Rare Germ Line CHEK2 Variants Identified in Breast Cancer Families Encode Proteins That Show Impaired Activation

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Abstract

Germ line mutations in CHEK2, the gene that encodes the Chk2 serine/threonine kinase activated in response to DNA damage, have been found to confer an increased risk of some cancers. We have previously reported the presence of the common deleterious 1100delC and four rare CHEK2 mutations in inherited breast cancer. Here, we report that predictions made by bioinformatic analysis on the rare mutations indicate that two of these, delE161 (483–485delAGA) and R117G, are likely to be deleterious. We show that the proteins encoded by 1100delC and delE161 are both unstable and inefficiently phosphorylated at Thr68 in response to DNA damage, a step necessary for the oligomerization of Chk2. Oligomerization is in turn necessary for additional phosphorylation and full activation of the protein. A second rare mutation, R117G, is phosphorylated at Thr68 but fails to show a mobility shift on DNA damage, suggesting that it fails to become further phosphorylated and hence fully activated. Our results indicate that delE161 and R117G encode nonfunctional proteins and are therefore likely to be pathogenic. The findings from the biochemical analysis correlate well with predictions made by bioinformatics analysis. In addition, the results imply that these mutations, as well as 1100delC, cannot act in a dominant-negative manner to cause cancer, and tumorigenesis in association with these mutations may be due to haploinsufficiency. (Cancer Res 2006; 66(18): 8966-70)

Introduction

The CHEK2 gene encodes the Chk2 serine/threonine kinase, which is a component of the signaling pathways activated in response to DNA damage. Here, it plays an important role in double-strand break responses leading to cell cycle checkpoint arrest, apoptosis, and DNA repair (1). The Chk2 protein consists of three distinct functional domains. The most NH2-terminal of these is the SQ/TQ cluster domain (residues 19-69), which contains Thr68, the site phosphorylated in response to DNA damage by the upstream kinase, ATM. Downstream of this is the fororkhead-associated domain (residues 115-165), which functions in trans to modulate protein-protein interactions and also in cis to affect other functional domains within the protein itself. A serine/threonine kinase domain (residues 225-490) occupies almost the entire COOH-terminal half of Chk2 (2). On DNA damage, oligomerization of Chk2 is promoted through phosphorylation of Thr68, which serves as a specific ligand for the fororkhead-associated domain of another Chk2 molecule (3). This allows the intermolecular trans phosphorylation of Chk2 on Thr383 and Thr387 resulting in the release of active Chk2 monomers, which proceed to function in DNA damage–induced cellular responses (4).

We have previously shown that 1100delC, which abolishes the kinase function of Chk2 (5), is a founder mutation, present in 1% of the normal healthy northern European population and in 5% of familial breast cancer cases without a mutation in the two known major breast cancer predisposition genes, BRCA1 and BRCA2 (6). This shows epidemiologically that 1100delC confers an increased risk of breast cancer. Further epidemiologic investigations carried out on other founder mutations, such as I157T and IVS2-G>A, have also shown that these confer an increased risk of a variety of cancers, indicating that CHEK2 is a multigorgan cancer susceptibility gene (7).

Analysis of the whole coding region of CHEK2 in breast and prostate cancer families and sporadic cases has led to the discovery of rare mutations in the gene (8–10). We have previously reported four rare, novel, germ line mutations in families with more than two breast cancer cases: delE161, R117G, R137Q, and R180H (9, 11). To characterize these mutations, both bioinformatic and biochemical investigations were undertaken. Bioinformatic investigations were used to analyze amino acid changes according to the biochemical severity of the amino substitution and its context within cross-species conservation of the protein sequence. Biochemical studies included analysis of the expression, stability, and phosphorylation of the mutant proteins. From these studies, we report that the rare germ line mutations delE161 and R117G are deleterious and likely to be pathogenic. The effect of these mutations, as well as 1100delC, on the phosphorylation of the protein suggests that these mutants cannot act in a dominant-negative manner to contribute to tumorigenesis.

