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The DNA damage-response pathway has been implicated in many human cancers and cancer-prone diseases. However, this area has been understudied in prostate cancer. In this study, we tested our hypothesis that genetic defects in this pathway may confer susceptibility to prostate cancer using a mutation screening of candidate gene approach. We screened for mutations in CHK2, a direct upstream regulator of p53, and identified a total of 28 (4.8%) germline *CHK2* mutations in 178 primary prostate tumors, 149 familial prostate cancer families, and 400 sporadic cases. Sixteen of the 18 unique *CHK2* mutations identified in this study were not detected among 423 unaffected men, suggesting that *CHK2* mutations have increased risk of developing prostate cancer. We analyzed loss of heterozygosity (LOH) in 21 prostate tumors harboring *CHK2* mutations and found 6 of them lost the wild-type alleles, suggesting that CHK2 is a tumor suppressor. By analysis of patients' EBV-transformed cell lines and by kinase activity assays of cell lines stably expressing the CHK2 mutants, we demonstrated that the mutant CHK2 reduced ability to response to DNA damage through either reduction of CHK2 protein level or reduction of CHK2 kinase activity. Collectively, our data provide evidence for the first time that the germline *CHK2* mutations confer susceptibility to prostate cancer. Mutations in CHK2 may promote tumorigenesis in prostate through altering CHK2 expression level and/or kinase activity.

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Table of Contents

Final Report of DOD grant DAMD17-02-1-0093

CHK2, A Candidate Prostate Cancer Susceptibility Gene

Wanguo Liu, PhD, Mayo Clinic, Rochester MN 55905

Introduction:

Prostate cancer is the second most common cancer and the second leading cause of cancer mortality in American men. Previous studies of family history and twins with prostate cancers have shown that genetics plays a critical role in the development of this disease. However, genetic components contributing to prostate cancer (MIM 300200) have been difficult to identify, largely due to the complexity of this disease and the presence of phenocopies in high-risk families. Regarding the difficulties in identifying high penetrant genes based on linkage analysis and positional cloning, it has been suggested that the pathogenesis of the disease is related at least, in part, to genomic mutations in multiple low-penetrant genes. Although less penetrant, such genes might play an important role at a population level. Our laboratories have recently applied a new approach to identify prostate cancer susceptibility gene(s) based on mutation screening of candidate genes involved in the DNA damage-signaling pathway and identified mutations in the ***CHK2*** gene. CHK2 is a key regulator in this pathway. It regulates a number of downstream effector proteins such as p53 and plays essential roles in coordinating DNA repair, cell cycle progression, transcriptional regulation and apoptosis in response to various DNA-damaging events. While p53 mutations are infrequent in prostate cancer yet common (more than 50%) in all other cancers, we **hypothesize** that CHK2, the upstream regulator of p53, could be a candidate prostate cancer susceptibility gene. Therefore, we **propose** to perform the following investigation:

1. We will screen for *CHK2* mutations in 163 familial prostate cancer families collected at the Mayo Clinic and determine whether the mutations segregate with prostate cancer in families.

2. We will perform functional analyses to determine the impact of mutant CHK2 in the DNA damage-signaling pathway using a kinase activity assay.

3. We will perform loss of heterozygosity (LOH) studies to determine if CHK2 functions as a tumor suppressor in prostate.

   These results will advance our understanding of the etiology of prostate cancer and may also enable us to develop diagnostic tools for the early detection and prevention of prostate cancer.

Body:

We have completely fulfilled all of the aims that we proposed in this proposal and the accomplishments in the grant period (Jan 1, 2003 – Jan 31, 2005) are summarized below:

Aim 1: To screen for *CHK2* mutations in familial prostate cancer families, collected at the Mayo Clinic, and determine whether the mutations segregate with prostate cancer in families.

We first screened all 14 exons of the *CHK2* gene for mutations in 298 samples from 149 families (two from each family) by PCR, DHPLC analysis, and direct sequencing, and we identified 5 different *CHK2* mutations in 9 families. All of the mutations identified in the familial cases were confirmed by direct sequencing of the RT- PCR products amplified from the RNA isolated from the EBV-transformed cell lines generated from the affected individuals in the families. We then collected DNA from 45 family members from the 9 families and analyzed them for *CHK2* mutations. The numbers of mutations identified in the affected men or unaffected family members in each family are summarized in **Table 1.**

Table 1: Germline *CHK2* Mutations in Prostate Cancer Families					
Family ID	***Mutation***	***Family members***	***Aff. men***	***Aff. men with mutation/age***	***Normals with mutations***
1	Ile157Thr	7	3	3 (50, 53, 54)	0/3M + 1F
2	1100del C	4	3	2 (72, 76)	0/1M
3	Ile157Thr	4	3	2 (54, 56)	0/1M
4	Ile157Thr	8	3	3 (56, 58, 62)	3/5F
5	Ile157Thr	5	3	2 (64, 70)	0/2F
6	Ile157Thr	11	4	2 (70, 70)	0/3M + 4F
7	Glu239Lys	2	2	1 (61)	0/0
8	Ile251Phe	2	2	1 (63)	0/0
9	IVS2+1G->A	2	2	1 (74)	0/0
		45	25	17 (63.5)	0/8M, 3/12F

We found 17 (63.5%) of 25 affected men in these families are *CHK2* mutation carriers but none of the eight unaffected men has *CHK2* mutations, suggesting that the *CHK2* mutations may associate with prostate cancer in these families. Interestingly, in the two families (#1 and #4) in which three out of three affected men harboring the *CHK2* mutations, the average age of onset is 55.5, much younger than that (66.4) of the entire familial cases. However, in the linkage analyses of the *CHK2* mutations in the 9 families under the assumption of an autosomal dominant model, we found no recombination between the underlying susceptibility locus and CHK2. Except in two families (#1 and 4), the other seven families showed evidence against cosegregation (**Figure 2 in Appendix A**). We, therefore, could rule out only cosegregation with a highly penetrant effect but we cannot rule out a weakly penetrant effect with our data.

Since the data from analysis of the familial prostate cancer families does not fully support CHK2 as a high-penetrant risk factor in prostate cancer, we performed association studies of the *CHK2* mutations among several groups of cases and controls. As shown (**Table 1 in Appendix A**), we detected *CHK2* mutations in approximately 4% of case groups but only 1.4% in the control group. Since the frequency of the Ile157Thr mutations did not appear to differ between the case (1.6%) and control (1.18%), we tested the significance of our mutation data with and without this alteration. A global test using Fisher's exact test showed a significant difference among all of the groups ($p = 0.002$). When the Ile157Thr mutation was omitted, the p value was <0.0001. Each of the four case groups was then compared individually to the pooled control groups. With all of the data included, only the first unselected tumor group and the sporadic cases showed a statistically significant increase in the frequency of *CHK2* mutations compared to the control group. When the Ile157Thr mutation was excluded, each of the three non-familial groups demonstrated statistically significant increases ($p < 0.0001$, 0.0003, and 0.008, respectively). In both analyses, the frequency of *CHK2* mutations in the familial group was not statistically different than the control group. When the mutations were broken down into 4 different categories (1100delC, all truncating mutations, all missense, and all missense except Ile157Thr), the associations between the mutations and prostate cancer risk were still significant, with the exception of 1100delC (**Table 2**).

Table 2. Germline *CHK2* Mutations in Men with or without Prostate Cancer

Mutations	Non-familial CaP cases	Unaffected Men	Fisher Exact Test
	n = 578	n = 423	
1100delC	5	0	0.077
All truncating mutations	7	0	0.023
All missense mutations	21	6	0.046
All missense excluding Ile157Thr	14	1	0.006
Total (Ile157Thr included)	28 (4.8%)	6 (1.4%)	0.0025
Total (Ile157Thr excluded)	21 (3.6%)	1 (0.2%)	0.0001

In addition, we realized that 16 of 18 unique *CHK2* mutations identified in cases were not found in the unaffected controls, suggesting a pathological effect of these *CHK2* mutations in prostate cancer development. Collectively, our data suggests that mutations in CHK2 contribute to prostate cancer risk. The detailed results from these studies were described in paper by Dong *et al.* (2003, Appendix A).

