intronic variants (MAF<0.1%) with 12 rare intronic variants seen in cases versus one variant in controls (RR=13.7, 95% CI 2.0, 591.3) listed in Supp. Table S2. This finding remained significant even when rare and common intronic variants were combined ($p=0.01$). Further *in silico* analysis of these intronic variants using Human Splicing Finder v2.4.1 (Desmet, et al., 2009) did not predict that they would have significant effects on splicing. Furthermore, when we examined the incidence of breast cancer in first degree relatives of carriers of rare intronic variants the rate was not elevated compared to non-carriers. Eleven of the 12 individuals were of non-Hispanic white ethnicity making an ethnic-specific effect unlikely. When the median age of onset of the second breast cancer was compared between the rare intronic group (51.8 years, $n=11$), carriers of deleterious mutations (56.6 years, $n=5$) and the rest of the cases (age 51.1, $n=543$) no significant difference was observed ($p=0.3$, Kruskal-Wallis test). There was no significant effect of having received radiation therapy among subsets of mutation carriers (number of events was too small to analyze deleterious variants independently; data not shown).

Discussion

A number of studies have implicated *PALB2* gene mutations as a rare, but important, contributing factor to hereditary breast cancer (Tischkowitz and Xia, 2010). The results

presented here support these findings in a population-based setting, and confirm that women with germline *PALB2* mutations also have an increased risk of CBC. Although only five clearly pathogenic mutations were identified in the group of 1124 women, all of these occurred among the cases. The penetrance of *PALB2* gene mutations was originally estimated to be associated with a 2.3-fold increased risk (Rahman, et al., 2007) but subsequent studies suggested this could be higher, at least for specific mutations (Erkko, et al., 2008; Southey, et al., 2010). The relative risk estimate of 5.3 from our study is indeed higher, although this estimate needs to be interpreted with caution given the small number of carriers and carrier relatives in our study and the correspondingly wide confidence intervals.

Despite a large number of published studies, to date no definitely pathogenic *PALB2* missense variants have been identified, suggesting that they are non-existent or rare. Our results are consistent with this literature. As an analogy, in the breast cancer predisposing genes *BRCA1* and *BRCA2* the vast majority of missense variants are neutral, but a few deleterious variants have been identified. The identification of isolated, individual deleterious mutations using case-control studies requires very large numbers of subjects, or evidence that particular types of missense variants are deleterious collectively, allowing aggregation of the data to create sufficient statistical power. Our hierarchical modeling (data not shown) was designed for this purpose, but did not uncover any trends of this nature. Thus, our study does not rule out the possibility that isolated missense *PALB2* mutations may be deleterious, but it offers no strong evidence that this is a likely possibility.

The clinical significance of our observation that rare intronic variants are more frequent in the CBC cohort is unclear as none of these variants were predicted to be pathogenic by affecting splicing and only one has been reported previously. Further investigations to address this would involve studying these variants in additional breast cancer cohorts and, at the cellular level, looking for possible functional effects such as diminished *BRCA2* binding capacity and reduced homologous recombination efficiency (Tischkowitz, et al., 2007). It should also be noted that we did not see an increased incidence of breast cancer in first degree relatives of carriers of rare intronic variants which would argue against these being pathogenic.

Our study was conducted using population-based series of breast cancers and as such it avoids the potential problem of ascertainment bias that can occur in cohorts derived from familial cancer clinics or other high-risk settings. Nevertheless, our study has some limitations. First, while a large number of women were screened for *PALB2* mutations, the relative rarity of these mutations meant that only a small number of pathogenic mutations were identified. Second, because the study excluded women with synchronous cancers and women who underwent prophylactic contralateral mastectomy following initial breast cancer, and included only women who survived their initial breast cancer, the results may underestimate the overall mutation prevalence. Lastly, we used High Resolution Melting analysis which, as with conventional sequencing, would miss detection of large exon deletions. However, based on our previous experience on the rarity of *PALB2* exon deletions (Tischkowitz, et al., 2009) it is unlikely that we would have missed a significant number of mutations.

In summary, we have confirmed that germline deleterious truncating *PALB2* mutations are associated with breast cancer in a population-based case-control study. Although *PALB2* mutations are a rare cause of breast cancer, their overrepresentation in the cohort of women with CBC is relevant to the clinical management of newly diagnosed women with breast cancer who are found to be *PALB2* mutation carriers as it implies a significant risk of developing a second breast cancer. Our study also provides suggestive evidence that rare intronic variants may be associated with risk, but this finding requires careful validation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Table 1

Characteristics of 559 Cases (women with contralateral breast cancer) and 565 Controls (women with unilateral breast cancer)

