Breast Cancer (2019) 26:11–28
https://doi.org/10.1007/s12282-018-0894-0

REVIEW ARTICLE

CDKN2A/P16INK4A variants association with breast cancer and their in-silico analysis

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Received: 30 March 2018 / Accepted: 13 July 2018 / Published online: 23 July 2018

Abstract

CDKN2A was first identified as melanoma predisposition tumour suppressor gene and has been successively studied. The previous researches have not established any noteworthy association with breast cancer. Therefore, through extensive literature search and in-silico analysis, we have tried to focus on the role of *CDKN2A* in breast cancer. *CDKN2A* variants in breast cancer were collected from different databases. The overall percentage of variants (approximately 5.8%) and their incidence frequency in breast cancer cases were found to be very low as compared to the number of samples screened in different studies. Exon 2 was identified as the major region of alternations. Approximately 42.8% were entire gene deletions, while 24.2% were missense mutations. These variants cannot be ignored because of their pathogenic effects as interpreted by the bioinformatics tools used in the present study. Earlier studies have shown that *CDKN2A* excludes the predisposition of germline variants, but interestingly shares common breast cancer germline variants with other carcinomas. Most of the data have revealed this gene as rarely mutated or deleted in breast cancer. However, few association studies have shown that in addition to being a 'multiple' tumour suppressor gene, it is mutated/deleted more in breast cancer cell lines as compared to breast cancer tissues or blood samples; thus, this gene cannot be neglected as a breast cancer candidate gene. The deletion/malfunctioning of *CDKN2A* in different tumours including breast cancer has recently led to the discovery of many clinical CDK inhibitors. Furthermore, these collected genetic variants will also be helpful in developing diagnostic, preventive, and treatment approaches for patients.

Keywords Breast cancer · Variant analysis · *CDKN2A* · P16

Introduction

Breast cancer is a type of a tumour in which cancerous cells originate from tissues of the breast. Males are at fewer risks of getting breast cancer, but like women, the chances of developing breast cancer increase with age [1]. It is the most common cancer in females worldwide with the highest incidence and mortality rate after lung cancer and has been ranked second among all of the cancers [1, 2].

In general, two genetic oscillations are involved in tumour development, one is activation of oncogenes and second is inactivation of tumour suppressor genes [3]. *CDKN2A* (cyclin-dependent kinase inhibitor 2A)/*p16INK4A*/*p14ARF* is an anti-oncogenic or tumour suppressor gene that was

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discovered as cyclin-dependent kinase inhibitor (CDKI) in 1993 [4, 5]. The cytological location is the short arm of chromosome 9 (9p21) in human and consists of three exons [6]. Lately, it is identified that first exon of *CDKN2A* is unique, i.e., 1α and 1β that shares exon 2 and 3 (Fig. 1a); therefore, *p16/ p16INK4A* and *p14/p14ARF/ARF* are transcribed by the exon 1α and exon 1β, respectively, from different promoters [4, 6]. The molecular data that supported p14 role in tumour suppression are rare, but mutations in *CDKN2A* lead to inactivation of both p16 and p14 proteins [7–9].

Both these proteins have distinct pathways; primarily, p16 plays role in G1-to-S-phase cell cycle checkpoint mechanism by its interaction in cyclin D/CDK/pRb pathway [4]. Focusing on the p16 protein that is 14.5 kDa has PDB ID 2A5E [10], this protein consists of four Ankyrin repeats (ANK-REP-I–IV) [10, 11]. In cyclin D/CDK/pRb pathway, p16 protein acts as an inhibitor and competes with cyclin D for binding to CDK4/6. P16 binding with CDK4/6 leaves the pRb non-phosphorylated. A non-phosphorylated pRb remains attached to the E2F and transcription does not occur [4, 6]. The ARF/p14 protein also acts as a tumour suppressor through its p53/MDM2/p21 pathway, as illustrated in Fig. 1. However, the role of *p14ARF* as a tumour suppressor gene needs to be further characterized, as some studies have suggested a fewer or not any a tumour suppressive role for *p14ARF* in breast carcinoma [8] as compared to *p16INK4A* [9].

CDKN2A/p16INK4A alternations have been described in many previous and recent studies [11–17]. To ascertain

