Analysis of *KLLN* as a high penetrance breast cancer predisposition gene

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Abstract

KLLN is a p53 target gene with DNA binding function and represents a highly plausible candidate breast cancer predisposition gene. We screened for predisposing variants in 860 high-risk breast cancer families using high resolution melt analysis. A germline c.339_340delAG variant predicted to cause premature termination of the protein after 57 alternative amino acid residues was identified in 3/860 families who tested negative for *BRCA1* and *BRCA2* mutations and in 1/84 sporadic breast cancer cases. However, the variant was also detected in 2/182 families with known *BRCA1* or *BRCA2* mutations and in 2/464 non-cancer controls. Furthermore, loss of the mutant allele was detected in 2/2 breast tumors. Our data suggests that pathogenic mutations in *KLLN* are rare in breast cancer families and the c.339_340delAG variant does not represent a high-penetrance breast cancer risk allele.

Keywords: familial breast cancer, germline, mutation, BRCAX, KILLIN

Introduction

The genetic cause of multiple-case breast cancer or breast/ovarian cancer families has only been elucidated in approximately one third of families, with the majority of these explained by germliene *BRCA1* or *BRCA2* mutations [1]. Additional moderate or low penetrance variants have been identified in genes such as *ATM* or *CHEK2*, but it is likely that additional gene variants contribute to the apparently highly penetrant genetic predisposition observed in many breast cancer kindreds negative for mutations in known predisposition genes.

KILLIN is a 20 kDa nuclear protein that is necessary for p53-induced apoptosis [2]. In addition, germline methylation of the *KLLN* promoter has been associated with an increased risk of breast and renal cancer in Cowden syndrome and Cowden syndrome-like individuals [3], providing a substantial biological basis for considering *KLLN* as a breast cancer predisposition gene. We screened for germline variants in the *KLLN* gene in multiple-case breast cancer families to assess this possibility. This is the first study to analyse the relationship of this gene to breast cancer risk.

Materials and Methods

Sample cohorts

Multiple-case breast cancer families were selected from the Kathleen Cunningham Foundation Consortium for Research into Familial Breast Cancer (kConFab), which recruits families from Familial Cancer Clinics in Australia and New Zealand [1]. An index case from each family was previously screened for mutations in *BRCA1* and *BRCA2* including for large deletions.

Additional breast cancer cases with features of a germline predisposition (age of onset under the age of 40, a family history of breast cancer (defined as two or more cases of breast cancer in first or second degree relatives) or bilateral breast cancer irrespective of age of onset or family history) were provided by Dr. Diana Eccles from patients presenting to hospitals in the south of England [4]. Sporadic breast cancers were provided by the Peter MacCallum Cancer Centre tissue bank or by Dr Nick Hayward (Queensland Institute for Medical Research, Brisbane, Australia). Unselected ovarian cancer cases were obtained from patients presenting to hospitals in the south of England, UK [5].

Non-cancer control DNA samples were obtained from kConFab (age- and ethnicity-matched best friend controls) and from the Princess Anne Hospital, Southampton UK [6].

Variant screening by High Resolution Melt (HRM)

Oligonucleotide primers were designed using AmplifX v 1.5.4 (http://ifrjr.nord.univ-mrs.fr/AmplifX) and Primer3 [7] to amplify the coding region of the *KLLN* gene (NM_0011126049.1) in four overlapping fragments (Table 1). DNA samples for HRM were whole-genome amplified using RepliG according to the manufacturer's instructions (Qiagen, Hilden, Germany). HRM analysis and DNA resequencing were performed as described previously [8]. All samples with variant HRM profiles were re-amplified from non-whole genome amplified DNA and Sanger sequenced.

Analysis of Loss of Heterozygosity (LOH)

Epithelial tumour cells were needle microdissected from 10 micron sections of formalin-fixed paraffin embedded tissue and DNA was extracted as previously described [9]. The variant of interest was PCR amplified and sequenced as above. LOH was estimated based on the

intensity of the variant nucleotide relative to the normal nucleotide in the sequence electrophoretogram.