Characteristic	Cases (n=559)		Controls (n=565)	
	N	%	N	%
Age at diagnosis, years				
<30	6	1.1	7	1.2
30-34	14	2.5	15	2.7
35-39	51	9.1	50	8.9
40-44	138	24.7	137	24.3
45-49	178	31.8	180	31.9
50-54	172	30.8	176	31.1
Median	47		47	
Range (interquartile)	43-51		43-51	
At-risk period, years				
<5	318	56.9	320	56.6
>=5	241	43.1	245	43.4
Race/ethnicity				
Non-Hispanic White	519	92.8	525	92.9
Hispanic White	14	2.5	17	3.0
Black	17	3.0	16	2.8
Japanese	1	0.2	1	0.2
Chinese	1	0.2	1	0.2
Filipino	5	0.9	5	0.9
Other Asian/other	2	0.4	0	0.0
Jewish ancestry				
No	504	90.2	519	91.9
Yes	42	7.5	37	6.5
Unknown	13	2.3	9	1.6
Family history of breast cancer				
No first-degree relatives	388	69.4	450	79.6

Characteristic	Cases (n=559)		Controls (n=565)	
	N	%	N	%
Any first-degree relatives	167	29.9	110	19.5
Adopted or unknown	4	0.7	5	0.9
Family history of ovarian cancer				
No first-degree relatives	538	96.2	545	96.5
Any first-degree relatives	17	3.0	15	2.7
Adopted or unknown	4	0.7	5	0.9
Geographic Region				
State of Iowa	89	15.9	89	15.8
Orange County and San Diego County, California	93	16.6	94	16.6
Los Angeles County	150	26.8	149	26.4
Three Western Washington Counties	85	15.2	86	15.2
Country of Denmark	142	25.4	147	26.0
Menopausal status				
Premenopausal	203	36.3	185	32.7
Postmenopausal, <45 years	133	23.8	142	25.1
Postmenopausal, ≥45 years	222	39.7	235	41.6
Unknown	1	0.2	3	0.5
Estrogen receptor status of first primary breast cancer				
Positive	290	51.9	310	54.9
Negative	126	22.5	113	20.0
Other or Unknown	143	25.6	142	25.1
Chemotherapy for first primary breast cancer				
No	322	57.6	273	48.3
Yes	237	42.4	292	51.7
Adjuvant hormone therapy for first primary breast cancer				
No	394	70.5	369	65.3
Yes	165	29.5	195	34.5
Unknown	0	0.0	1	0.2

Characteristic	Cases (n=559)		Controls (n=565)	
	N	%	N	%
Radiation therapy				
No	291	52.1	176	31.2
Yes	268	47.9	389	68.8
Oophorectomy status				
Yes	35	6.3	50	8.8
No	523	93.6	514	91.0
Unknown	1	0.2	1	0.2

Table 2Primer sequences and PCR conditions used for *PALB2* analysis

Exon	Primer Sequences		Annealing temperature (°C)
	Forward	Reverse	
1	GGATTTAATTGGCCGAGTTTA	ACACAAAGCCAGGCCTAAAAC	66 ^a
2+3	CTGACTCCACCTTTCCACTTG	TACCTGGGAAATGAATAATAAGCA	66
4.1	GCCTGAATGAAATGTCAGTATT	ATGTCCTCTTCTGCTGCTTCTT	64
4.2	GGCCCAGGAGGATTACCTAT	TGGGCAGTTGGTGGGAATTA	66
4.3	TAATTCCACCAACTGCCCA	GTTATTGTAGGTGAGTTCATTTAGAGAACA	66
4.4	TGTTCTCTAAATGAACTCACCTACAATAAC	AATTTCACTTTGGTCAGTTTCCTC	64
4.5	CAGAGTCATTTGGATGTCAAGAAA	TGTTAACAATCGACAGGCTAGAAGTT	66
4.6	CATCAGATCATTGTGAACCACT	GCCAGGCAAATAGTAATTGTAACTT	66
5.1	TTTGTGCATCAGTGAAACAGATTGTC	GACTCAGTTCCTCTGGAAAAATACA	65 ^b
5.2	TTAAGCTTGAAAAAGTGAAGTCCTG	GTTCGTCCAGCAACTTCTGTAGAT	66
5.3	GCTTTCCCCATCTTAGGTACTACTC	ACAGAGTCACAGTCACAGGTAGGTT	66
5.4	AACCTACCTGTGACTGTGACTCTGT	GCTGTTTACATCTACTAAGGCATTT	64
6	TAGACATTTTGATGAGTGGGTAATG	CCAAATCTGTTTTTCTGAATCTGTT	64
7	TCACTTTCATACCTTATGCTTT	GGTAAGCTGCCCATCTACATTATC	64
8	TTGGAAAATCTGGATTAAACAAAAA	ACTTAAAACCAGCTGACAGAGACAA	64
9	TTAAAAGGTTACTCCTCACATCAC	AACGAGATCCTAGTTACCCAACTTT	66
10	CCTAGAGACTGCTTTAGTGCAAAGT	TCTTCACAACAACCCTGTAAAATTAG	66
11	GTCACCTCCTAAGACATGCTATGAT	TGAGACCAACAGTAACACACAAAGT	64
12	TGTCTCTGCCAGATCTTTATTTTTC	TTTCCATTCTTCTAAGTGACACAAA	64
13.1	GTTTTGGGAACATGGTTTTGAC	TTTAAAGTGTCATTCTAGATATTCTCC	64 ^b
13.2	CTGGATATATTGGGCCTCTTAGTA	AAAGAATGGGAAACAATAACATGC	65 ^b