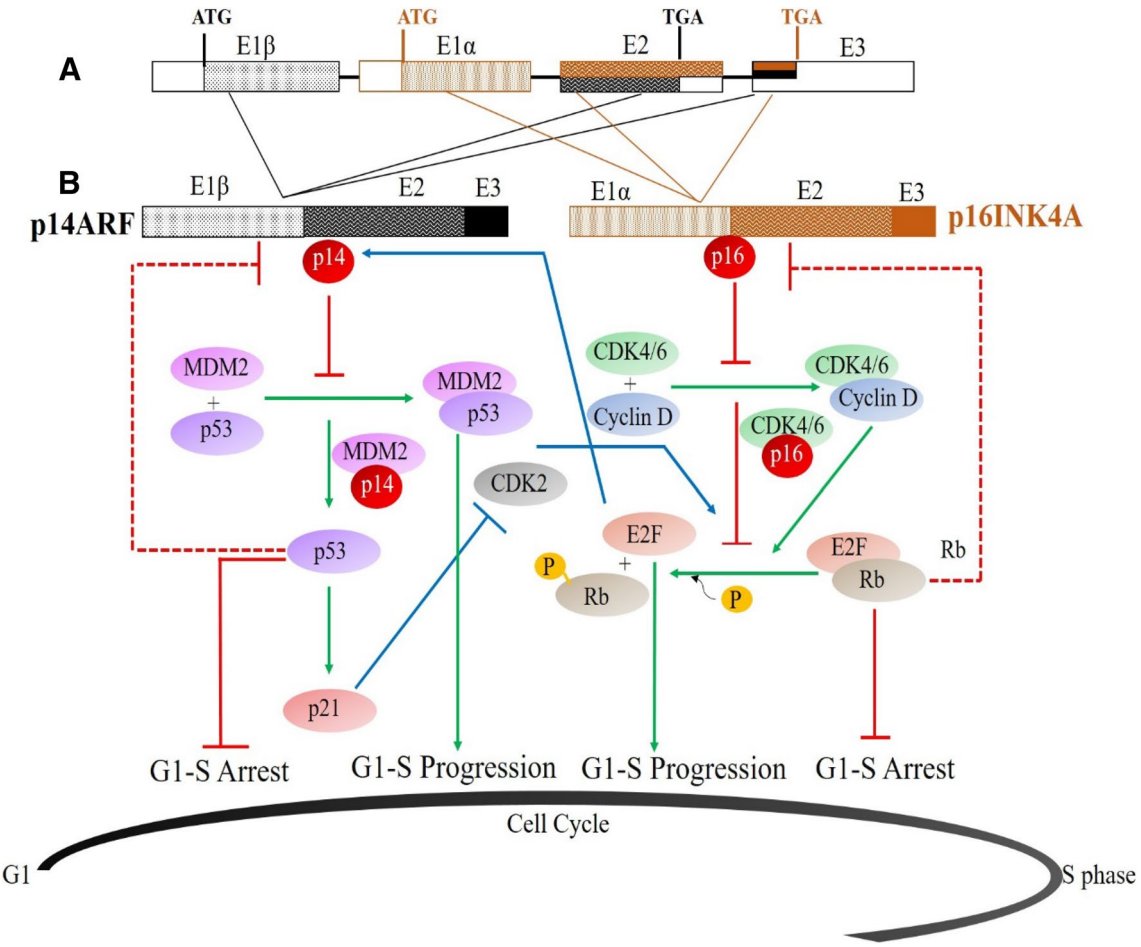


Fig. 1 Brief illustration of *CDKN2A* pathway. **a** Structure of *CDKN2A* and its two unique transcripts, i.e., *p14ARF* and *p16INK4A*. **b** Two tumour suppressor pathways p14/MDM2/p53 and p16/CDK4–6/Rb. The stimulatory signals as shown with green arrows and inhibitory with the red interrupted line. The crosstalk between both pathways, shown with blue arrows and interrupted line. P14 activates p53 by inhibiting the Murine double minute 2 (MDM2)–p53 complex formation to arrest G1-to-S-phase cell cycle progression. Similarly, P16 inhibits the Rb phosphorylation to keep E2F confined to arrest G1–S-phase cell cycle progression. In crosstalk, p21 deactivates the CDK2-mediated Rb phosphorylation, while it has been predicted that p16 loss could trigger the p53 by stimulation of E2F-dependent transcription that would increase the p14 level. Hence, CDk4–6/Cyclin D and MDM2-p53 are stimulatory complexes, and E2F-Rb is inhibitory complex for cell cycle progression

whether this gene has any association with breast cancer or not, we have compiled human *p16INK4A* variants associated with breast cancer. The sources of variants are germlines, somatic cell lines as well as breast cancer variants shared with other tumours. These alterations are then subjected to in-silico analysis to predict their pathogenic effect. The findings of this study have shown the prevalence level of *p16INK4A* genetic alternations in breast cancer. Furthermore, through in-silico analysis, we have predicted the pathogenic impact of associated alternations on splicing, and their structural and functional roles on the protein. We have also tried to discuss and relate the *p16INK4A* variants possible applications in clinical preventions and therapies against breast cancer.

CDKN2A-cancer genetics

CDKN2A has been reported in controlling tumour growth to be directly involved in cell cycle regulation. Chromosome 9p21 allele has shown a higher frequency for loss of heterozygosity (LOH) [16–18]. Apart from this, it contains inversions, translocations, and homozygous deletions in a number of tumours. This gene was not only found to be involved in different somatic mutations, but also has shown germline association in many tumours [6, 18–21].

Loss of function of *CDKN2A* in multiple tumours was reported first by two separate groups in 1994 [22, 23]. *CDKN2A* association with cancer was also reviewed in detail by different authors since 1994 [6, 9, 15, 23]. The best-associated cancer is melanoma followed by prostate carcinoma, head and neck carcinoma, non-small lung carcinoma, oesophageal, ovarian, and renal cancer [6, 13, 19, 24]. Low frequency of *CDKN2A* mutations was also found in colon cancer, breast cancer, and bladder cancer [6, 25].

The rate of alteration in *CDKN2A* varies depending on the type of cancer, i.e., it is more vulnerable in melanoma and entails histone modifications in addition to DNA methylation [18, 19, 26, 27]. Gastric cancer has a significant co-relation with *CDKN2A* hypermethylation [28–30]. Homozygous deletions have been reported in high frequency in primary samples and cell lines derived from breast, lung, bladder, bone, brain, skin, ovary, and lymphocytes, while frameshift, non-sense, and miss sense mutations have been widely reported in melanoma cell lines [18, 21, 26, 31]. Thus, like previous studies, we emphasize the character of *CDKN2A* as a 'multiple' tumour suppressor gene [21, 23].

The previous studies reported rare *CDKN2A* mutations in breast cancer [32–35]. Sorensen and Hovig stated somatic and germline mutations in *CDKN2A* for other cancers, but did not find any association with breast cancer [36], while some studies showed *CDKN2A*-breast cancer association with some extent [8, 18, 37, 38]. This point has gained our interest to co-relate genetic association of *CDKN2A* with breast cancer from the literature.

In the previous studies, *CDKN2A* was found to be mutated and deleted in breast cancer cases, while LOH and methylation were found to be other factors associated with carcinoma [8]. Exon 1 was evaluated to be a region of CpG island in different studies [8, 39, 40], which has shown a methylated form in the normal and hypermethylated form in tumour cases [41]. A recent study has also shown hypermethylation of exon 2 in breast cancer [42]. Furthermore, hemizygous was the second cause, followed by a homozygous minor role in downregulation of p16, while substitutions and indels were absent [17].

Variants collection

To correlate *p16INK4A* in breast cancer, we have collected *P16INK4A* variants that were evaluated in different breast cancer samples. The collected variants are illustrated in Table 1 according to their sources, i.e., somatic (tissue source), cell line, and germline (blood) source. Therefore, the variant collection was based on a search from different databases and published studies. Mainly, the Human Gene Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk/ac/search.php), Leiden Open (source) Variation Database (LOVD) (http://www.lovd.nl/3.0/home), the Catalogue Of Somatic Mutations In Cancer (COSMIC) (http://cancer.sanger.ac.uk/cosmic), Domain Mapping of Disease Mutation (DMDM) (http://bioinf.umbc.edu/dmdm/), Cancer genetic web (http://www.cancerindex.org/geneweb/) and Mutations, Oncogenes and Knowledge & Cancer (MOKCa) (http://strubiol.icr.ac.uk/extra/mokca/) were accessed.

The type of variants and their number of identification from different researches are also given in Table 1b. The deletion of *CDKN2A* (30/70) from chromosome was found to be a major factor for inactivation of this gene in breast cancer.

In-silico characterization of collected *p16INK4A* variants

The collected variants (Table 1) were analyzed to predict their structural and functional effect on protein through computational analysis. To show the impact of possible variants on protein, we have applied the combination of tools to improve predictive accuracy [43].

Splice site variants analysis

Through data collection we have found only one splice site variants (c.151-1G > C) and to know the impact of splice

Table 1 Identified *CDKN2A* variants in breast cancer, collected via literature and database search

