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PRECLINICAL STUDY

Deleterious *CHEK2* 1100delC and L303X mutants identified among 38 human breast cancer cell lines

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Abstract The CHEK2 protein plays a major role in the regulation of DNA damage response pathways. Mutations in the *CHEK2* gene, in particular 1100delC, have been associated with increased cancer risks, but the precise function of *CHEK2* mutations in carcinogenesis is not known. Human cancer cell lines with *CHEK2* mutations are therefore of main interest. Here, we have sequenced 38 breast cancer cell lines for mutations in the *CHEK2* gene and identified two cell lines with deleterious *CHEK2* mutations. Cell line UACC812 has a nonsense truncating mutation in the CHEK2 kinase domain (L303X) and cell line SUM102PT has the well-known oncogenic *CHEK2* 1100delC founder mutation. Immunohistochemical analysis revealed that the two *CHEK2* mutant cell lines expressed neither CHEK2 nor P-Thr^{68} CHEK2 proteins, implying abrogation of normal CHEK2 DNA repair functions. Cell lines UACC812 and SUM102PT thus are the first human CHEK2 null cell lines reported and should therefore be a major help in further unraveling the function of *CHEK2* mutations in carcinogenesis.

Keywords Cancer susceptibility · Cell lines · Gene mutation · *CHK2* · *p53*

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Introduction

Cell cycle checkpoint kinase 2 (CHEK2, also known as CHK2, MIM604373) is a key regulator of DNA damage response pathways [1–3]. In response to DNA double strand breaks, CHEK2 is activated through ATM-mediated phosphorylations in the N-terminal regulatory domain, specifically at residue Thr^{68}. CHEK2 kinase is then fully activated by homodimerisation, intermolecular phosphorylation and subsequent release of active CHEK2 monomers. Activated CHEK2 kinase may control cell cycle arrest through phosphorylation of its substrates CDC25A and CDC25C, promote DNA repair through phosphorylation of BRCA1 and/or regulate apoptosis through phosphorylation of p53 [1, 2].

We and others identified the protein truncating *CHEK2* 1100delC mutation as a low-risk breast cancer susceptibility allele [4–6]. We recently have shown that *CHEK2* 1100delC also is a colorectal cancer susceptibility allele (MW and MS, manuscript submitted for publication). Although classified as a low-risk cancer allele, the *CHEK2* 1100delC mutation tends to be more prevalent in families with a high-risk cancer inheritance pattern. In addition, the *CHEK2* 1100delC mutation only partially segregates with the cancer phenotype in these families. Together, these observations have suggested a synergistic model in which the *CHEK2* 1100delC mutation acts in concert with another as-yet-unknown cancer susceptibility allele to confer high

cancer risks [4–6]. Apart from the *CHEK2* 1100delC mutation, four other allelic variants have convincingly been associated with cancer risks: the non-synonymous I157T and S428F mutations, the IVS2+1G>A splice site mutation and the truncating del5395 mutation [6]. Such a limited *CHEK2* mutation profile renders the gene particularly amenable for a polygenic cancer susceptibility model that involves biological synergy among partners. However, thorough investigation of the biological mechanism of *CHEK2*-related carcinogenesis is hampered by the lack of human cancer cell lines with proven oncogenic *CHEK2* mutations. Here, we report two novel deleterious *CHEK2* mutant cell lines that were identified through an extensive *CHEK2* mutation analysis of 38 human breast cancer cell lines.

Materials and methods

Breast cancer and colon cancer cell lines

The 38 human breast cancer cell lines used in this study are listed in Table 1 and have been described previously by Wasielewski et al. [7]. The SUM102PT cell line is generated in the Ethier laboratory (available at www.asterand.com) and the UACC812 cell line is obtained from American Type Culture Collection (available at www.lgcpromochem-atcc.com). The twelve evaluated human colon cancer cell lines included Caco-2, COLO205, DLD-1, HCT15, HCT116, HT29, NCI-H716, RKO, SW403, SW480, SW620 and SW1116. All colon cancer cell lines were obtained from American Type Culture Collection. Cell lines DLD-1 and HCT15 were independently established from the same tumor [8].