Aim 2: To perform functional analyses of mutant CHK2 to determine the impact of the mutants in the DNA damage-signaling pathway using a kinase activity assay.

To explore the functional importance of the *CHK2* mutations in prostate cancer development, we analyzed mutant CHK2 proteins by two different approaches. First, using Western blot analysis of four different CHK2 mutations in the patients' EBV-transformed cell lines, we found dramatic reduction of CHK2 protein levels in the cell lines harboring the truncating mutations (1100delC and IVS2+1 G→A) (**Figure 1**).

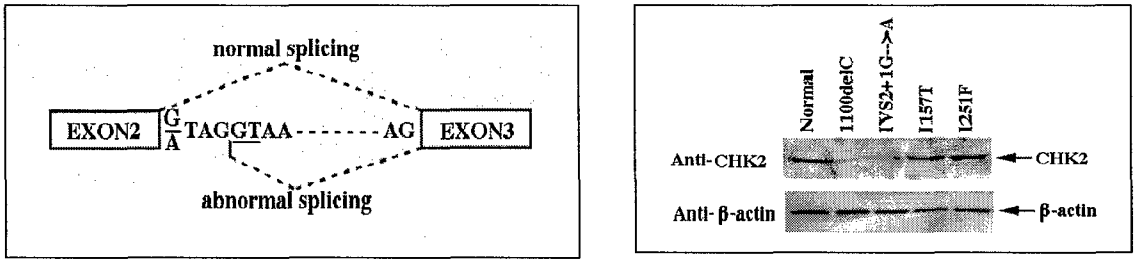


Fig.1. Abnormal splicing and abnormal protein syntheses of the two CHK2 truncating mutations. Left, Schematic representation of the abnormal splice for the IVS2+1G->A mutant. A 4-bp insertion is created due to the usage of the new splice donor site. Right, Western blot analysis showing the reduction of CHK2 in cell lines carrying the two truncating mutations (1100delC and IVS2+1 G→A).

For missense *CHK2* mutations, we examined their kinase activities before and after IR. We generated and analyzed stable cell lines expressing nine *CHK2* mutants (**Table 3**) and found that the kinase activities of the majority of the *CHK2* missense mutations were greatly reduced and one (E321K) was completely abolished (**Figure 2**). Thus, it is very likely that these *CHK2* mutations identified in prostate cancer affect some steps in CHK2 activation after the initial phosphorylation of CHK2 at the Thr-68 site by ATM. Since reduction of CHK2 protein level or reduction of CHK2 kinase activity impairs the function of CHK2 in response to DNA damage, our data suggests that the CHK2 mutations identified in CaP may lose the capability to maintain genomic stability resulting in neoplastic transformation in prostate cancer. The manuscript describing the detailed functional analysis of the CHK2 mutations has been submitted to *Am J Hum Genet* (**Appendix B**).

Table 3. *CHK2* mutations identified in prostate cancer for kinase activity assay

Mutants	*Mutation*	*Amino acid change*	*Domain*	
1	G190A	E64K	S/TQ-rich	germline
2	A349G	R117G	FHA	somatic
3	G434C	E145P	FHA	germline
4	T479C	I157T	FHA	germline
5	C541T	R181C	Unknown	germline
6	G715A	E239K	Kinase	germline
7	G961A	E321K	Kinase	somatic
8	A967C	T323P	Kinase	germline
9	C1427A	T467K	Kinase	germline

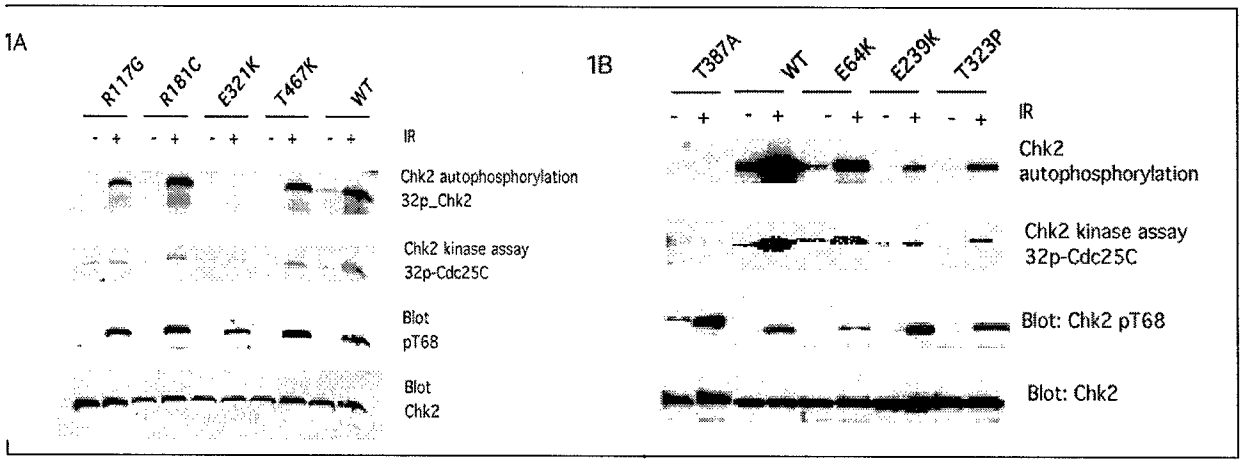


Fig. 2. CHK2 mutants in prostate cancer patients reduce CHK2 kinase activity. A) HCT15 cell lines stably expressing WT and 4 CHK2 mutants were treated with 10Gy of IR. Cells were collected one hr later and lysed. CHK2 was IP with anti-HA antibody and CHK2 kinase assays were performed (upper panel). WB blots with anti-pT68 and anti-CHK2 antibodies were included (lower two panels). B) Other 4 CHK2 mutants were analyzed similar to that described in A.

Aim 3: To perform loss of heterozygosity (LOH) analyses to determine if CHK2 functions as a tumor suppressor in prostate.

Preparation of tumor DNA for LOH analysis:

To determine whether *CHK2* is a tumor suppressor gene in prostate cancer development, we analyzed the primary tumors harboring *CHK2* mutations for evidence of LOH. As we reported before, we have identified a total of 18 germline and 2 somatic *CHK2* mutations in tumor or blood samples from 43 patients with prostate cancer. Tumor specimens for 21 of these patients were available for LOH analysis (**Table 4** in the next page). For patients whose *CHK2* mutations were originally identified in tumor specimens, the freshly frozen tumor tissues and the matched normal adjacent tissues were used for LOH study. For those whose mutations were identified in blood samples, the paraffin-embedded primary tumor specimens (indicated by *) and the blood DNA were used for this analysis. Laser capture microdissection (LCM) was performed on the slides from the tumor specimens for enrichment of the tumor cells. The adjacent normal tissues were also examined for any trace of tumor tissue contamination before slicing for DNA isolation. DNA isolated using this approach warranted accurate LOH analyses and results.

Markers and LOH analysis:

Two types of markers were used for LOH analyses. We searched microsatellite markers in the flanking regions of the *CHK2* gene in human genome and identified five potential markers for testing. They are D22S277, D22S283, D22S1177, D22S272, and D22S275. The marker D22S275 resides between exons 4 and 5 within the *CHK2* gene and the others reside about 25-33 kb telomeric or centromeric to the gene. For some of the tumor samples, we also used the mutation itself as a marker for LOH analysis. LOH investigation was carried out by PCR on tumor and matched normal DNA using fluorescent-labeled primers. The PCR products were electrophoresed using ABI 377 and analyzed by Gene-Scan software. A signal reduction in one allele of at least 70% was taken as the threshold for recognition of LOH. The tumor was scored as having LOH when at least one of the five microsatellite markers lost wild-type allele. Six tumors were found to have LOH (**Table 4**).