(a) Details of variants/mutations

Variant source	Sample name[a]	Variants		Location	Variant class/type	Histological sub-type	Zygosity	Variants findings/studies[b] (*n*)	Database	References[c]
		Protein change	cDNA change							
Somatic	929692	p.M52K	c.155T>A	Exon 2	Missense	NS	Heterozygous	1/35	Cosmic, Mokca	[62]
	926634		c.151_457del307	Exon 2	Deletion	NS	Homozygous	3/100	Cosmic	[17]
	926676									
	926683									
	TCGA-A8-A09I-01	p.T137fs*.5	c.407_408insG	Exon 2	Frameshift	NS	NA	NA	Cosmic	[63]
	TCGA-LQ-A4E4-01	p.T79fs*40	c.235_236delAC	Exon 2	Frameshift	NS	NA	NA	Cosmic	[63]
	PD11341a	p.D108N	c.322G>A	Exon 2	Missense	Ductal carcinoma	NA	1/560	Cosmic	[64]
	933014	No protein	No gene	KO	Whole gene deletion	Ductal carcinoma	Homozygous	3/18	Cosmic	[8]
	933015									
	933016									
	S88890	p.P75L	c.224C>T	Exon 2	Missense	NS	Homozygous	1/164	Cosmic, Mokca	[35]
	S88892	No protein	c.151_457del307	Exon 2	Deletion	NS	Homozygous	1/164	Cosmic	[35]
	S88891	p.V106V	c.318G>A	Exon 2	Silent	NS	Homozygous	1/164	–	[35]
	PD11336a	p.H83Y	c.247C>T	Exon 2	Missense	Ductal carcinoma	NA	NA	Cosmic	[64]
	PD18733a	p.R80*	c.238C>T	Exon 2	Non-sense substitution	Ductal carcinoma	NA	NA	Cosmic	[64]
	7-MBC	p.R80*	c.238C>T	Exon 2	Non-sense	Metaplastic carcinoma	NA	1/5	Cosmic	[65]
	1107901	p.P48L	Not confirmed	Exon 1	Missense	Phyllodes tumour	NA	1/42	Cosmic Mokca	[66]
	Tumor A1	p.H83Y	c. 247C>T 14	Exon 2	Missense	NS	NA	1/22	Cosmic	[67]
	Tumor A13	p.A73A	Not confirmed	Exon 2	Silent mutation	NS	NA	1/22	–	[67]
	2158001	No protein	c.1_471del471	KO	Gene deletion	Benign & malignant phyllodes tumour	Homozygous	2/20	Cosmic	[68]
	2158006									
	2394252	No protein	c.1_471del471	KO	Gene deletion	Metaplastic carcinoma	Homozygous	2/3	Cosmic	[69]
	2556845	No protein	c.1_471del471	Whole gene	Gene deletion	Metaplastic carcinoma	Homozygous	4/20	Cosmic	[70]
	2556850									
	2556852									
	2556857									
	SC-her2-043	p.G122V	c.365G>T	Exon 2	Missense	ER-positive carcinoma	NA	1/36	Cosmic	[71]

Table 1 (continued)

(a) Details of variants/mutations

Variant source	Sample name[a]	Variants		Location	Variant class/type	Histological sub-type	Zygosity	Variants findings/studies[b] (*n*)	Database	References[c]
		Protein change	cDNA change							
Cell lines	MDA-MB-231	No protein	c.151_457del307	Exon 2	Deletion	NS	Homozygous	2/6	Cosmic	[35]
	MCF-7 2									
	MDA-MB-361	p.M52I	c.156G > C	Exon 2	Missense	Luminal carcinoma	Homozygous	1/20	Cosmic Mokca	[34]
	MCF-7	No protein	c.1_471del471	Whole deletion	Gene deletion	NS	Homozygous	5/20	Cosmic	[34]
	Hs-S78T									
	MDA-MB-330									
	MDA-MB-231									
	BT-20									
	MCF-1OF	No protein	c.151_457del307	Exon 2	Deletion	NS	NA	2/4	–	[67]
	MCF-12F									
	184A1	p.E88*	c.262G > T	Exon 2	Non-sense	NS	NA	1/4	–	[67]
	SUM52PE	p.A68V	c.203C > T	Exon 2	Missense	Luminal carcinoma	Homozygous	1/41	Cosmic	[72]
	MDA-MB-361	p.M52I	c.156G > C	Exon 2	Missense	Luminal carcinoma	Homozygous	1/41	Cosmic	[72]
	OCUB-F	p.A76fs*70	c.225delC	Exon 2	Deletion	Luminal carcinoma	Homozygous	1/41	Cosmic	[72]
	MCF-7	No protein	c.1_471del471	KO	Gene deletion	Luminal carcinoma	Homozygous	1/41	Cosmic	[72]
	BT-20	No protein	c.1_471del471	KO	Gene deletion	Basal Carcinoma	Homozygous	8/41	Cosmic	[72]
	Hs-578-T									
	MDA-MB-231									
	SK-BR-7									
	SUM102PT									
	SUM1315MO2									
	SUM149PT									
	SUM229PE									
	MDA-MB-361	p.D108H	c.332G > C	Exon 2	Missense	HER-positive carcinoma	NA	1/46	Cosmic	[73]
	MCF7	p.G6E	Not available	Exon 1	Missense	ER–PR-positive carcinoma	NA	1/46	Cosmic	[73]
	CAL-148	p.G124E	Not available	Exon 2	Missense	Triple negative	NA	1/46	–	[73]
	Hs-578-T 30	No protein	c.1_471del471	KO	Gene deletion	NS	Homozygous	2/5	Cosmic	[74]
	MCF-7									
	BT-20	No protein	c.1_471del471	KO	Gene deletion	NS	Homozygous	3/9	Cosmic	[75]
	MCF-7									
	MDA-MB-231									
	MCF-7 35	No protein	c.1_471del471	KO	Gene deletion	NS	Homozygous	1/5	Cosmic	[76]

Table 1 (continued)

(a) Details of variants/mutations

Variant source	Sample name[a]	Variants		Location	Variant class/type	Histological sub-type	Zygosity	Variants findings/studies[b] (*n*)	Database	References[c]
		Protein change	cDNA change							
Germline[e]	Family 543	–	c.151-1G > C	Intron 1	Splice site	NS		1/31	–	[77]
	Lund M13	p.113insR	c.336_337insCGT	Exon 2	Insertion	NS	NA	6/9[d]	–	[21]
	Lund M9									
	Lund M12									
	Lund M49									
	Lund M62									
	Lund M2									
	Case 5	p.A85T	Not available	Exon 2	Missense	NS	NA	1/31	–	[33]
	Case 16	p.V59G	Not available	Exon 2	Missense	NS	NA	1/31		[33]
	CO1	p.L16P	c.47T > G	Exon 1	Missense	NS	NA	NA	–	[78]
	D26	p.P81T	c.241C > A	Exon 2	Missense		NA	NA		[78]

(b) Total number of different types of variants in association with breast cancer

Missense mutations	17
Non-sense mutations	3
Indels (few nucleotides)	9
Exon 2 region deletions	8
Entire gene deletion	30
Silent mutations	2
Intronic splice region mutations	1
Total mutations reported/total number of samples screened	70 / 1191

A.A amino acid, *NS* not specified, *NA* not available

[a]Sample name or ID taken from database or literature

[b]Number of studies mentioned in literature work

[c]References include first author or other studies (irrespective of the first author)

[d]Nine malignant melanoma families carried 113insArg mutation in them, out of them six families contains breast cancer cases, and all six carried that mutation

[e]The germline source mutations that were identified to be common with other cancer type in a patient or family

Table 2 In-silico analysis to determine the possible functional impact of *CDKN2A* variants in breast cancer association

(a) Impact of CDKN2A Intronic mutations on splicing.

Variants	HSF[a] HSF matric score range (1–100)				Sroogle[b] Range (1–100)		Netgene2[c] Range (0–1)		NNsplice[d] Range (0–1)		Prediction of possible functional impact[l]
	WT	Mutant	Variation %	Interpretation	WT	Mutant	WT	Mutant	WT	Mutant	
c.151-1G>C	91.45	62.51	−31.65	Probably effecting splicing	12.09 (Max entropy) 88.10 (PSSM)[b]	4.03 (Max entropy) 64.66 (PSSM)	1.00 A.S[c]	No site generated	0.95 A.S	No site generated	Possibly highly deleterious

(b) Impact of *CDKN2A* missense mutations on protein stability and function.