Breast cancer families and control population

The *CHEK2* L303X mutation was screened in blood-derived DNA from the youngest diagnosed breast cancer case of 290 breast cancer families from the southwestern Netherlands. All families had at least two breast cancer cases in first or second-degree relatives of whom at least one was diagnosed before age 60 years. Breast cancer families were ascertained through the Rotterdam Family Cancer Clinic at Erasmus MC. The 171 control cases were spouses of heterozygous cystic fibrosis mutation carriers ascertained through the department of Clinical Genetics at Erasmus MC. DNA of 69 European American and African American control cases were from the Coriell Human Variation Collection and were a kind gift by Dr. André G. Uitterlinden. All breast cancer cases gave informed consent

Table 1 *CHEK2* mutation analysis of 38 human breast cancer cell lines

Breast cancer cell lines N = 38	*CHEK2* Variant[a]	CHEK2 protein expression	CHEK2 Thr^{68} protein expression
BT20	wt	+++	+++
BT483	2,7	+++	+++
BT549	7	+++	+++
CAMA-1	7	++	++
DU4475	2,7	+/−	++
EVSA-T	2,7	+++	+++
HCC1937	wt	+++	+++
Hs578T	7	+++	+++
MCF-7	7	+++	+++
MDA-MD-134VI	2,7	+	++
MDA-MD-157	7	+++	++
MDA-MD-175VII	7	+++	+++
MDA-MD-231	7	+/−	+/−
MDA-MD-330	1,7	+/−	+/−
MDA-MD-361	7	++	++
MDA-MD-415	7	+++	+++
MDA-MD-435S	7	++	++
MDA-MD-436	7	+++	+++
MDA-MD-453	7	++	++
MDA-MD-468	7	+	+
MPE600	7	+	+
OCUB-M	2,7	n.d.	+++
SK-BR-3	7	+/−	++
SK-BR-5	wt	+/−	+
SK-BR-7	7	+++	++
SUM52PE	7	+++	+++
SUM102PT	2,**6**,7	−	−
SUM149PT	1,4,7,8	+/−	+
SUM159PT	7	+++	+++
SUM185PE	2,7	+++	++
SUM190PT	2,7	+++	+++
SUM225CWN	7	+++	++
SUM229PE	7	+	+
SUM1315M02	7	+++	++
T47D	2,7	+++	+++
UACC-812	3,**5**	−	−
UACC-893	7	+++	+++
ZR75-1	3	+++	+++

[a] Identified *CHEK2* gene variants are detailed in Table 2; n.d., not determined; +++, nuclear staining in >75% of cells; ++, nuclear staining in 50–75% of cells; +, nuclear staining in 25–50% of cells; +/−, nuclear staining in <25% of cells; −, no nuclear staining detected

Cell lines and variants in bold indicate deleterious mutants

to screen for susceptibility genes, and the medical ethical committee of Erasmus MC approved this study.

CHEK2 mutation analysis

The complete coding sequence, including intron/exon boundaries, of *CHEK2* (ENSG00000183765) was analyzed for genetic alterations. Exons 1 through 8 were amplified from genomic DNA by standard PCR [7]. Amplification of exons 9 through 14 was performed by long-range PCR and the ~9.2 kb product served as template for nested PCR's for each of exons 9 through 14. *CHEK2* amplification products were sequenced using BigDye™ Terminator v3.0 Cycle Sequencing mix (Applied Biosystems) and were analyzed on an ABI 3100 capillary sequencer. Unique sequence alterations were confirmed at least once on an independently generated (long-range) PCR product. Primer sequences and reaction conditions are available upon request.

Azacytidine treatment

Promoter hypermethylation was evaluated by treating exponentially growing cells with the demethylating agent 5-aza-2′-deoxycytidine (Sigma). Cells were cultured for 3 days with daily addition of 10 μM azacytidine. RNA of azacytidine-treated cells was isolated at the fourth day using RNeasy kit (Qiagen). *CHEK2* transcript expression was determined by RT-PCR, using Qiagen OneStep RT-PCR kit and *CHEK2*-specific primers annealing to sequences in exon 1 and exons 10–11.