Table 4: LOH analysis of prostate tumor samples carrying *CHK2* mutations				
Samples	Mutations	Domains		LOH positive markers
1	G190A (Glu64Lys)	S/TQ-rich	Germline	
2	A349G (Arg117Gly)	FHA	Somatic	D22S275
3	G434C (Arg145Pro)	FHA	Germline	
4	T470C (Ile157Thr)	FHA	Germline	
5*	T479C (Ile157Thr)	FHA	Germline	
6*	T479C (Ile157Thr)	FHA	Germline	
7*	T479C (Ile157Thr)	FHA	Germline	
8	C538T (Arg180Cys)	Unknown	Germline	D22S283
9	C538T (Arg180Cys)	Unknown	Germline	
10	C541T (Arg181Cys)	Unknown	Germline	
11*	G715T (Glu239Stop)	Kinase	Germline	D22S275
12	G715A (Glu239Lys)	Kinase	Germline	
13*	A751T (Ile251Phe)	Kinase	Germline	
14	G954A (Arg318His)	Kinase	Germline	
15	G961A (Glu321Lys)	Kinase	Somatic	D22S275/D22S272
16	A967C (Thr323Pro)	Kinase	Germline	
17	1100delC (Frameshift)	Kinase	Germline	
18	1100delC (Frameshift)	Kinase	Germline	D22S275
19	1100delC (Frameshift)	Kinase	Germline	D22S272
20*	1100delC (Frameshift)	Kinase	Germline	
21	C1427A (Thr476Lys)	Kinase	Germline	

To confirm the above LOH results, we also analyzed LOH in these tumors using DHPLC technology. Briefly, the exon containing the *CHK2* mutation was PCR amplified from the tumor and matched normal DNA. The PCR products were then subjected to DHPLC analysis. As shown in **Figure 3**, when one allele is reduced, the peak of the heteroduplex is also reduced. By comparison between the DHPLC profiles generated by analyzing normal or tumor DNA, LOH in the six prostate tumors were determined. These data confirmed that the LOH results produced using the microsatellite markers were accurate.

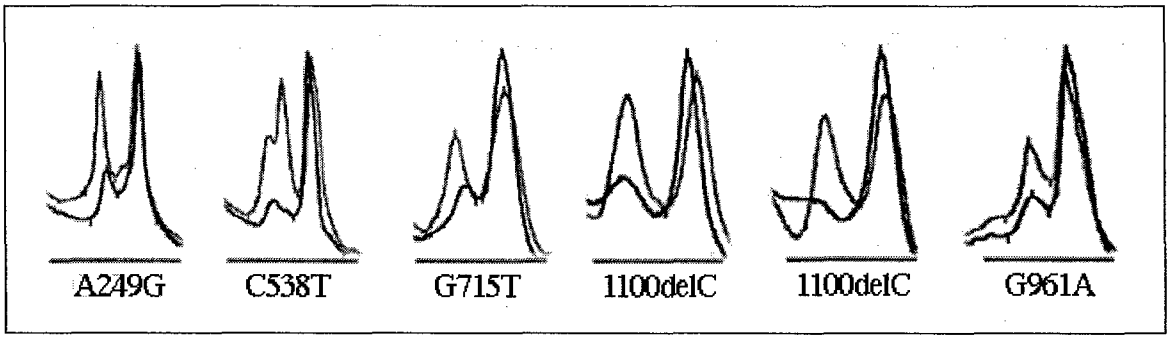


Fig. 3. LOH analysis of six prostate tumors carrying *CHK2* mutations by DHPLC. From left to right are six overlaid DHPLC profiles generated from tumor samples #2, 8, 11, 18, 19, and 15 listed in table 3. The blue chromatogram in each profile represents the DHPLC result generated by analyzing the tumor DNA while the red represents the result from the matched normal DNA.

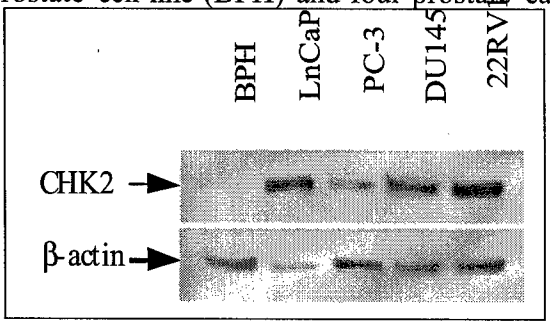
LOH results:

Six of the 21 tumors carrying *CHK2* mutations were found to lose the wild-type alleles by LOH analysis (**Table 4 and Figure 3**). Both tumors carrying somatic *CHK2* mutations lost the wild-type alleles while 4 of 19

tumors harboring germline *CHK2* mutations also lost wild-type alleles. Moreover, 2 of 4 tumors carrying 1100delC mutations were demonstrated to have LOH in *CHK2* locus. LOH has also been reported in breast cancer patients carrying this *CHK2* mutation. Our results support the functional significance of CHK2 mutations in prostate cancer and suggest that CHK2 is probably a tumor suppressor in prostate, particularly in tumors carrying somatic *CHK2* mutations or 1100delC germline mutations.

CHK2 expression in prostate cancer:

CHK2 was activated after DNA damage. Activated CHK2 subsequently phosphorylates p53 to induce cell cycle regulation, apoptosis, and DNA repair pathways to prevent neoplastic transformation. Many lines of evidence have suggested that prostate cancer probably result from DNA damage. However, the CHK2 expression level in prostate cancer has never been investigated. We compared CHK2 protein levels in one benign prostate cell line (BPH) and four prostate cancer cell lines (LnCaP, PC-3, DU-145, and 22RV). Western blot data showed that CHK2 was activated in all 4 tumor cell lines but not in the benign cell line (**Figure 4**). This preliminary data suggests that CHK2 is probably necessary to be activated in prostate tumor tissues to respond to DNA damage. Mutations in CHK2 may impair the integrity of the DNA damage-response pathway and thus induce tumorigenesis. We are now planning to perform Western blot analysis on a large set of primary prostate tumor tissues and compare the CHK2 protein levels between tumors and their matched normal adjacent tissues including tumors carrying *CHK2* mutations to test the above hypothesis.



Key Research Accomplishments:

1. We analyzed *CHK2* mutations in prostate cancer patient blood or tumor samples and identified germline *CHK2* mutations in 4.8% (28/578) of the patients but in only 1.4% (6/423) of unaffected men, suggesting a 3-4 fold increased risk for male *CHK2* mutation carriers to develop prostate cancer. If the Ile157Thr alteration is omitted, the increased risk for male carriers will raise to more than 15-fold (21/578 vs. 1/423). In addition, we also identified 2 (2.4%) *CHK2* somatic mutations in 84 prostate tumor samples.

2. We analyzed *CHK2* mutations in 149 of familial prostate cancer families and identified 9 families who carry *CHK2* mutations. We found that the age of onset of the affected men in two families with complete segregation of *CHK2* mutations and prostate cancer was very young. Since 7 of 9 families showed evidence against cosegregation, we could rule out only cosegregation with a highly penetrant effect; but we could not rule out a weakly penetrant effect with our data.

3. We established several EBV-transformed cell lines from patients carrying *CHK2* mutations and generated stable cell lines expressing nine *CHK2* mutations identified in prostate cancer patients or tumors. Using these cell lines, we demonstrated that the abilities of CHK2 mutant's response to IR were greatly reduced through either reduction of protein level (for truncating *CHK2* mutations) or reduction of kinase activities (majority of missense *CHK2* mutations).

4. We performed LCM on 21 prostate tumor specimens harboring *CHK2* mutations and investigated LOH on these tumors. We found that wild-type alleles were lost in 6 of 21 prostate tumors, particularly in those carrying *CHK2* somatic mutations and those harboring 1100delC germline mutations. Our results suggest that CHK2 is a tumor suppressor in prostate.

5. We also analyzed CHK2 protein level in prostate cancer cell lines demonstrating that CHK2 is activated in tumors but not in benign tissues. This result suggests that the elevation of CHK2 protein level might be necessary for proper response to DNA damage in prostate tissue; reduction of CHK2 protein level due to CHK2 mutations or LOH could be one of the triggers to induce prostate tumorigenesis.