^a = plus Q solution (Qiagen, Valencia, California)^b = plus 25mM MgCl₂

Table 3

Summary of variants identified in cases and controls

All variants			Rare variants (MAF less than or equal to 1%)			
Variant category	CBC cases	UBC controls	RR (95%CI) p-value	Variant category	CBC Cases	UBC controls
Wild type	275	316		Wild type	275	316
Deleterious	5	0	-- p=0.04 *	Deleterious	5	0
Intronic	255	211	1.4 (1.1, 1.8) p=0.01 *	Intronic	12	1
Synonymous	35	30	1.3 (0.8, 2.3) p=0.32 *	Synonymous	5	9
Missense	128	116	1.3 (0.9, 1.7) p=0.14	Missense	23	21
<i>SIFT</i>				<i>SIFT</i>		
Wild type	275	316	1.0	Wild type	275	316
Tolerated	92	95	1.1 (0.8, 1.6)	Tolerated	18	18
Affect protein function	35	21	1.9 (1.1, 3.5) p=0.04#	Affect protein function	5	3
<i>Polyphen</i>				<i>Polyphen</i>		
Wild type	275	316	1.0	Wild type	275	316
Benign	60	60	1.1 (0.8, 1.7)	Benign	6	10
Possibly Damaging	1	1		Possibly Damaging	1	1
Probably Damaging	66	55	1.4 (0.9, 2.0) p=0.1#	Probably Damaging	16	10
<i>GV</i> [†]				<i>GV</i>		
Wild type	275	316	1.0	Wild type	275	316
GV>0	95	96	1.1 (0.8, 1.6)	GV>0	21	19
GV=0	32	20	1.8 (1.0, 3.5) p=0.05#	GV=0	2	2
<i>GD</i> [‡]				<i>GD</i>		
Wild type	275	316	1.0	Wild type	275	316
GD>0	95	96	1.1 (0.8, 1.6)	GD>0	21	19
GD=0	32	20	1.8 (1.0, 3.5) p=0.05#	GD=0	2	2

All variants			Rare variants (MAF less than or equal to 1%)				
Wild type	275	316	1.0	Wild type	275	316	1.0
GD<65	97	96	1.1 (0.8, 1.6)	GD<65	22	20	1.3 (0.6, 2.5)
GV>=65	31	19	1.9 (1.0, 3.6) p=0.05 [#]	GV>=65	1	1	1.1 (0.0, 90.5) p=0.6 [#]

^{*} P-values using Fisher's exact test evaluating the risk of developing CBC among carriers of different mutation types relative to wild-type PALB2 subjects.

[#] Exact two-sided Cochran-Armitage trend test p-value. Missense variants were further characterized using SIFT (Kumar, et al., 2009), Polyphen(Adzhubei, et al., 2010) and Align GVG (Tavtigian, et al., 2008).

[†] GV, or Grantham Variation, is a measure of range of variation at an amino acid position in a protein multiple sequence alignment. GV=0 is indicative of an invariant position, and higher GVs are indicative of greater variability (Tavtigian, et al., 2008).

[‡] GD, or Grantham Deviation, is a measure of the severity of a missense substitution with respect to the range of variation at its position in a protein multiple sequence alignment. GD 65 is indicative of a substitution that is non-conservative with respect to the observed range of variation (Tavtigian, et al., 2008).

Table 4
Summary of the deleterious mutations and tumor characteristics of the five *PALB2* carriers with CBC

Exon	Mutation	Type	Tumor characteristics				Family history of BC	Previously reported
			Age	Type	Grade	ER	PR	
4	c.1592delT, p.Leu531Cysfs*	Deletion frameshift	47	Med IDC	3/3 3/3	neg neg	neg	(Erkko, et al., 2007)
			55				neg	
9-1	c.2835-1G>C	splice	46	IDC IDC	3/3 2/3	neg pos	neg	no
			53				pos	
10	c.3113G>A, p.Trp1038*	stop/ splice	38	IDC IDC/Lob	U U	pos pos	pos	(Casadei, et al., 2011; Garcia, et al., 2009; Rahman, et al., 2007; Southey, et al., 2010; Wong, et al., 2011)
			47				pos	
11+1	c.3202+1G>C	splice	46	IDC IDC	2/3 U	pos pos	pos	no
			60				pos	
13	c.3549C>G, p.Tyr1183*	stop	53	IDC IDC	U 1/3	pos pos	pos	(Hofstatter, et al., 2011; Rahman, et al., 2007; Reid, et al., 2007)
			68				neg	

ER= estrogen receptor status, PR = progesterone receptor status, IDC = infiltrating ductal carcinoma, Med= medullary, Lob = lobular, U= unknown, pos = positive, neg = negative (Reference sequence NM_024675.3)