CHEK2 immunohistochemistry

A cell line tissue microarray (TMA) was constructed by arraying triplicate 1-mm punches of paraffin embedded cell lines in a recipient paraffin wax block, using an automatic tissue micoarray system (Beecher). Normal placenta, colon and kidney tissues were arrayed as controls. Paraffin sections (4-μm) of the cell line TMA were mounted on starfrost glass slides (Knittel Gläser), deparaffinized and dehydrated. Epitopes were retrieved in 0.1 M Tris–EDTA pH 9.0 for 30 min at 100°C in a microwave oven and slides were blocked with 2% BSA in PBS for 30 min at RT. CHEK2 staining was performed for 1 h at RT with mouse monoclonal antibody NCL-CHK2 (clone DCS 270.1, 1:40; Novocastra Laboratories). Staining of phosphorylated CHEK2 at residue Thr^{68} was done overnight at 4°C with rabbit polyclonal antibody CHK2 phosphoT68 (clone E126, 1:100; Abcam). Isotype-matched mouse monoclonal antibody X0943 (1:40; Dako) and normal rabbit IgG sc-2027 (1:1000; Santa Cruz Biotechnology) were used as negative controls. Positive control antibodies were Vimentin for SUM102PT (clone V9, 1:4500; Dako) and Cytokeratin 8/18 for UACC812 (clone NCL5D3, 1:50; Biogene). Reactions were visualized using the EnVision+® System-HRP (DAB) kit (DakoCytomation) as described [9].

Results and discussion

CHEK2 mutation analysis in human breast cancer cell lines identifies two deleterious mutants

Sequencing of the *CHEK2* gene identified eight unique sequence alterations among 38 breast cancer cell lines (Tables 1 and 2). Six known single nucleotide polymorphisms included five intronic and one synonymous (E84E) sequence alterations that together were identified 48 times, varying from once to 33 times. The two non-synonymous sequence alterations c.908T>A and c.1100delC were each identified once.

The hemizygous *CHEK2* c.908T>A mutation was identified in cell line UACC812, predicting the substitution of a leucine to a termination codon at amino acid position 303 that is located in the kinase domain of the CHEK2 protein (p.L303X; Tables 1, 2). The patient from whom UACC812 was derived was first diagnosed with breast carcinoma at age 39 years [10]. The original tumor or blood-derived DNA were however unavailable for analysis, precluding confirmation of the mutation. Although the cancer history of the family was not known and *CHEK2* L303X has never been reported, the early age at diagnosis might be suggestive for a germ line mutation. We therefore genotyped the L303X mutation in 290 Dutch familial breast cancer cases and in 171 geographically-matched controls, but no positive cases were identified. As *CHEK2* L303X was identified in a cell line of American origin, we also screened 69 European American and African American controls for the mutation, but again none were positive. Together, these results suggest that the L303X mutation is not a common variant among Dutch and American populations. It also is possible that the mutation has been acquired during in vitro propagation of UACC812 cells. Be what it may, the homozygous deleterious nature of the L303X mutation renders the UACC812 cell line the first human CHEK2 null cell line.

The heterozygous *CHEK2* c.1100delC mutation was identified in cell line SUM102PT, predicting a shift in the *CHEK2* reading frame with an insertion of 14 new amino acids after codon 367 followed by a termination codon (p.T367fsX15; Tables 1, 2). Cell line SUM102PT was established from a patient who was diagnosed with in situ ductal breast carcinoma at age 56 years [11]. The family of the patient had a hereditary cancer inheritance pattern, but no details are known. Analysis of blood-derived DNA from

Table 2 *CHEK2* sequence variants identified among 38 human breast cancer cell lines

CHEK2 variant	Location	Nucleotide change[a]	Predicted protein change[b]	Type of variant[c]	Number of cell lines
1	Exon 1	c.252A>G	p.E84E	SNP (rs1805129)	2
2	Intron 1	c.319+38insA	–	SNP (rs3841692)	9
3	Intron 5	c.793-127T>C	–	SNP (rs9625541)	2
4	Intron 5	c.793-11G>A	–	SNP (rs5997387)	1
5	Exon 7	c.908T>A	p.L303X	Oncogenic	1
6	Exon 10	c.1100delC	p.T367fsX15	Oncogenic	1
7	Intron 11	c.1375+78C>G	–	SNP (rs5762749)	33
8	Intron 13	c.1542+11T>A	–	SNP (rs17881716)	1