Reportable outcomes:

1. Men carrying *CHK2* mutation have increased risk of developing prostate cancer by 3-4 fold.
2. Both germline and somatic *CHK2* mutations identified in prostate cancer patients or tumors impair CHK2 expression level or kinase activity.
3. Loss of heterozygosity (LOH) was observed in 6 of 21 prostate tumors analyzed, suggesting that CHK2 is a tumor suppressor in prostate.
4. CHK2 is activated in prostate cancer.

Conclusions:

Germline *CHK2* mutations are present in both familial and sporadic prostate cancers but with very low frequency in unaffected men, suggesting that mutations in the *CHK2* gene is associated with prostate cancer risk. Mutant CHK2 lost ability to respond to DNA damage through either reduction of protein level, reduction of kinase activity, or deletion of the wild-type allele due to LOH. Our study strongly supports the notion that the integrity of the DNA damage-response pathway is essential for the prevention of neoplastic transformation in prostate and genetic defects in CHK2 and probably in many other DNA damage-response genes may play an important role in the development of prostate cancer.

Appendices:

A. Dong X, Wang L, Taniguchi K, Wang X, Cunningham JM, McDonnell SK, Qian C, Marks AF, Slager SL, Peterson BJ, Smith DI, Cheville JC, Blute ML, Jacobsen SJ, Schaid DJ, Tindall DJ, Thibodeau SN & Liu W (2003) Mutations in *CHEK2* Associated with Prostate Cancer Risk. ***Am J Hum Genet*** 72:270-280.

B. Xianglin Wu, Xiangyang Dong, Wanguo Liu, and Junjie Chen. Characterization of *CHEK2* mutations in prostate cancer (submitted to ***Am J Hum Genet***)

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Mutations in *CHEK2* Associated with Prostate Cancer Risk

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The DNA-damage–signaling pathway has been implicated in all human cancers. However, the genetic defects and the mechanisms of this pathway in prostate carcinogenesis remain poorly understood. In this study, we analyzed CHEK2, the upstream regulator of p53 in the DNA-damage–signaling pathway, in several groups of patients with prostate cancer. A total of 28 (4.8%) germline *CHEK2* mutations (16 of which were unique) were found among 578 patients. Additional screening for *CHEK2* mutations in 149 families with familial prostate cancer revealed 11 mutations (5 unique) in nine families. These mutations included two frameshift and three missense mutations. Importantly, 16 of 18 unique *CHEK2* mutations identified in both sporadic and familial cases were not detected among 423 unaffected men, suggesting a pathological effect of *CHEK2* mutations in prostate cancer development. Analyses of the two frameshift mutations in Epstein Barr virus–transformed cell lines, using reverse-transcriptase polymerase chain reaction and western blot analysis, revealed abnormal splicing for one mutation and dramatic reduction of CHEK2 protein levels in both cases. Overall, our data suggest that mutations in *CHEK2* may contribute to prostate cancer risk and that the DNA-damage–signaling pathway may play an important role in the development of prostate cancer.

Introduction

Genetic components contributing to prostate cancer (MIM 300200) have been difficult to identify, largely because of the complexity of this disease and the presence of phenocopies in high-risk families. Current genetic studies, using linkage analysis of "high-risk families" followed by positional cloning approaches, have identified more than six susceptibility loci (Ostrander and Stanford 2000). Only two studies have shown any success with the cloning of candidate susceptibility genes from these regions: *HPC1* (MIM 601518) and *HPC2/ELAC2* (MIM 605367), localized to chromosomes 1q and 17p, respectively (Tavtigian et al. 2001; Carpten et al. 2002). However, follow-up studies for *HPC2/ELAC2* have failed to replicate the original findings (Wang et al. 2001; Xu et al. 2001) or have suggested only a limited role in hereditary prostate cancer (Rebbeck et al. 2000; Wang et al. 2001). Since prostate cancer is heterogeneous in nature, and because of the difficulty in identifying highly penetrant susceptibility genes, it may be that the pathogenesis of the disease is related, at least in part, to genomic mutations in multiple low-penetrance genes. Although less penetrant, such genes might play an important role at a population level.

Genomic instability is a common feature of many human cancers (Hoeijmakers 2001). The DNA-damage–signaling pathway plays a critical role in maintaining genomic stability in response to a variety of DNA-damaging events (Khanna and Jackson 2001). Disruption of this pathway has been shown to be pivotal in cancer development, since several proteins involved in this pathway (such as BRCA1 [MIM 113705], TP53 [MIM 191170], and ATM [MIM 208900]) are frequently mutated in human cancers and in several heritable cancer-prone syndromes, such as Li-Fraumeni syndrome (LFS [MIM 151623]) and ataxia telangiectasia (MIM 208900) (Malkin et al. 1990; Miki et al. 1994; Savitsky et al. 1995). Evidence that the DNA-damage–signaling pathway is also important in prostate cancer development comes from several studies. Adenovirus-mediated antisense *ATM* gene transfer has been shown to sensitize prostate cancer cells to radiation (Fan et al. 2000), and mutation in p53 is associated with amplification of the androgen receptor (MIM 313700) gene in prostate cancer (Koivisto and Rantala 1999). In addition, a low fre-

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quency of germline mutations in the breast cancer predisposition genes *BRCA1* and *BRCA2* (MIM 600185) has been identified in familial prostate cancer (Gayther et al. 2000). Moreover, the male mutation carriers in these families had been shown to have a 3.3-fold increased risk for prostate cancer, relative to the general population (Ford et al. 1994). Cumulatively, these data support the notion that the integrity of the DNA-damage–signaling pathway is essential for the prevention of prostate cancer. Since mutations in TP53, the key regulator of the DNA-damage–signaling pathway, are infrequent in prostate cancer but common in all other cancer types, we hypothesized that other components in this pathway could be mutation targets in prostate cancer.

CHEK2 (MIM 604373) is a mammalian homologue of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1, both of which are involved in the DNA-damage–signaling pathway (Paulovich and Hartwell 1995; Sanchez et al. 1996; Boddy et al. 1998). CHEK2 is phosphorylated in response to various DNA-damage agents in an ATM-dependent fashion (Matsuoka et al. 1998). Activated CHEK2, along with other DNA-damage–activated protein kinases, stabilizes TP53 or enhances degradation of Cdc25A (MIM 116974) in the cell-cycle checkpoint control (Matsuoka et al. 1998; Hirao et al. 2000; Falck et al. 2001), through coordination of DNA repair, cell-cycle progression, and apoptosis (Caspari 2000; Bulavin et al. 2001). Recently, heterozygous germline mutations in the *CHEK2* gene have been identified in patients with LFS, a highly penetrant familial cancer phenotype usually associated with inherited mutations in TP53 (Bell et al. 1999). One of the *CHEK2* germline mutations (1100delC) identified in LFS was also identified in 5.1% of noncarriers of *BRCA1* or *BRCA2* mutations in families with breast cancer, suggesting its involvement in familial breast cancer, as well (Meijers-Heijboer et al. 2002). Subsequently, somatic *CHEK2* mutations were also found in a subset of the primary tumors of LFS, such as sarcoma, breast cancer, and brain tumors, but were rare in other tumors (Allinen et al. 2001; Miller et al. 2002).

In this study, we examined DNA from patients with both sporadic and familial prostate cancers for mutations in *CHEK2*. We compared the frequency of the *CHEK2* mutations in these two prostate-cancer groups with that in an unaffected control group, to determine whether defects in CHEK2 play a role in the development of prostate cancer.

Material and Methods

Ascertainment of Patients with Prostate Cancer

Tissue.—Two separate sets of primary prostate tumor samples were collected at the Mayo Clinic and used in this study. The first set of tumor tissues ($n = 84$) was unselected and was collected between 1997 and 1998. The second set ($n = 92$) was selected for young age at diagnosis (<59 years) and was collected between 2000 and 2001. For these patients, neither family history information nor blood was available.