Materials and Methods

Bioinformatic studies. A protein multiple sequence alignment of exons 1 to 4 from nine CHEK2 orthologues was made using the multiple sequence alignment engine 3DCoffee (12). The Chk2 crystal structure used in the alignment was 1GXC.pdb (13). Sequences used were human (Homo sapiens), NP-009125.1; mouse (Mus musculus), NP-057890; dog (Canis familiaris), XP-543464.1; cow (Bos taurus), AAX46445; gray short-tailed opossum (Monodelphis domestica), sequence deduced from the opossum genome sequence assembly BROAD63; scaffold-14857; chicken (Gallus gallus), sequence deduced from the February 2004 chicken genome sequence assembly; chromosome 15 (there is a chicken CHEK2 cDNA sequence in GenBank, but we found it to be chimeric); frog (Xenopus laevis), AF326574.1; pufferfish (Fugu rubripes), sequence deduced from the August 2002 F. rubripes genome sequence assembly; and sea urchin (Stongylocentrotus purpuratus), sequence deduced from the November 2004
Rare Germ Line CHEK2 Variants Encode Inactive Proteins

S. purpuratus genome assembly, contig 611723. Duplicated pufferfish and sea urchin sequences were searched by BLASTP against the mammalian protein sequences in GenBank to confirm orthology. In both cases, the best hits were unambiguously against CHEK2. The 3DCCoffee alignment was run with the options Malign-id-pair, Mslow-pair, Mclusaalw, and Mfugue-pair. Cross-species sequence variability within the alignment was measured by calculating the Grantham variation (GV) of each position in the alignment, and the fit between each missense substitution and the observed range of variation was measured both by calculating the Grantham deviation (GD) of each missense substitution and by obtaining Sorting Intolerant from Tolerant scores for each substitution (14, 15).

**Mutant constructs.** Wild-type (wt) full-length CHEK2 cDNA was cloned into pcDNA3.1/His C (Invitrogen, Paisley, United Kingdom). QuickChange Site-Directed Mutagenesis (Stratagene, La Jolla, CA) was used to generate mutant constructs encoding the naturally occurring 1100delC, R117G, R137Q, R180H, delE161, R145W, and synthetically derived R117A mutants as recommendations of the manufacturer. The oligonucleotides used were 1100delC (forward, gactcttacttacttacttactccttacctcctg; reverse, gttttctggctgttgctgatgtttggtatttatctgttc), R117G (forward, gacaatacgttgggggagaacagtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtgtggtg
human to frog. In analysis of BRCA1 missense substitutions, the set of substitutions that fall within the range of variation observed from human to frog is highly enriched for neutral variants (15); consequently, R137Q is relatively unlikely to cause notable loss of function. The last substitution, R180H, falls at a moderately variable position (GV to frog = 71.0; GV to sea urchin = 81.6). The substitution is slightly outside the range of variation from human to frog but within the range of variation from human to sea urchin, leading to an inconclusive result.

Expression of Chk2 mutants. Our biochemical studies were initiated by comparing the expression of wt Chk2 with different Chk2 mutants exogenously expressed in U2OS cells (Fig. 2). R145W and artificially derived R117A, which have previously been characterized (3, 16), were used as positive controls. delE161 and 1100delC, also previously observed (16), were found to be expressed at low levels. The expression levels of R137Q and R117A were the same as that of wt Chk2 whereas R117G and R180H were present at reduced levels but were not as low as delE161, 1100delC, or R145W.

Stability of Chk2 mutants. The low expression of the R145W mutant has been shown to be due to the instability of the encoded protein (16). We therefore investigated if the low expression of delE161, R117G, and 1100delC was also caused by reduced protein stability. Equal amounts of wt and mutant Chk2 proteins were expressed in U2OS cells followed by cycloheximide treatment to block new protein synthesis. The results show that the half life of wt Chk2 was ~6 hours as previously reported (16) and that its degradation was rescued fully in the presence of the proteasome inhibitor N-acetyl-Leu-Leu-Norleau-al (Fig. 3). The stability of R117A and R137Q was similar to that of the wt protein. The three mutants, delE161, R145W, and 1100delC, were found to be very unstable, with a half-life of <1 hour (Fig. 3). Inhibition of proteasome-dependent degradation only partially rescued the loss of these mutants, which suggests that they may also be degraded by other means. The R117G and R180H mutants were more stable than these three mutants but less stable than the wt.
protein. The level of instability of the mutants reflects their expression levels when cells are transfected with equal amounts of DNA (Figs. 2 and 3).