[a] Numbering of nucleotide changes according *CHEK2* ensemble gene ID ENSG00000183765

[b] Frame shift mutation is indicated by the first changed codon and the number of newly encoded codons, including premature termination codon X

[c] Oncogenic; variant associated with cancer susceptibility [3] or presumed oncogenic as it predicts a premature termination codon (L303X). Both *CHEK2* mutant cell lines did not express CHEK2 and P-Thr^{68} CHEK2 proteins

the SUM102PT patient confirmed that the *CHEK2* 1100delC mutation was present in her germ line. The *CHEK2* 1100delC mutation is indeed a well-known oncogenic founder mutation that has been associated with an increased breast cancer risk [4–6]. Thus, the SUM102PT cell line is the first oncogenic *CHEK2* cell line reported thus far.

The two deleterious *CHEK2* mutant cell lines do not express CHEK2 and P-Thr^{68} CHEK2 proteins

To address biological consequences of the identified mutations, we analyzed the 38 breast cancer cell lines as well as the *CHEK2* mutant colorectal cancer cell line HCT15 for CHEK2 and P-Thr^{68} CHEK2 protein expression by immunohistochemistry (Table 1, Fig. 1, [12, 13]). All 36 *CHEK2* wild-type breast cancer cell lines showed CHEK2 and P-Thr^{68} CHEK2 protein expression, where the level of CHEK2 protein expression generally was in concordance with the expression level of P-Thr^{68} CHEK2. Cell line HCT15 had weak CHEK2 and P-Thr^{68} CHEK2 protein expression, consistent with its reported impaired CHEK2 function due to bi-allelic *CHEK2* missense mutations (Fig. 1, Table 4, [12, 13]). In contrast, neither CHEK2 nor P-Thr^{68} CHEK2 protein expression was detected in the *CHEK2* mutant cell lines SUM102PT and UACC812 (Fig. 1), implying complete loss of CHEK2 function in these cell lines.

No *CHEK2* promoter hypermethylation in SUM102PT cells

Absence of CHEK2 and P-Thr^{68} CHEK2 protein expression in cell line SUM102PT was somewhat unexpected as this cell line had retained a *CHEK2* wild-type allele. To exclude *CHEK2* promoter hypermethylation as an additional mechanism for *CHEK2* inactivation in cell line SUM102PT, we treated SUM102PT cells with the demethylating agent azacytidine. Treatment with azacytidine did however not upregulate *CHEK2* transcript levels in SUM102PT cells, suggesting that *CHEK2* promoter hypermethylation is not underlying its decreased transcript expression and absent protein expression in cell line SUM102PT.

CHEK2 and *p53* mutations are mutually exclusive in human cancer cell lines

It is well established that CHEK2 is an upstream activator of p53 proteins but it is as yet unclear whether both proteins also function in the same tumor suppressor pathway [1, 2]. Such functional interaction predicts that *CHEK2* and *p53* mutations occur mutually exclusively in a single tumor. We have evaluated the *CHEK2* and *p53* mutation status in a cohort of 54 human cancer cell lines. This cohort included the here described 38 breast cancer cell lines [7], five previously reported prostate cancer cell lines [14] and 11 unique colon cancer cell lines with known *p53* mutation status [15–17] that we also have analyzed for *CHEK2* mutations (Table 3). Forty-two cell lines were *p53* mutant and five cell lines were *CHEK2* mutant. The five *CHEK2* mutant cell lines included the here reported SUM102PT and UACC812 breast cancer cell lines, the colorectal cancer cell lines HCT15 and HCT116 and the prostate cancer cell line LNCaP (Table 4). Cell line HCT15 has bi-allelic inactivation of the *CHEK2* gene as it has the R145W mutation in the fork head-associated domain of CHEK2 and the A247D mutation in the kinase domain [12, 13]. Cell lines HCT116 and LNCaP carry the heterozygous missense mutations L355P and T387N in the CHEK2 kinase domain, respectively [14, 18]. The *CHEK2* R145W