Blood.—For a third group, blood was collected from patients with prostate cancer ($n = 400$) with no family history of prostate cancer. These patients with sporadic prostate cancer were collected at the Mayo Clinic and were selected from respondents to a family history survey who reported no family history of prostate cancer (Wang et al. 2001). They were matched by year of diagnosis, age at diagnosis, and number of brothers in the familial group, which is described below. All but 11 of these men were treated surgically for their prostate cancer.

Familial Prostate Cancer Ascertainment

Families with familial clustering of prostate cancer were ascertained as described elsewhere (Berry et al. 2000). These families have occurrence of prostate cancer in a minimum of three men over at least two generations. Blood was collected from as many family members as possible. All men with prostate cancer who contributed a blood specimen had their cancers verified by review of medical records and pathologic confirmation. One family had Hispanic ancestry, and the remainder were white. Two affected members (the proband and one randomly selected affected man from the family) from each of 149 families were initially selected for mutation analysis. When mutations were identified, the other available family members were also screened for the specific mutation.

Unaffected Control Individuals

From a sampling frame of the local population, provided by the Rochester Epidemiology Project (Melton 1996), 475 men were randomly selected for a clinical urologic examination (Oesterling et al. 1993). This exam included digital rectal examination (DRE) and transrectal ultrasound (TRUS) of the prostate, abdominal ultrasound for post-void residual urine volume, serum prostate-specific antigen (PSA) and creatinine measurement, focused urologic physical examination, and cryopreservation of serum for subsequent sex-hormone assays. Any patient with an abnormal DRE, elevated serum PSA level, or suspicious lesion on TRUS was evaluated for prostatic malignancy. If the DRE and TRUS were unremarkable and the serum PSA level was elevated (>4.0 ng/ml), a sextant biopsy (three cores from each side) of the prostate was performed. An abnormal DRE or TRUS result, regardless of the serum PSA level, prompted a biopsy of the area in question. In addition, a sextant

biopsy of the remaining prostate was performed. Those men who were found to be without prostate cancer on the basis of this extensive work-up, at baseline or at any of the follow-up exams through 1994, were used for the control population (n = 372). To make up for study attrition, the sample was augmented with random samples from individuals in the population who were subjected to an identical workup (n = 138), resulting in a total sample of 510 men without evidence of prostate cancer (Roberts et al. 2000). Three hundred and thirty-one of these individuals gave informed consent to participate in this particular study. The second group of normal control DNA samples was obtained from 92 men participating in an ongoing NCI prostate cancer chemoprevention trial, all of whom were free of evidence of prostate cancer at the time blood was collected, on the basis of DRE and PSA (PSA level <3 ng/ml). The mean age for the 86 men with age data available was 65 years (range 57.6–75.9 years). These two groups of control individuals were combined for the analysis. This study was approved by the Mayo Clinic institutional review board.

Genomic PCR and Mutation Analyses

DNA and RNA isolation from blood, tumor tissues, and cell lines were performed following the manufacturer's protocol (QIAGEN). Thirteen pairs of intronic primers covering 14 exons of the *CHEK2* gene (GenBank accession number XM_009898) were designed (available upon request). Primers used for amplification of exons 10–14 were particularly designed so that either one or both primers for each set of primers had a base mismatch in the most 3′ nucleotide, compared with sequences from nonfunctional copies of *CHEK2*. The primers thus preferentially amplified the functional *CHEK2* on chromosome 22 rather than nonfunctional copies elsewhere in the genome. PCR amplification was performed in a volume of 12.5 μl containing 25 ng of genomic DNA, each primer at 0.2 μM, each dNTP at 0.2 mM, 2.0 mM $MgCl_2$, 0.5 U of *Taq* polymerase (Ampli*Taq* Gold, Perkin Elmer), and 1 × buffer provided by the manufacturer. Denaturing high-performance liquid chromatography (DHPLC) analyses and direct sequencing of the PCR products were performed as described elsewhere (Liu et al. 1997).

RT-PCR

Lymphoblastoid cell lines from the proband of each family were established on the basis of standard procedures. Lymphocytes from peripheral blood were transformed with Epstein-Barr virus (EBV) and were cultured in RPMI-1640 medium containing 10% fetal bovine serum. All transformed cells were frozen in liquid nitrogen for future use. *CHEK2* germline mutations in these cell lines were confirmed by direct sequencing of genomic DNA. The two pairs of primers used for RT-PCR analysis of the mutations are as follows: CHK2F2 (5′-AAAAGAACAGATAAATACCGAACAT-3′) and CHKR2 (5′-TCTGCCTCTCTTGCTGAACC-3′), covering the mutations T470C, G715A, and A751T; and CHK2F3 (5′-AATTGATGGAAGGGGGAGAGCTGT-3′) and CHK2R3 (5′-TAGGTGGGGGTTCCACATAAGGT-3′), covering the 1100delC mutation. For RT-PCR analysis of the abnormal splicing products in the IVS2+1G→A mutant, one pair of exonic primers covering nucleotides 367 (in exon 2) to 564 (in exon 3) were designed (forward, 5′-TATTGCTTTGATGAACCACTGC-3′; reverse, 5′-TTCAGAATTGTTATTCAAAGGAC-3′). RT-PCR products were cloned into pGEM-T easy vector, according to the manufacturer's protocol (Promega). The abnormal splicing products were detected by DHPLC and were then directly sequenced.

Western Blot Analysis

CHEK2 proteins in the cell lines with *CHEK2* mutations were analyzed by western blot analysis. In brief, total protein from each cell line was harvested, denatured in Laemnli buffer (Bio-Rad), and separated on 8% polyacrylamide gels with prestained protein Benchmark (Gibco/BRL). After being transferred onto Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech), CHEK2 protein was visualized with rabbit polyclonal anti-CHEK2 antibody raised against N-terminal residues of human CHEK2 (kindly provided by Dr. J. Chen) by the ECL Western Blotting System (Amersham Pharmacia Biotech). The mouse monoclonal anti-β-actin antibody (clone AC-15, Sigma) was used as an internal control.

Statistical Methods

The frequencies of mutation carriers were compared among different groups, through use of Armitage's test for trend. For statistical comparisons of patients with familial disease versus control subjects, a test for trend in the number of variant alleles, analogous to Armitage's test for trend in proportions (Sasieni 1997) but with the appropriate variance to account for the correlated family data, was used (Slager and Schaid 2001).

Results

CHEK2 *Mutation Screening*

In this study, we screened the *CHEK2* gene for mutations in several groups of men with prostate cancer. For the first two groups, only tissue (tumor and matched normal) was available for study (clinic tumors 1 and 2 in table 1). In the 178 patients with available tissue, 13 *CHEK2* mutations were identified (table 1; fig. 1). Nine

Table 1

***CHK2* Germline Mutations Identified in Men with Prostate Cancer and in Unaffected Control Individuals**

Mutation Number	Mutation	Amino Acid Change	Exon	Domain	Clinic Tumors 1 (n = 84)[a]	Clinic Tumors 2 (n = 94)[b]	Individuals with Sporadic Prostate Cancer (n = 400)[c]	Individuals with Familial Prostate Cancer (n = 298)[d]	Unaffected Men (n = 423)[e]
1	G190A	Glu64Lys	1	S/TQ-rich	1	0	1	0	0
2	245del15 bp	del DQEPE	1	S/TQ-rich	0	0	1	0	0
3	G434C	Arg145Pro	2	FHA	0	0	1	0	0
4	IVS2+1G→A	Frameshift	2	FHA	0	0	0	1	0
5	T470C	Ile157Thr	3	FHA	1	0	6	7	5
6	G499A	Gly167Arg	3	FHA	0	0	1	0	0
7	C538T	Arg180Cys	3	Unknown	0	2	0	0	1
8	G539A	Arg180His	3	Unknown	0	0	1	0	0
9	C541T	Arg181Cys	3	Unknown	1	0	0	0	0
10	G542A	Arg181His	3	Unknown	0	0	1	0	0
11	G715T	Glu239Stop	5	Kinase	0	0	1	0	0
12	G715A	Glu239Lys	5	Kinase	1	0	0	1	0
13	A751T	Ile251Phe	5	Kinase	0	0	0	1	0
14	G954A	Arg318His	8	Kinase	0	1	0	0	0
15	A967C	Thr323Pro	8	Kinase	1	0	0	0	0
16	A980G	Tyr327Cys	8	Kinase	0	0	1	0	0
17	1100delC	Frameshift	10	Kinase	3	1	1	1	0
18	C1427A	Thr476Lys	12	Kinase	1	0	0	0	0
Total					9 (10.7%)	4 (4.3%)	15 (3.75%)	11 (3.7%)	6 (1.4%)
P value (Ile157Thr included)[f]					<.0001	.07	.03	.08	
P value (Ile157Thr excluded)[g]					<.0001	.0003	.008	.11	

[a] Unselected prostate-cancer tumor samples collected in 1997 and 1998.