	Polyphen-2[e]		SIFT[f]		FATHMM[g]	Align-GVGD[h]			Prediction of possible functional impact[l]
Variants	Score (range 0–1)	Prediction	Score (range 0–1)	Prediction	Prediction threshold is −0.75	GV[d]	GD[d]	Prediction	
p.M52K	0.994	Probably damaging	0.05	Damaging	−1.14	0.00	94.49	Class C65	Possibly highly deleterious
p.D108N	1.00	Probably damaging	0.1	Tolerated	−5.59	0.00	23.01	Class C15	Possibly deleterious
p.P75L	1.00	Probably damaging	0.27	Tolerated	−5.74	0.00	97.78	Class C65	Possibly highly deleterious
p.H83Y	1.00	Probably damaging	0.6	Tolerated	−1.60	0.00	83.33	Class C65	Possibly highly deleterious
p.P48L	1.00	Probably damaging	0.03	Damaging	−5.74	0.00	97.78	Class C65	Possibly highly deleterious
p.G122V	1.00	Probably damaging	0.00	Damaging	−5.97	0.00	108.79	Class C65	Possibly highly deleterious

site mutation, the in-silico tools were accessed, i.e., Human Splicing Finder HSF (http://www.umd.be/HSF3/), Sroogle (http://sroogle.tau.ac.il/), Netgene2 (http://www.cbs.dtu.dk/services/NetGene2/), and NNSplice 0.9 version were used from server Berkeley Drosophila Genome Project (BDGP) (http://www.fruitfly.org/seq_tools/splice.html) (Table 2a). By assessing the default settings, the damaging effect was predicted by the magnitude of the difference of wild-type

Table 2 (continued)

p.M52I	0.589	Possibly damaging	0.16	Tolerated	−0.99	0.00	10.12	Class C0	Possibly less deleterious
p.A68V	1.00	Probably damaging	0.00	Damaging	−2.91	0.00	64.43	Class C55	Possibly highly deleterious
p.D108H	1.00	Probably damaging	0.00	Damaging	−5.66	0.00	81.24	Class C65	Possibly highly deleterious
p.G6E	1.00	Probably damaging	0.33	Tolerated	−2.30	0.00	97.85	Class C65	Possibly highly deleterious
p.A85T	1.00	Probably damaging	0.00	Damaging	−2.51	0.00	58.02	Class C55	Possibly highly deleterious
p.V59G	0.998	Probably damaging	0.00	Damaging	−1.26	0.00	108.79	Class C65	Possibly highly deleterious
p.L16P	1.00	Probably damaging	0.00	Damaging	−3.13	0.00	97.78	Class C65	Possibly highly deleterious
p.P81T	1.00	Probably damaging	0.00	Damaging	−1.66	0.00	37.56	Class C35	Possibly highly deleterious

(c) Impact of *CDKN2A* substitution mutations on protein domain.

Variants	SMART[i]	InterPro[j]		ScanProsite[k]		Prediction of possible functional impact[l]
		Amino acid	Domain predicted	Amino acid	Scores	
p.M52K	No Ank[A] repeat domain predicted	12–148	Ank repeat containing domain	16–130	19.598	Possibly less deleterious
p.D108N	No effect on Ank repeats	12–148	Ank repeat containing domain	16–130	19.412	Possibly not deleterious

Table 2 (continued)

	domain					
p.P75L	No effect on Ank repeats domain	12–148	Ank repeat containing domain	16–130	19.545	Possibly not deleterious
p.H83Y	No Ank repeat domain predicted	12–148	Ank repeat containing domain	16–130	18.563	Possibly deleterious
p.P48L	No Ank repeat domain predicted	11–136	Ank repeat containing domain	16–130	18.324	Possibly deleterious
p.G122V	No effect on Ank repeats domain	12–148	Ank repeat containing domain	16–130	18.245	Possibly less deleterious
p.M52I	No effect on Ank repeats domain	12–148	Ank repeat containing domain	16–130	19.678	Possibly not deleterious
p.A68V	No effect on Ank repeats domain	12–148	Ank repeat containing domain	16–130	19.014	Possibly not deleterious
p.D108H	No effect on Ank	12–148	Ank repeat containing	16–130	18.855	Possibly less deleterious

Table 2 (continued)

	repeats domain		domain			
p.G6E	No effect on Ank repeats domain	12–148	Ank repeat containing domain	16–130	19.678	Possibly not deleterious
p.A85T	No Ank repeat domain predicted	12–148	Ank repeat containing domain	16–130	18.643	Possibly deleterious
p.V59G	No Ank repeat domain predicted	12–148	Ank repeat containing domain	16–130	18.908	Possibly deleterious
p.L16P	No effect on Ank repeats domain	12–148	Ank repeat containing domain	19–130	19.173	Possibly not deleterious
p.P81T	No Ank repeat domain predicted	12–148	Ank repeat containing domain	16–130	18.616	Possibly deleterious

(WT) and mutant scores. The score range provided by each database is mentioned in Table 2a. Furthermore, the HSF matric with the percentage less than 10 evaluates that mutation may break the acceptor site. The greater variation between WT and mutant scores predicted to be damaging for splice site. The c.151-1G > C variant may altered the acceptor site and cause an effect on splicing.

Analysis of substitution mutations effect on protein stability

From the collected data, we have found 14 missense mutations. These may cause deleterious effects on protein structure and function, and can be predicted by online tools (Table 2b). For any deleterious effect of non-synonymous

Table 2 (continued)

wt wild type

[a]HSF = Human splicing finder, HSF matric scores difference btw WT and mutant, variation % < −10% predicting mutation breaks the splice site [79]

[b]Sroogle = Predicting effect on splicing through difference between wild-type and mutant scores. The matrices which showed difference in score were selected in above table. PSSM; position-specific scoring matric, http://sroogle.tau.ac.il/

[c]Netgene2 = neural network prediction server, predicts the acceptor site (A.S) and donor site (D.S), score near to 1 regarded as more strong splice site. http://www.cbs.dtu.dk/services/NetGene2/output.php

[d]NNsplice = NNsplice version 0.9 work on NNSPLICE algorithm, predicts the acceptor site (A.S) and donor site (D.S), score near to 1 regarded as more strong splice site. http://www.fruitfly.org/seq_tools/splice.html

[e]Polyphen-2 = Polymorphism Phenotyping v2, predictive scale 'benign, possibly damaging and, probably damaging'. Scores near to 1 predicted to have probably damaging mutation (http://genetics.bwh.harvard.edu/pph2/)

[f]SIFT = Sorting Intolerant from Tolerant (SIFT) SIFT version 4.0, predictive scale either 'tolerating or damaging' (http://sift.jcvi.org/)

[g]FATHMM = Functional Analysis through Hidden Markov Models (v2.3) (FATHMM), mutation with threshold < −0.75 are interpreted to be associated with cancer. (http://fathmm.biocompute.org.uk/)