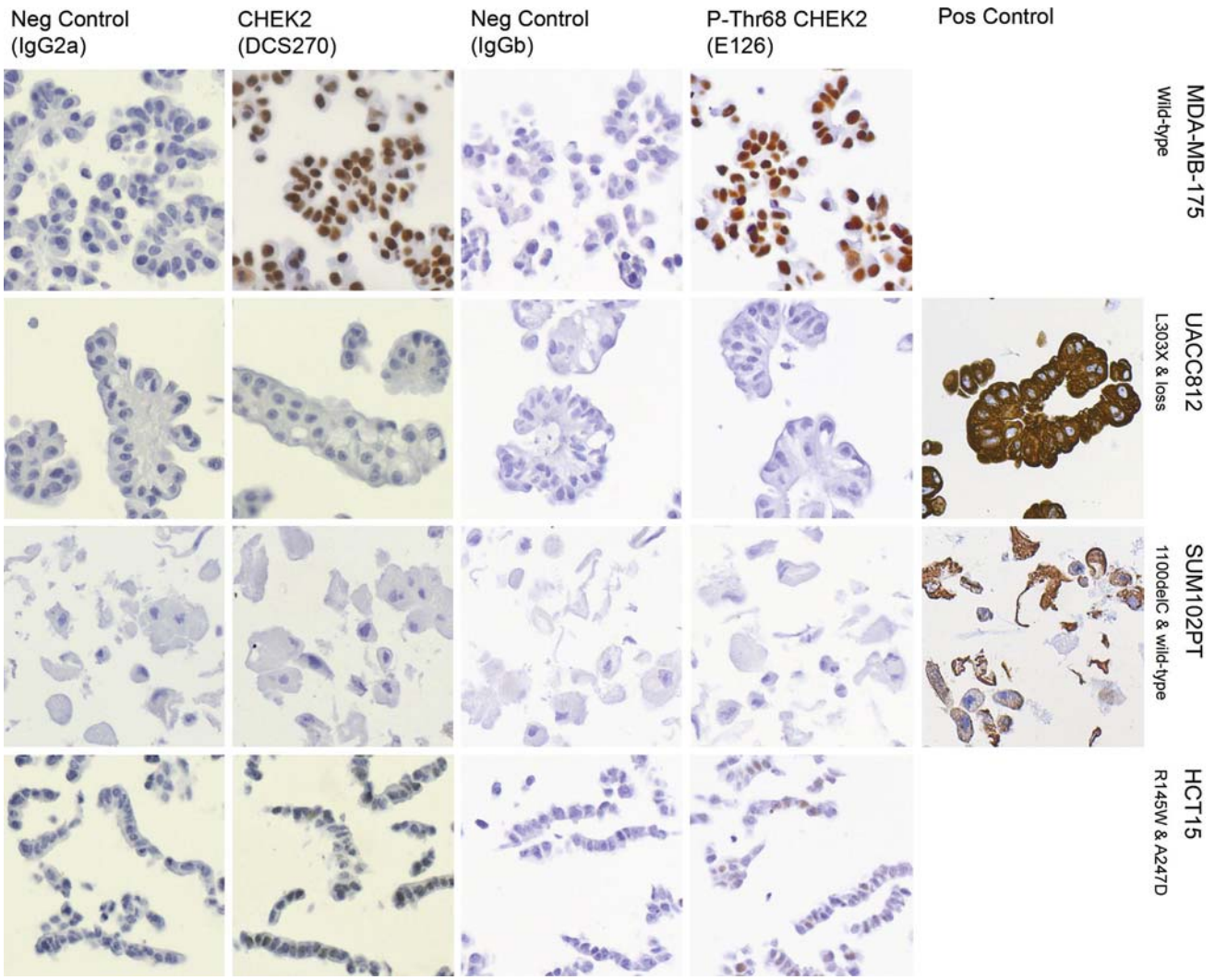

Fig. 1 Absence of CHEK2 protein expression in mutant *CHEK2* breast cancer cell lines. *CHEK2* mutant breast cancer cell lines UACC812 and SUM102PT do not express CHEK2 or P-Thr^{68} CHEK2 protein, in contrast to *CHEK2* wild-type cell line MDA-MB-175. *CHEK2* mutant colorectal cancer cell line HCT15 shows weak CHEK2 and P-Thr^{68} CHEK2 protein expression. Negative control antibodies were isotype-matched for CHEK2 and P-Thr^{68} CHEK2 antibodies. Positive control antibodies were Vimentin for SUM102PT and Cytokeratin 8/18 for UACC812