[b] Prostate-cancer tumor samples, with a younger age at onset (age <59 years), collected in 2000 and 2001.

[c] Blood samples from patients without a family history of prostate cancer.

[d] Two affected men from each of 149 families were screened.

[e] Population-based control group (n = 331) with a mean age at diagnosis of 53.4 years (range 42–83 years), an average PSA value of 0.9 (range 0.15–9.1), and normal TRUS and DRE results, plus a group of unaffected men (n = 92) enrolled in an ongoing NCI prostate cancer chemoprevention trail, who were free of clinically evident prostate cancer as assessed by DRE and PSA (<3).

[f] P values comparing each group with controls, using Armitage's test for trend. Control data: 6 (1.4%) with mutation, 417 (98.6%) with no mutation.

[g] P values comparing each group with controls, using Armitage's test for trend. Control data: 1 (0.2%) with mutation, 422 (99.8%) with no mutation.

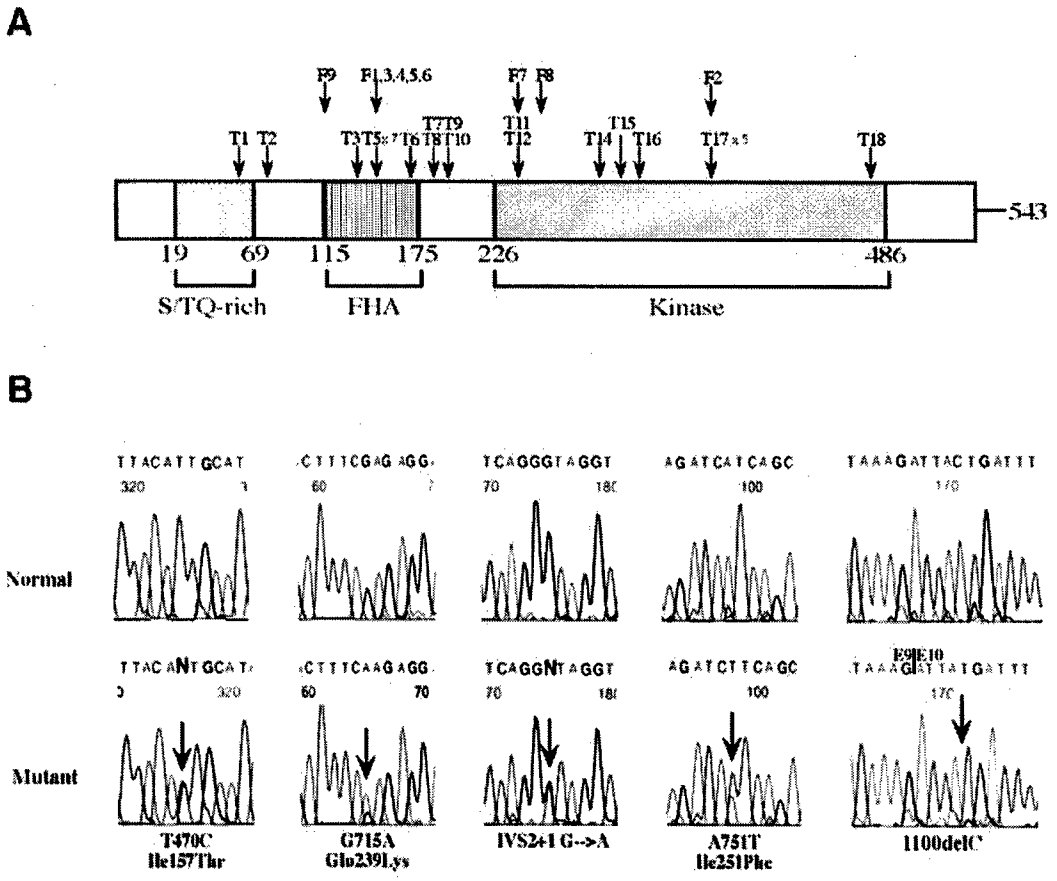

Figure 1 *CHEK2* germline mutations in prostate cancers. *A*, Mutations found in the *CHEK2* gene. "T" indicates the clinic or sporadic prostate tumor samples (numbers as shown in table 1), and "F" indicates families with prostate cancer in which *CHEK2* mutations were identified (numbers as indicated in fig. 2). *B*, Sequence analysis shows the five *CHEK2* germline mutations identified in families with familial prostate cancer. DNA sequence analyses were performed on either genomic DNA (*first four pairs of panels*) or cDNA (*right-most panels*). Sequences are presented in the 5′→3′ direction, and arrows mark the location of each mutation. The upper panels depict the regions from wild-type alleles and the lower panels show the respective sequences with the mutations. All mutations were detected with genomic DNA and were confirmed with cDNA.

(10.7%) were detected among the 84 unselected patients with prostate cancer, and 4 (4.3%) were detected among the 94 patients with early-onset cancer. These included eight different missense mutations and 1-bp deletion mutations at nucleotide 1100 (1100delC). All of the mutations altered evolutionarily conserved amino acids, with the exception of the Arg181Cys mutation in exon 3. These mutations were considered to most likely be germline mutations, since they were present in both tumor and matched normal prostate tissues. However, since DNA from blood was not available for analysis, there is a possibility that they may represent very early somatic events. In an effort to address this concern, DNA from blood leukocytes was obtained from an additional 400 patients with prostate cancer without a family history of prostate cancer. Fifteen *CHEK2* mutations were identified in this third group (3.75%) (table 1). Although there are differences in the frequency of *CHEK2* mutations among the three prostate cancer groups, the overall incidence of *CHEK2* mutations present in these patients (28/578, 4.8%) suggest that *CHEK2* germline mutations are likely to be associated with development of a subset of prostate cancer.

To investigate whether the *CHEK2* mutations are also present in familial prostate cancer, we screened two affected members from each of 149 families with familial prostate cancer collected at the Mayo Clinic (Berry et al. 2000). Five different *CHK2* mutations in nine families were identified (table 1; fig. 1). Three were missense mutations—one in exon 3 (T470C, Ile157Thr) and two in exon 5 (G715A, Glu239Lys and A751T, Ile251Phe). The other two were frameshift mutations, including the 1100delC mutation and a splice-site mutation in intron 2 (IVS2+1G→A). All five mutations changed amino ac-

ids in either the FHA (forkhead homology-associated) or the kinase activation domain of CHK2, which have previously been shown to be important for protein-protein interaction and phosphorylation of p53 in DNA-damage–signaling (Durocher et al. 2000; Shieh et al. 2000; Li et al. 2002). The presence of these mutations in such important functional domains further suggested that these *CHK2* mutations could be deleterious.