[h]Align-GVGD = Grantham difference (GD) and Grantham variation (GV). Prediction series from C65 (most likely damaging) to C0 (less likely damaging) and ≥ C25 were predicted as deleterious (http://agvgd.hci.utah.edu/)

[i]SMART[a] = Simple Modular Architecture Research Tool (SMART), (http://smart.embl-heidelberg.de/)

[j]InterPro[b] = https://www.ebi.ac.uk/interpro/

[k]ScanProsite[c] = http://prosite.expasy.org/scanprosite/

Wt p16 protein Scores and amino acid coverage by [A]anky/ank (Ankyrin) repeat domain predicted by; SMART = Ank-1 from 44 to 72 amino acid (a.a), Ank-2 from 77 to 106 a.a; InterPro = 12–148 a.a, Ankyrin repeat containing domain; ScanProsite = amino acid coverage sequence by Ank repeats region = 16–30 a.a, score = 19.678

[l]Prediction scale

Possibly highly deleterious = mutation predicted to be highly damaging via all tools

Possibly deleterious = mutation predicted to be damaging via two tools

Possibly less deleterious = mutation predicted to be less damaging via only one tools

Possibly not deleterious = mutation predicted to have no impact

mutations polyphen-2 (http://genetics.bwh.harvard.edu/pph2/) was run, the predictive scale was 'benign, possibly damaging and, probably damaging' and score range was from 0 to 1. Only one substitution M52I was marked as 'possibly damaging', while all others were 'probably damaging' with a score near or equal to 1. The other in-silico tool, Sorting Intolerant From Tolerant (SIFT) version 4.0 (http://sift.jcvi.org/) was used for analyzation. In addition, prediction is classified as either 'tolerated' or 'damaging'. Functional Analysis Through Hidden Markov Model (v2.3) (FATHMM) (http://fathmm.biocompute.org.uk/) was run with default threshold of −0.75; according to this software, the prediction threshold below this value would be interpreted as associated with cancer. Furthermore, Grantham difference (GD) relative to the evolutionary difference (Grantham variation, GV) was also examined using Align-GVGD (http://agvgd.hci.utah.edu/), its output was in an ordered series from class C65 (most likely) to C0 (less likely), and variants having class ≥ C25 were considered deleterious [43]. If three or four tools assign variants to be damaging, then a variant is predicted as possibly "highly deleterious" (Table 2b). The predictive results of all four tools were found to be consistent, mutation M52I was showed to be very less deleterious, while 12/14 mutations were predicted as highly damaging and can be designated as possibly 'pathogenic'.

Analysis of missense mutations effect on protein domains

The impact of these 14 substitution variants on the domain of p16 protein was evaluated using three online tools (Table 2c). **S**imple Modular Architecture Research Tool (SMART) (http://smart.embl-heidelberg.de/), InterPro: protein sequence analysis and classification (https://www.ebi.ac.uk/interpro/), and ScanProsite (http://prosite.expasy.org/scanprosite/) were used with default settings. Sequence analysis was done for variant effect on ankyrin domains of the p16 protein. The scores and domains of WT protein predicted from these tools were compared with each variant. A large difference between scores and missing domains was predicted to have a damaging effect on the protein domain and stability. According to the expected score, it was predicted that these 14 variants may not have a high deleterious effect on the protein domains.

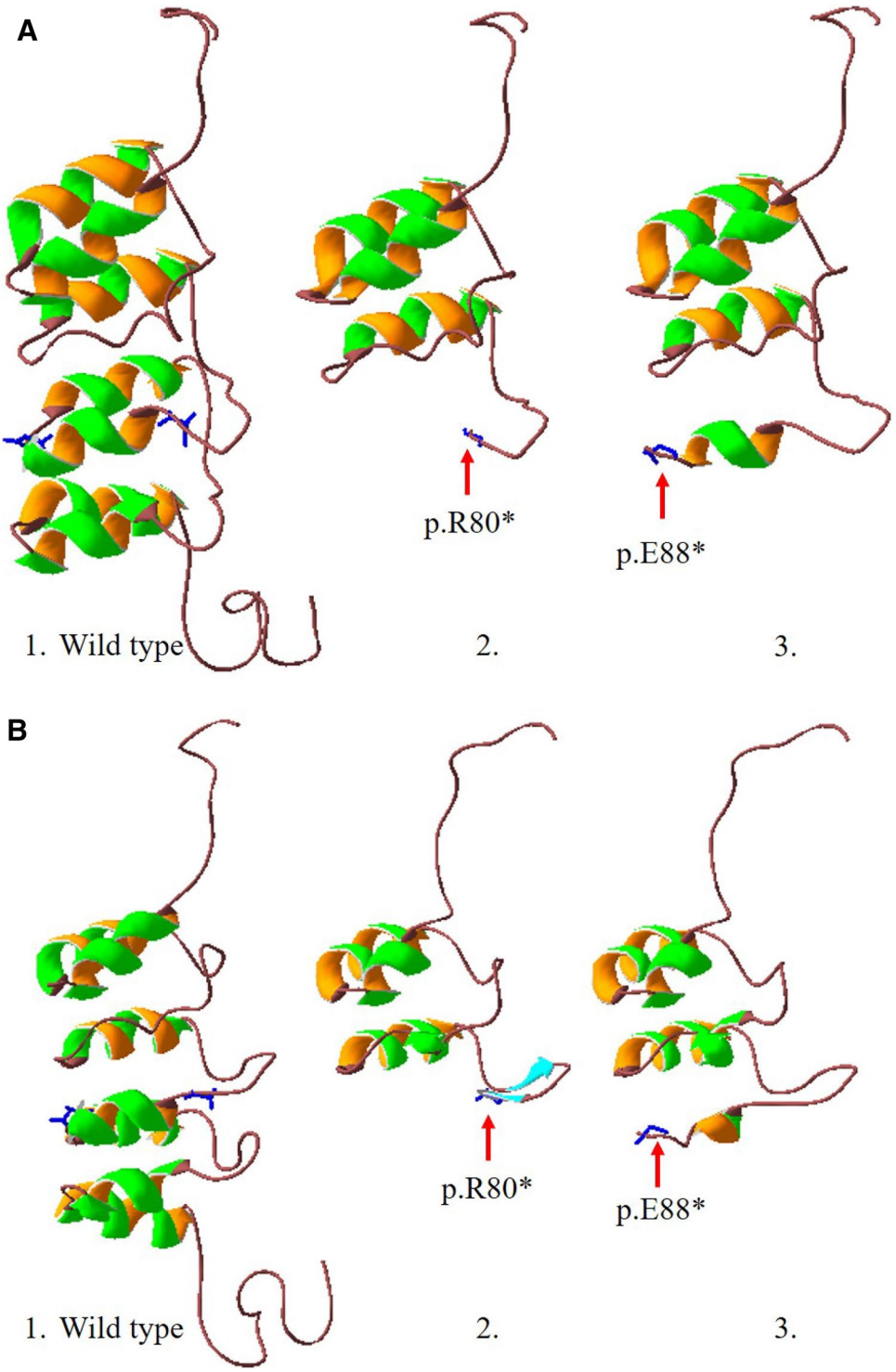


Fig. 2 Wild type and mutant model of the p16 protein. **a** Structure 1 is the wild-type p16 PDB model structure (PDB ID 2a5e), structure 2 and 3 are the Swiss generated model of non-sense mutation p.R80* and p.E88*, respectively. **b** Structure 1 is the wild-type PDB p16 model compared with CPH model of mutants p.R80* and p.E88*, respectively. Both the models are clearly representing the truncated mutant protein structure