The R117G, R137Q, R145W, and delE161 mutations all reside in the forkhead-associated domain of Chk2, which mediates protein–protein interactions. The crystal structure of the forkhead-associated domain reveals that it consists of a multiple strand β-sandwich containing short α-helical loops that extend and make contact with phosphothreonine-containing peptides (13). Mutations of residues, believed to form the β-sandwich structure of the forkhead-associated domain, destabilize the protein (3). This is consistent with what has been documented for R145W, which lies in the β-sandwich and was also observed in this investigation (3, 16). Accordingly, the results obtained in this investigation showed that the delE161 mutant, which also lies in the β-sandwich (3), renders the protein unstable.

Effect of Chk2 mutants on activation in response to etoposide-induced DNA damage. To investigate if the Chk2 mutants we identified in breast cancer families prevent efficient activation of Chk2 in response to DNA damage, we studied the phosphorylation of Thr68 in response to etoposide-induced DNA damage and compared the changes in mobility of the expressed mutant protein with the wt. The p53 tumor suppressor is phosphorylated and stabilized in response to DNA damage (17); thus, increased expression of p53 protein was examined to verify that DNA damage had taken place on etoposide treatment. This was confirmed by Western blot analysis (Fig. 4A and B).

On etoposide-induced DNA damage, Thr68 phosphorylation of the delE161 mutant is reduced compared with wt Chk2 (Fig. 4A). A slower electrophoretic mobility of Chk2 in response to DNA damage is a consequence of intermolecular trans-phosphorylation of Thr383 and Thr387 (3, 4). The lack of a mobility shift suggests that this mutant is not phosphorylated on Thr383 and Thr387 in response to DNA damage (3). A similar observation was made with R145W, which suggests that both these mutants fail to oligomerize (3), a step necessary for further phosphorylation and full activation of the protein. The R117G mutant is phosphorylated on Thr68 but does not show an altered mobility of the protein in response to DNA damage. This suggests that R117G, as reported for the R117A mutant, cannot oligomerize, which allows further phosphorylation (3). R180H and R137Q resemble wt Chk2 in their response to DNA damage. Interestingly, the 1100delC does not become phosphorylated at Thr68, although the SQ/TQ domain, where Thr68 resides, is intact in this mutant (Fig. 4B). Previously, a kinase dead Chk2 mutant, D368N, has been shown to become phosphorylated at Thr68 on DNA damage (3), indicating that an active Chk2 kinase domain is not necessary for this event. It is possible that a residue(s) lost in the truncated protein encoded by 1100delC is necessary for Thr68 phosphorylation or the way the protein is folded in the mutant prevents this phosphorylation.

R117G, delE161, and R145W are predicted to be deleterious by bioinformatic analysis. R137Q is predicted to be neutral and R180H is unclassified according to Align-GVGD (Fig. 1B). The results from the biochemical analysis show that mutants R117G and delE161, like R145W, are not efficiently activated in response to DNA damage and therefore dysfunctional. Although slightly unstable, R180H is fully activated like R137Q and, therefore, these mutants are unlikely to be pathogenic. R180H may have a subtle effect on the function of the protein and may be classified as neutral or of little functional significance. The results obtained in the biochemical analysis compare well with the predictions made from bioinformatic analysis.

Classically, the mechanism of tumorigenesis in association with tumor suppressor genes in inherited cancers involves the loss of the wt allele by loss of heterozygosity (LOH) or by dominant-negative mode of action whereby the wt allele is prevented from carrying out its function by binding to the mutant allele. We and others have shown that the mechanism of tumorigenesis in association with CHEK2 variants may not involve LOH (9, 18). The results from this investigation indicate that some germ line Chk2 mutations identified in breast cancer families and described herein are nonfunctional. This is because they are either very unstable or fail to become efficiently phosphorylated on Thr68 and thus cannot oligomerize and become fully activated through further phosphorylation. This mechanism of inactivation would also imply that they cannot act in a dominant-negative manner. Furthermore, an individual homozygous for the 1100delC mutation has been reported (19), and also chk2−/− knockout mice are viable and appear normal (20), indicating that loss of function of Chk2 is not lethal. There is increasing evidence that haploinsufficiency of tumor suppressors also leads to tumorigenesis (21). From the work presented here and other studies, it is therefore proposed that the mechanism by which some Chk2 mutants may contribute to tumorigenesis is haploinsufficiency.
Haploinsufficiency in this instance may be due to low level of expression and/or lack of phosphorylation/oligomerization of the Chk2 protein.

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