To evaluate the association between the *CHEK2* mutations and prostate cancer risk, we screened a group of unaffected men ($n = 423$). This group was comprised of two individual sets (table 1). One set contained 331 population-based unaffected control men (Wang et al. 2001), and the other was comprised of 92 control men free of evidence of prostate cancer. Within this control group, two different mutations among six individuals were detected: Arg180Cys ($n = 1$) and Ile157Thr ($n = 5$) (table 1). For the six unaffected men with *CHEK2* alterations, there was no evidence of disease at the time of blood collection. However, the mean age of these individuals at the time of collection was only 59.6 years (range 45.5–67.0 years), much younger than 71 years, the mean age at diagnosis of prostate cancer for whites in the United States (Bell et al. 1999). Although it is possible that these individuals may develop prostate cancer or other malignancies occurring in LFS or LFS-like syndromes later in life, it is also likely that the Ile157Thr alteration represents a polymorphism rather than a causative mutation.

Among the mutations detected, the frequency of the Ile157Thr mutation did not appear to differ between case (1.6%) and control (1.18%) individuals. We therefore tested the significance of our mutation data with and without this alteration. A global test using Fisher's exact test showed a significant difference among all of the groups ($P = .002$). When the Ile157Thr mutation was omitted, the P value was $<.0001$. Each of the four case groups was then compared individually with the pooled control groups. With all of the data included, only the first unselected group and the sporadic case group showed a statistically significant increase in the frequency of *CHEK2* mutations, compared with the control group (table 1). When the Ile157Thr mutation was excluded, each of the three nonfamilial groups demonstrated statistically significant increases ($P < .0001$, .0003, and .008, respectively). In both analyses, the frequency of *CHEK2* mutations in the familial group was not statistically different than the control group. When the mutations are broken down into four different categories (1100delC, all truncating mutations, all missense mutations, and all missense mutations except Ile157Thr), the associations between the mutations and prostate cancer risk are still significant, with the exception of 1100delC. However, the numbers within each category are too small to allow conclusions to be drawn. The 1100delC mutation has been proposed to confer a low penetrant risk associated with breast cancer risk. Whether it is also a risk factor for prostate cancer or other cancers remains to be elucidated.

CHEK2 *Mutations Present in Families with Familial Prostate Cancer*

To determine whether *CHEK2* mutations cosegregated with prostate cancer in the nine families (families 1–9 in fig. 2), we analyzed the DNA from all available family members for *CHEK2* mutations, including both affected and unaffected individuals. Families 1, 3, 4, 5, and 6 had the Ile157Thr mutation. This alteration was present in all affected individuals in two of the five families (families 1 and 4). Family 1 had six prostate cancer cases in two generations. The Ile157Thr mutation was present in all three affected men, including two brothers (individuals 12 and 13) and their cousin (individual 11), and was absent from three unaffected male siblings (individuals 9, 10, and 14). Although not tested directly, the proband's father (individual 7) and paternal uncle (individual 4) are also expected to be carriers of this mutation, since both are affected and have affected sons with a mutation. In family 4, all the individuals affected with prostate cancer carried the Ile157Thr mutation. However, three sisters also carried the mutation but had no evidence of cancer.

Four families (families 2, 7, 8, 9) had mutations other than Ile157Thr. Family 2 is a family with multiple cancers, including five prostate cancers, two breast cancers, two uterine cancers, three skin cancers, one lung cancer, one bladder cancer, and one lip cancer, in six males and five females in three generations (fig. 2). The proband harbors the 1100delC mutation. Analysis of the available DNA from three affected men and one unaffected man of this family revealed the mutation in two affected men but not in the unaffected brother. The proband's daughters, one of whom was diagnosed with breast cancer and two of whom were diagnosed with uterine cancer, were not available for study. In the other three families, the *CHEK2* mutation was detected in only one of the two affected brothers. Overall, analysis of *CHEK2* mutations in available family members from all nine families revealed that 17 of 25 (68%) affected men harbored *CHEK2* mutations, whereas none of the unaffected men ($n = 8$) carried the mutation (fig. 2).

To test for cosegregation of *CHEK2* mutations with prostate cancer, we performed linkage analyses under the assumption of an autosomal dominant model (Smith et al. 1996) and no recombination between the underlying susceptibility locus with *CHEK2*. Although seven of nine families showed evidence against cosegregation, we could rule out only cosegregation with a highly penetrant effect; we cannot rule out a weakly penetrant effect with our data.

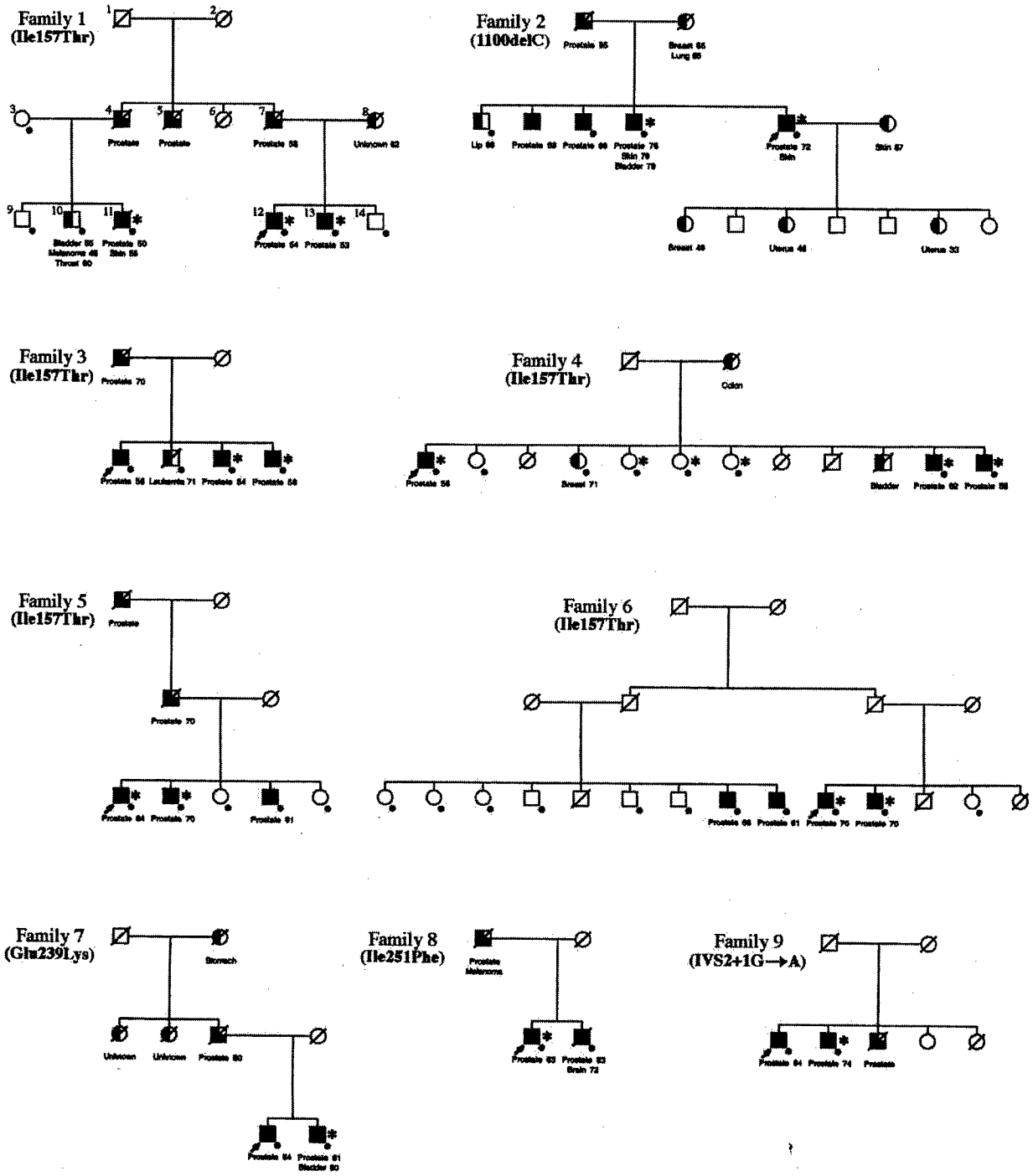

Figure 2 Segregation of *CHEK2* mutations in nine families with prostate cancer (families 1–9). Where known, the individual's age is indicated to the right side of each cancer. A dot (●) is present at the lower right corner of the symbol if a blood sample was available and was analyzed. An asterisk (*) to the right of the symbol indicates the presence of the *CHEK2* mutation carriers in each family. The individual indicated with a dot but without an asterisk has tested negative for *CHEK2* mutation. Arrows (↗) indicate probands. Squares denote males; circles denote females; completely blackened symbols denote patients with prostate cancer for whom pathology records were available; 3/4 blackened symbols denote patients with prostate cancer for whom records were unavailable; 1/2 blackened symbols denote patients with other types of cancer; all symbols with a diagonal denote deceased individuals. The cancer type for each individual is shown underneath each symbol.