Protein homology modelling

The comparative modelling of mutant proteins was done by Swiss model (https://swissmodel.expasy.org/) and CPH model 3.2 server (http://www.cbs.dtu.dk/services/CPHmodels/). The mutant's 3D structure was analyzed and compared with the WT p16 protein standard PDB (ID 2A5E) structure in Swiss PDB viewer v.4.10. The selected 16 variant structures, including 14 missense substitutions and 2 non-sense substitutions (p.E88* and p.R80*), were modelled. The non-sense variants have displayed the truncated protein structure (Fig. 2). However, all 14 missense

substitutions have shown no visual difference in protein structure, but they may have a pathogenic effect as predicted by other in-silico tools (Table 2b, c).

Multiple sequence alignment

Multi-sequence alignment of p16 protein was done with Clustal Omega program using Align tool of UniProt (http://www.uniprot.org/align/). Alignment of human p16 protein was performed with other nine species, i.e., *Pan troglodytes* (Chimpanzee), *Nomascus leucogenys* (Northern white-cheeked gibbon), *Gorilla gorilla* (Western lowland gorilla), *Macacamulatta* (Rhesus macaque), *Callithrix jacchus* (White-tufted-ear marmoset), *Pan troglodytes* (Chimpanzee), *Tarsiussyrichta* (Philippine tarsier), *Susscrofa* (Pig), and *Bostaurus* (Bovine). The protein structure was found to be highly selective and conservative in nature (Fig. 3). Moreover, the region of subjected variants was also evolutionary conserved. All the variants, especially the variants lying in second exon (151–457 amino acid), were found to be in conserved sequence. While substitutions in exon 1 were shown as non-conservative mutations. Most of the variants were located in secondary structure consisting of beta strands, turns, bends, and alpha helix, while very few were identified in primary structure; thus, these variants were found to be more deleterious to protein structure and may play a critical role in association with breast cancer.

Physical and chemical properties of amino acid substitutions

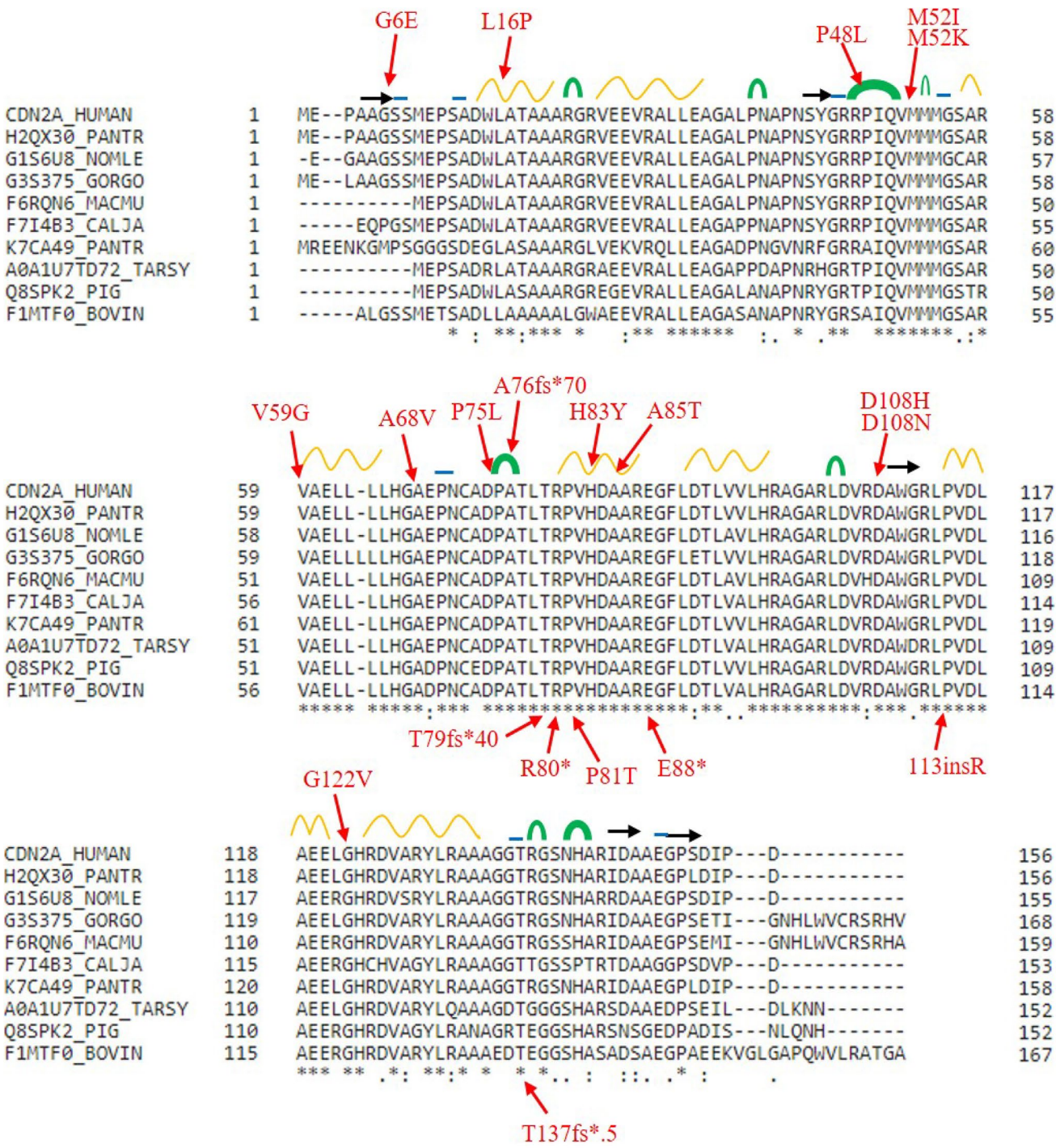
Substitution is characterized by physical and chemical properties of amino acid which may affect the protein stability and binding activity. The change in size, charge, and hydrophobicity changes the protein reactivity, binding site, and physical properties [44]. We looked at the physiochemical properties of amino acid substitution from the literature and interpreted the amino acid substitution preference (Table 3) [44]. According to the evaluation, 8 out of 14 substitutions have a 'disfavored' effect on protein properties.