Results

We screened the entire *KLLN* coding region for germline variants in 556 individuals from 460 multiple-case breast cancer families with no known pathogenic mutations in *BRCA1* or *BRCA2* from the Australasian kConFab cohort using HRM. We identified 5 non-synonymous sequence variants and 1 indel (Tables 2 and 3). The latter variant, a 2 bp frame shifting deletion, c.339_340delAG, was observed in 3 families and is predicted to lead to alteration of the C-terminal of the protein with 57 novel amino acids substituted from residue 114 prior to premature termination (p.(Ala115Serfs*58)).

We screened for the c.339_340delAG variant in a number of other cohorts. It was observed in one sporadic breast cancer case (1/84, 1.2%) but not in the index cases of an additional 399 breast cancer families from kConFab or 379 individuals diagnosed with breast cancer selected on the basis of: age of onset under the age of 40, a family history of breast cancer or bilateral breast cancer irrespective of age of onset or family history [4]. The variant was also observed in non-cancer controls at a similar frequency (2/463, 0.4%), and also in 2/182 families (1.1%) known to carry *BRCA1* or *BRCA2* mutations. As the genetic basis of breast and ovarian cancer is sometimes shared in families, we also assessed a cohort of unselected ovarian cancers, but no germline or somatic mutations were detected in any of these cases (n=108).

Classic tumor suppressor genes such as *BRCA1* are very frequently associated with bi-allelic inactivation in tumors, consistent with the “two-hit hypothesis” [10]. LOH, where the wild-type allele is lost in the tumor, is one mechanism whereby cancer cells acquire bi-allelic

inactivation of a tumor suppressor [11]. We sequenced the full *KLLN* gene in two available tumors from different individuals from the same family who both carried the c.339_340delAG variant (2202 and 2191). Both tumors showed clear loss of the variant allele and retention of the wild-type allele (Figure 1). No inactivating somatic mutations were identified in the retained allele in these cases.

We tested the predicted effect of the 5 non-synonymous variants using SIFT [12], PolyPhen-2 [13], MutPred [14], PMUT [15], MutationTaster [16] and SNAP [17]. P.Gly44Arg was predicted to have a damaging effect in 4/6 of these in silico prediction tools (Table 3), however this variant has been observed in the 1000 Genomes project [18] and in Asian populations at a frequency of ~1% [19]. One of two linked variants, p.Trp149Arg, was called deleterious in 2/6 predictors, but both this and the linked Asn131Ser allele were previously identified as polymorphisms. The other two variants, p.Cys99Trp and p.Pro104Ser were predicted to be damaging in 1/6 and 2/6 algorithms respectively. These variants were screened in our control cohorts but were not observed.

Discussion

KILLIN is a 178 amino acid residue protein that was identified through a screen for p53 target genes [2]. It functions as a DNA binding protein that is thought to cause stalled replication forks and thus S-phase arrest, and is essential for p53-mediated apoptosis. Accordingly, KILLIN represents a novel mechanism of p53 arrest distinct from p21-mediated G1 arrest, and one that is linked to apoptosis. While the mechanism for KILLIN-mediated apoptosis is not entirely elucidated, it is thought to be connected to the DNA damage response pathway, including CHK1 and the breast cancer predisposition gene CHK2. Thus, *KLLN* represents a

highly plausible candidate breast cancer tumor suppressor, as it intersects with the key hereditary predisposition pathway of DNA damage and repair.

We identified a likely inactivating mutation in *KLLN* in multiple-case breast cancer families that was predicted to alter the C-terminal portion of the protein after residue 114. However, functional domain mapping of the KILLIN protein suggested that only amino acid residues 8-49 are required for induction of apoptosis and the critical DNA binding activity [2]. Thus, in spite of the predicted disruption of the final third of the protein, the c.339_340delAG variant may still provide functional KILLIN protein.