variant was also found in a Li-Fraumeni patient with a sarcoma and a breast carcinoma but was not detected among 2,520 additionally genotyped cases and controls [13, 19, M. Wasielewski and M. Schutte unpublished data]. The *CHEK2* A247D mutation was absent in 187 genotyped controls and the L355P and T387N variants have not been further evaluated [13]. The significance of these four mutations in CHEK2-related carcinogenesis is thus still unclear. However, CHEK2 function has been shown to be impaired in cell lines HCT15 and LNCaP, suggesting that the R145W, A247D and T387N *CHEK2* mutations may be oncogenic. Two of five mutant *CHEK2* cell lines also had a heterozygous *p53* sequence alteration (Table 4). Cell line LNCaP carried the silent *p53* P152P polymorphism and cell line HCT15 had the *p53* S241F missense mutation that is likely oncogenic as this residue is crucial for interaction of wild-type p53 proteins with DNA [15, 20]. Cell line HCT15 also was reported to have the *p53* P153A missense mutation [17]. Yet, this mutation could not be confirmed by us nor by others that had sequenced the *p53* gene in HCT15 and/or DLD1 [15]. Thus, only one of the 54 human cancer cell lines (HCT15) had oncogenic mutations in both *CHEK2* and *p53* ($P = 0.005$ using Fisher's Exact Test, Table 4), suggesting that coincident *CHEK2* and *p53* mutant cells do not have a selective survival advantage compared to either *CHEK2* or *p53* mutant cells. Our observation is in concordance with a report on 84 primary prostate cancers that had been screened for mutations in the *CHEK2* and *p53* genes [14]. Those results had not been significant because too few mutant tumors had been

Table 3 Mutual exclusiveness of *CHEK2* and *p53* mutations among 54 human cancer cell lines (*CHEK2* and *p53* mutational status was determined in 54 breast, colorectal and prostate cancer cell lines. Note that only one of 54 cell lines is *CHEK2* and *p53* mutant ($P = 0.005$))

CHEK2 and *p53* oncogenic mutations	Human cancer cell lines			Total
	Breast cancer	Colorectal cancer	Prostate cancer	
CHEK2 wild-type and *p53* wild-type	6	1	0	7
CHEK2 wild-type and *p53* mutant	30	8	4	42
CHEK2 mutant and *p53* wild-type	2	1	1	4
CHEK2 mutant and *p53* mutant	0	1	0	1
Total	38	11	5	54

p53 sequence variant reported in Ref. 15

Table 4 Mutual exclusiveness of *CHEK2* and *p53* mutations among 54 human cancer cell lines (Overview of identified *CHEK2* variants in human cancer cell lines)

Cell line	*CHEK2* mutation	*p53* mutation
Breast cancer		
SUM102PT	T367fsX15	wild-type
UACC812	L303X	wild-type
Colorectal cancer		
HCT15/DLD-1	A247D, R145W	S241F[a]
HCT116	L335P	wild-type
Prostate cancer		
LNCaP	T387N	wild-type

p53 sequence variant reported in Ref. 15

identified (11 *CHEK2* mutant and 11 *p53* mutant; $P = 0.8$). However, combining both datasets conclusively shows that mutations in the *CHEK2* and *p53* genes are mutually exclusive ($P = 0.02$). Our analysis thus implies that CHEK2 and p53 proteins indeed operate in the same tumor suppressor pathway and that a main oncogenic function of CHEK2 involves p53 mediated G1 cell cycle arrest.

In conclusion, we here report two breast cancer cell lines with deleterious *CHEK2* mutations, L303X and 1100delC. Both mutations generate a premature termination codon in the encoded *CHEK2* transcripts leading to loss of CHEK2 and P-Thr^{68} CHEK2 protein expression. These mutation data, together with our previous mutation reports of the *BRCA1*, *E-cadherin*, *p53*, *PIK3CA*, *PTEN* and *RAS* genes, provide a mutation profile of human breast cancer cell lines that will be a useful tool in functional and in vitro breast cancer studies [7, 9, 21, 22].

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