The significance of *CDKN2A* and its variants in prognosis, prevention and therapeutics

The inactivation of tumour suppressor genes has proven to be more challenging to the drug therapy [45]. Different approaches presented many potential therapeutic targets which can overcome the loss of tumour suppressor [46, 47]. The best approaches can be: restoring gene function, using a dominant negative variant that can serve as an inhibitor, shutting down the pathway that has been abnormally activated due to the loss of tumour suppressor gene activity, the reactivation of silenced tumour suppressor gene, or inhibition of the epigenetic repression of the gene [20, 46, 47]. Such methods were applied to the malfunctioned *CDKN2A* recently [20, 46, 48, 49] which are summarized in the flowchart (Fig. 4). Moreover, the reactivation of *CDKN2A* has been achieved using regulatory gene such as FOXA1 successfully. The genistein has also shown remarkable properties of the chemo-preventive agent by epigenetic regulation of *CDKN2A*. In addition, the use of other reagents like doxorubicin and FUMI (5-fluorouracil and mitomycin C) has resulted in the demethylation of the *CDKN2A* promoter in breast cancer suppression [20]. Above all, the malfunction/loss of *CDKN2A* in breast cancer patients has been very efficiently fulfilled by the discovery of FDA approved CDK4/6-inhibitors, i.e., palbociclib (Feb 2015), ribociclib (Match, 2017) and abemaciclib (Sep 2017) [50–52]. These inhibitors can shut down the upregulation of cyclin D-CDK4/6 complex by inhibiting the CDK4/6. Hence, the loss of *CDKN2A* has made CDK4/6 a possible therapeutic target [53, 54]. The phase 1 clinical trials of drug ilorasertib (inhibitor of Aurora kinases) for CDKN2A deficient patients in solid tumours are also under progress (trial ID NCT02540876) [55]. A question may arise if *CDKN2A* does not show any breast cancer association while proven to be a principal regulator of cyclin D pathway, then why recent CDK inhibitors are showing remarkable clinical therapeutic results in breast cancer patients? This is likely to be due to the other factors apart from *CDKN2A* loss that may cause the overexpression or disruption of the cell cycle regulators like cyclins, CDK, or endogenous CDK inhibitors in breast cancer [50, 56].

CDKN2A is still under consideration as therapeutic target, but has been used significantly as a prognostic marker [48]. As the previous studies have shown that *CDKN2A* is hypermethylated in many cancers, which plays a magnificent role as a prognostic marker [57–60]. Interestingly, the *CDKN2A* was also reported overexpressed in different kinds of cancers due to the oncogenic stress mediated by Rb disruption [48] and this elevated level has been recognised as a prognostic marker for breast ductal carcinoma as well as sub-type of the breast cancer such as basal breast cancer. It has been shown that even a single marker can differentiate a tumour from different subtypes of breast cancer [48, 61]. Furthermore, it was also noticed that cancer patients with raised *CDKN2A* level, when treated with chemotherapeutic drugs, show more tumour suppression outcome as compared to the patients treated with cytostatic therapies (Fig. 4) [48].

We could not find any clinical importance of the collected variants in cancer therapy previously. The reason may be due to the low frequency of these variants incidents. However, the rate of gene deletion in breast cancer


G6E
L16P
P48L
M52I
M52K
CDN2A_HUMAN 1 ME--PAAGSSMEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRPIQVMMMGSAR 58
H2QX30_PANTR 1 ME--PAAGSSMEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRPIQVMMMGSAR 58
G1S6U8_NOMLE 1 -E--GAAGSSMEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRPIQVMMMGCAR 57
G3S375_GORGO 1 ME--LAAGSSMEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRPIQVMMMGSAR 58
F6RQN6_MACMU 1 ----------MEPSADWLATAAARGRVEEVRALLEAGALPNAPNSYGRRPIQVMMMGSAR 50
F7I4B3_CALJA 1 -----EQPGSMEPSADWLATAAARGRVEEVRALLEAGAPPNAPNSYGRRPIQVMMMGSAR 55
K7CA49_PANTR 1 MREENKGMPSGGGSDEGLASAAARGLVEKVRQLLEAGADPNGVNRFGRRAIQVMMMGSAR 60
A0A1U7TD72_TARSY 1 ----------MEPSADRLATAAARGRAEEVRALLEAGAPPDAPNRHGRTPIQVMMMGSAR 50
Q8SPK2_PIG 1 ----------MEPSADWLASAAARGREGEVRALLEAGALANAPNRYGRTPIQVMMMGSTR 50
F1MTF0_BOVIN 1 -----ALGSSMETSADLLAAAAALGWAEEVRALLEAGASANAPNRYGRSAIQVMMMGSAR 55
V59G
A68V
P75L
A76fs*70
H83Y
A85T
D108H
D108N
CDN2A_HUMAN 59 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDL 117
H2QX30_PANTR 59 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDL 117
G1S6U8_NOMLE 58 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLAVLHRAGARLDVRDAWGRLPVDL 116
G3S375_GORGO 59 VAELLLLLHGAEPNCADPATLTRPVHDAAREGFLETLVVLHRAGARLDVRDAWGRLPVDL 118
F6RQN6_MACMU 51 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLAVLHRAGARLDVHDAWGRLPVDL 109
F7I4B3_CALJA 56 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLVALHRAGARLDVRDAWGRLPVDL 114
K7CA49_PANTR 61 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDL 119
A0A1U7TD72_TARSY 51 VAELL-LLHGAEPNCADPATLTRPVHDAAREGFLDTLVALHRAGARLDVRDAWDRLPVDL 109
Q8SPK2_PIG 51 VAELL-LLHGADPNCEDPATLTRPVHDAAREGFLDTLVVLHRAGARLDVRDAWGRLPVDL 109
F1MTF0_BOVIN 56 VAELL-LLHGADPNCADPATLTRPVHDAAREGFLDTLVALHRAGARLDVRDAWGRLPVDL 114
T79fs*40
R80*
P81T
E88*
113insR
G122V
CDN2A_HUMAN 118 AEELGHRDVARYLRAAAGGTRGSNHARIDAAEGPSDIP---D----------- 156
H2QX30_PANTR 118 AEELGHRDVARYLRAAAGGTRGSNHARIDAAEGPLDIP---D----------- 156
G1S6U8_NOMLE 117 AEERGHRDVSRYLRAAAGGTRGSNHARRDAAEGPSDIP---D----------- 155
G3S375_GORGO 119 AEELGHRDVARYLRAAAGGTRGSNHARIDAAEGPSETI---GNHLWVCRSRHV 168
F6RQN6_MACMU 110 AEERGHRDVARYLRAAAGGTRGSSHARIDAAEGPSEMI---GNHLWVCRSRHA 159
F7I4B3_CALJA 115 AEERGHCHVAGYLRAAAGGTTGSSPTRTDAAGGPSDVP---D----------- 153
K7CA49_PANTR 120 AEELGHRDVARYLRAAAGGTRGSNHARIDAAEGPLDIP---D----------- 158
A0A1U7TD72_TARSY 110 AEELGHRDVARYLQAAAGDTGGGSHARSDAAEDPSEIL---DLKNN------- 152
Q8SPK2_PIG 110 AEERGHRDVAGYLRANAGRTEGGSHARSNSGEDPADIS---NLQNH------- 152
F1MTF0_BOVIN 115 AEERGHRDVARYLRAAAEDTEGGSHASADSAEGPAEEKVGLGAPQWVLRATGA 167
T137fs*.5
β- Strands
α-Helix
Turns
Bend
(*) conserved sequence
(:) conservative mutation
(.) semi-conservative mutation
( ) non-conservative mutation