We sought further genetic evidence for *KLLN* as a breast cancer predisposition gene by analyzing for the frameshift variant in several cohorts of individuals, including multiple-case breast and breast/ovarian cancer families, non-familial breast cancer including an early age-of-onset group, non-familial ovarian cancer and two control cohorts. Additional individuals carrying the c.339_340delAG variant were found at a low frequency (0 – 1.2%). However, the frequency of the c.339_340delAG variant was similar in the cancer and control groups (~0.5%) and was also detected in two families where known pathogenic mutations in *BRCA2* explained all affected individuals in each pedigree. In addition, the analysis of LOH in two available tumor samples indicated that the wild-type allele was retained in both cases, contrary to the expectation of bi-alleleic inactivation. Thus, it appears very unlikely that the c.339_340delAG variant in *KLLN* is a high-penetrance tumor suppressor allele. In addition, despite analysis of a large number of index cases from breast cancer families, no other deleterious mutations were identified suggesting that the *KLLN* is unlikely to play a significant role in high risk breast cancer families.

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Statement of author contributions

IGC and GM conceived of the study; ERT and DYHC carried out experiments and analysed data; KLG interpreted data and generated the figure; IGC, KLG and ERT were involved in writing the paper. All authors had final approval of the submitted and published versions.

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238 **Table 1. Oligonucleotide PCR primers**

Primer Name	**Primer sequence (5'-3')**	**Location in cDNA relative to coding start site (bp)**
Killin_1F	TCGGTTCTCAGAGACCACCT	-59 - 84
Killin_1R	GCTTCCTACCGTTCCGTACTT	
Killin_2F	GGGGTTACCGGGTTGAGT	54 - 278
Killin_2R	CGAGCAAAGGAAGAAGACGA	
Killin_3F	TTCCCACTCCCCAGTGATAG	226 - 416
Killin_3R	GGATGTGGGTGCTTGTGTAA	
Killin_4F	GAACCCCAACCCTTCCTG	306-580
Killin_4R	GCGCAGAATAGGTCGATGTAG	

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Table 2. Frequency of the c.339_340delAG variant in sample cohorts

Cohort	Frequency
Multiple-case breast cancer families without known mutations	3/860
Multiple-case breast cancer families with known *BRCA1* or *BRCA2* mutations	2/182
Breast cancer cases under 40 years old	0/379
Non-familial breast cancer cases	1/84[1]
Non-familial ovarian cancer	0/108[2]
Controls	
Total	2/464
Best friend controls	2/227
Southampton controls	0/237

1. n=32 tumor DNA only analysed

2. n=9 tumor DNA only analysed

Table 3. Other variants

Variant[1]	dbSNP (v.135)	Protein	Mutation Taster	PMUT	MutPred	SIFT	PolyPhen	SNAP	kConFab	Controls	Early onset breast
c.130G>C	rs186106725	p.Gly44Arg	Polymorphism, protein may be affected	Neutral	Neutral	Damaging	Probably damaging	Non-neutral	7/446	nd	nd
c.297C>G	-	p.Cys99Trp	Polymorphism	Neutral	Neutral	Tolerated	Probably damaging	Neutral	2/860	0/464	1/379
c.310C>T	-	p.Pro104Ser	Polymorphism	Neutral	Neutral	Damaging	Probably damaging	Neutral	1/860	0/464	0/379
c.392A>G and c.445T>A	rs147932146 and rs144811392	p.Asn131Ser and p.Trp149Arg	Polymorphisms	Neutral	Neutral	Tolerated/ Damaging	Benign/ Possibly damaging	Neutral	2/447	nd	nd

1. Position relative to NM_0011126049.1

Legends to Figures

Figure 1. LOH analysis of the c.339_340delAG variant. Sequencing of c.339_340delAG variant in wild type individual, germline DNA from individual 2191 heterozygous for the variant, tumor DNA from individual 2202 showing complete loss of variant allele, tumor DNA from individual 2191 showing loss of variant allele (with some contamination from normal DNA present).