◂**Fig. 3** Sequence alignment of human CDKN2A/p16 protein (156 amino acid) with ten species, by Clustal Omega program using Align tool from UniProt (http://www.uniprot.org/align/). Secondary structure is indicated on the top of alignment [10] aided by the key below left. The reported missense, non-sense substitutions and indels are shown in red writing positioned with red arrows

is higher and CDK inhibitors are showing remarkable therapeutic results [52]. In the future, if the association studies can potentially link these reported variants with breast cancer by reasonable frequency, then they can serve as the target for the personalized medicines. Finally, a high efficient gene editing tool such as CRISPR/Cas9 system

Table 3 Chemical and physical characteristic change of amino acids and their impact on protein stability

Variants	Properties of amino acid substitution	Amino acid substitution preferences
p.M52K	From big, hydrophobic, non-polar, neutral, to big, hydrophilic, polar, positive charged amino acid change	Disfavoured
p.D108N	From small, hydrophilic, polar, negative charged to small, hydrophilic, polar, neutral amino acid change	Favoured
p.P75L	From small, hydrophobic, non-polar to hydrophobic, non-polar, amino acid change	Disfavoured
p.H83Y	From big, hydrophilic, polar, positive charged to big, hydrophilic, polar, neutral amino acid change	Favoured
p.P48L	From small, hydrophobic, non-polar, neutral to big, hydrophobic, non-polar, neutral amino acid change	Disfavoured
p.G122V	From tiny, hydrophobic, non-polar, neutral to small hydrophobic, non-polar, neutral amino acid change	Favoured
p.M52I	From big, hydrophobic, non-polar, neutral, to big, hydrophobic, non-polar, neutral amino acid change	Favoured
p.A68V	From tiny, hydrophobic, non-polar, neutral to small, hydrophobic, non-polar, neutral amino acid change	Favoured
p.D108H	From small, hydrophilic, polar, negative charged to big, hydrophilic, polar, positive charged amino acid change	Disfavoured
p.G6E	From tiny, hydrophobic, non-polar to hydrophilic, polar, negatively charged amino acid change	Disfavoured
p.A85T	From tiny, hydrophobic, non-polar, to big, hydrophilic, polar, neutral charged amino acid change	Disfavoured
p.V59G	From small, hydrophobic, non-polar to tiny, hydrophobic, non-polar amino acid change	Favoured
p.L16P	From big, hydrophobic, non-polar to small, hydrophobic, non-polar amino acid change	Disfavoured
p.P81T	From small, hydrophobic, non-polar to small, hydrophilic, polar, neutral charged amino acid change	Disfavoured

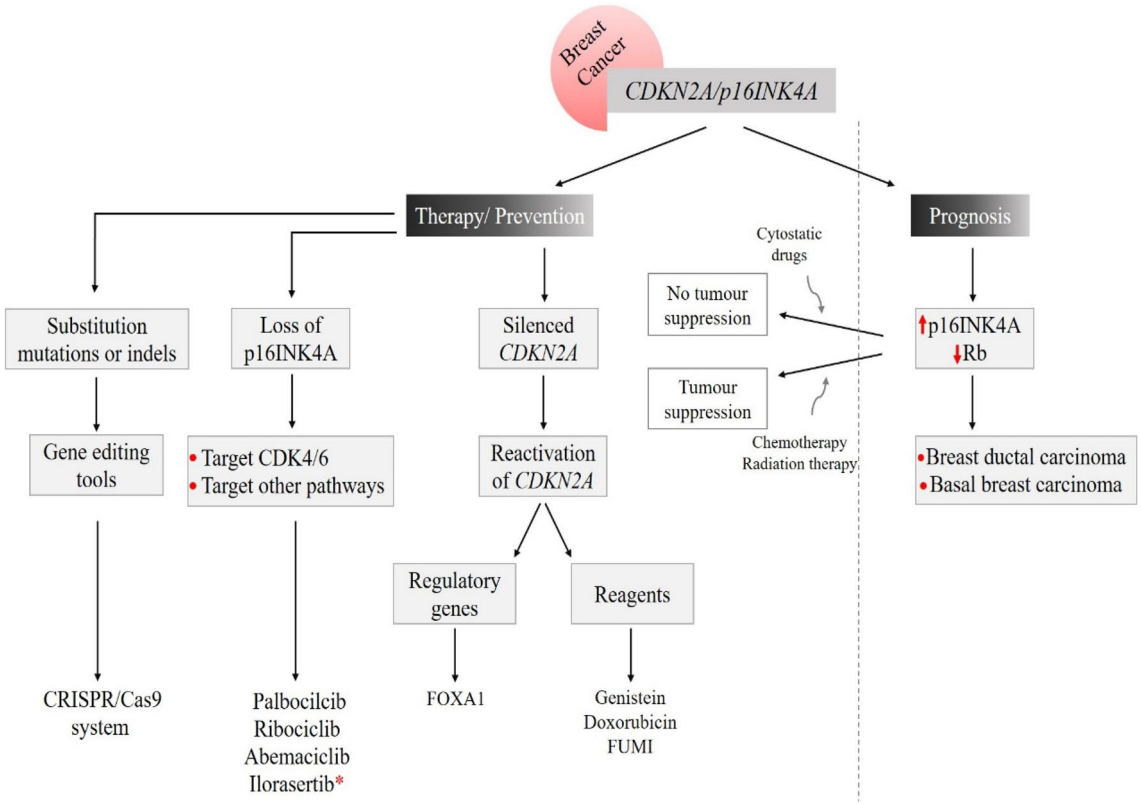


Fig. 4 Flowchart illustrating the *CDKN2A* recent or expected clinical prognosis, therapies and preventions in breast cancer. The dotted line separating the therapy/prevention from prognostic features of *CDKN2A*. Asterisk indicates ilorasertib is under phase 1 clinical trials for *CDKN2A*-deficient cancer patients

can be the expected therapy for small substitutions and indels [49].

Conclusion

CDKN2A plays a classical role in cell cycle progression and known as multiple tumour suppressor gene for so long. Previously, research was done to evaluate and characterize *CDKN2A* role in breast cancer, but very fewer mutations were identified in this area so far. According to the data collected in this study, approximately 5.8% (70 variants out of 1191 sample studies) of the breast cancer samples have shown alternations, where exon 2 being the major region of alternation. Cell lines have shown more alternations in breast cancer as compared to tissue or blood samples. The entire gene deletion was found to be more happening, i.e., 42.8%, missense occurred at 24.2%, while non-sense and frameshift were least occurring with the percentage of 4.1. Very less number of alternations were reported; however, they occupy an evolutionary conserved *CDKN2A* region in different species and these may run into offspring from the affected patient and may cause a deleterious effect. Moreover, in-silico studies done in this review predicted these variants as highly damaging, suggesting a significant association of *CDKN2A* variants with breast cancer. This article highlighted the overall research on *CDKN2A* association with breast cancer and concluded that this gene is not being frequently mutated, but a single mutation may have a dramatic change to protein function. The further association studies and characterization of these collected *CDKN2A* variants by animal models will lead to better therapeutic approaches to progress clinical course of various fatal cancer.

Compliance with ethical standards

Conflict of interest Authors have no conflict of interest.

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