Mutant CHEK2 *Altered Protein Expression*

The functional importance of the *CHEK2* mutations in prostate cancer development was explored by examination of the mutant gene products. RT-PCR analysis of the EBV-transformed cell lines that were established from the leukocytes of each proband confirmed that all *CHEK2* mutations were present in their transcripts, including the 1100delC mutation (fig. 1). The splice-site mutation (IVS2+1G→A) results in a 4-bp insertion due to an abnormal splicing using an alternative splice donor site in intron 2 (fig. 3*A* and 3*B*). This mutation creates a premature termination codon in exon 3 and eliminates part of FHA domain and the entire kinase activation domain of CHEK2. Western blot analysis of the two frameshift mutations in the patients' cell lines showed dramatic reduction of CHEK2 protein levels in both cases (fig. 3C). Reduction of CHEK2 protein has been shown to reduce the kinase activity of CHEK2 in response to DNA damage (Matsuoka et al. 2001). Altogether, our data provide evidence that some of the *CHEK2* mutations identified in the patients with prostate cancer whom we studied lead to disruption of CHEK2 expression.

Discussion

In the present study, we identified 18 unique germline *CHEK2* mutations among 28 (4.8%) individuals with prostate cancer and in nine families with familial prostate cancer. With the exception of two mutations (1100delC and Ile157Thr) that were previously reported in LFS (Bell et al. 1999), all *CHEK2* mutations identified in the present

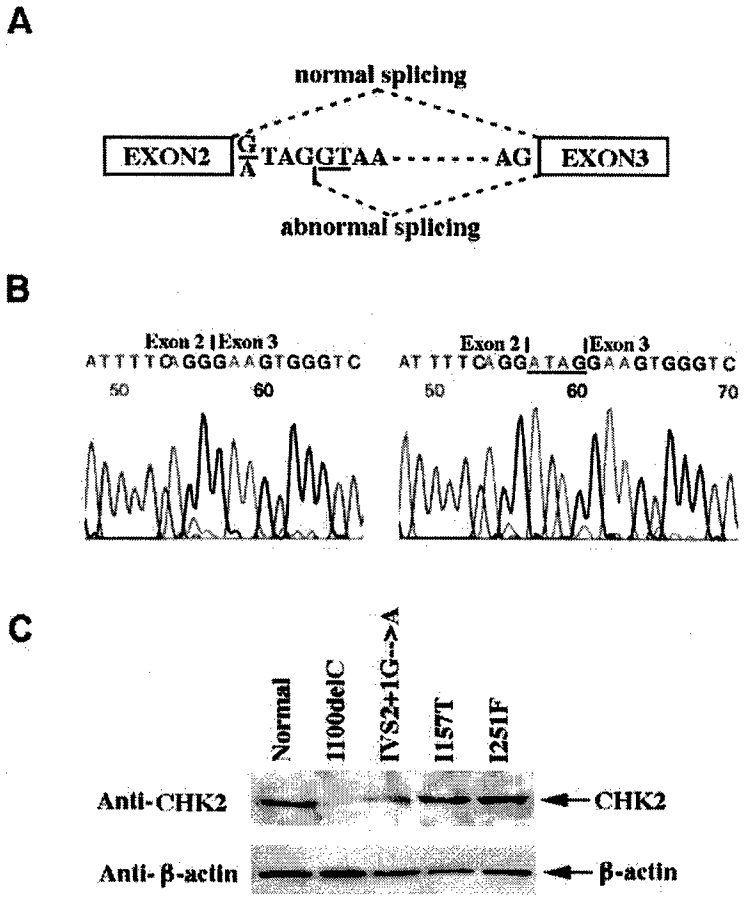

Figure 3 Abnormal splicing and abnormal protein syntheses of the two *CHEK2* frameshift mutations. *A*, Schematic representation of the abnormal splice for the IVS2+1G→A mutant. A 4-bp insertion is created in the mutant transcript because of the usage of the new splice donor site (*underlined*). *B*, Sequences of the wild-type (*left*) and mutant (*right*) *CHEK2* transcripts (between exons 2 and 3) from the cell line established from the affected men carrying the IVS2+1G→A germline mutation. *C*, Western blot analyses showing the reduction of CHEK2 in the cell lines carrying the frameshift mutations, compared with the normal lymphocyte cells and the cells carrying *CHEK2* missense mutations.

study are unique to prostate cancer. Moreover, some of the mutations presumed to be deleterious are represented by two new truncation mutations (IVS2+1G→A and Glu239Stop), which are predicted to lose their kinase activities.

Association studies between patients with sporadic disease and unaffected control individuals indicated an increased risk of developing prostate cancer in men harboring *CHEK2* mutations. The risk appears to be higher when the Ile157Thr mutation is excluded. In contrast, the frequency of *CHEK2* mutations in the familial group was not significantly different from that in the control group (table 1). Although the small sample size may account for this, the finding may reflect the presence of more-highly-penetrant genes in the familial group, compared with the other groups. In addition, the patients having *CHEK2* mutations in the familial cases may themselves represent phenocopies—that is, the prostate cancer in these patients may be due to *CHEK2* mutations and not due to other highly penetrant susceptibility genes segregating within the family. We recognize that well-designed epidemiological studies of large sample sets will be necessary to determine the relevance of these mutations in families with familial prostate cancer.

Germline *CHEK2* mutations were first reported by Bell et al. (1999) in patients with classic LFS and wild-type p53. LFS is a highly penetrant familial cancer syndrome, classically associated with germline mutations of *TP53*. The spectrum of cancers in this syndrome includes breast cancer, soft tissue sarcoma, brain tumors, osteosarcoma, leukemia, and adrenocortical carcinoma (Birch et al. 1984, 1990). Recently, the germline 1100delC mutation was identified in noncarriers of *BRCA1* or *BRCA2* mutations from families with breast cancer, the primary cancer in LFS (Meijers-Heijboer et al. 2002). This mutation is thought to confer a low penetrance for breast cancer. Allinen et al. (2001) screened the *CHEK2* gene in 79 Finnish families with hereditary breast cancer that did not have mutations in *BRCA1, BRCA2,* or *TP53*. However, they found only the Ile157Thr alteration, which was also present in 6.5% of control DNA samples. To date, other than somatic *CHEK2* mutations, there is no germline *CHEK2* mutation reported in other primary tumors of LFS (Miller et al. 2002). It is important to point out that there was no evidence, on the basis of published criteria, that the nine families with familial prostate cancer in which we detected germline *CHEK2* mutations had LFS or LFL syndrome (Li et al. 1988; Birch et al. 1994). Ascertainment of these families included collection of family history through telephone interviews and construction of formal pedigrees.

The most common *CHEK2* mutation identified in our study was Ile157Thr. The role of this mutation, however, is controversial, even though both genetic and biochemical data from previous studies suggest that this mutation is deleterious (Bell et al. 1999; Falck et al. 2001; Li et al. 2002). On the other hand, this mutation was found in 2.1% (2/95) of healthy population control individuals in Finland and was proposed as a polymorphism (Vahteristo et al. 2001). Other reports also indicate that this mutation is relatively common in normal healthy control individuals (Allinen et al. 2001; Meijers-Heijboer et al. 2002). In our current study, the frequency of this variant was not significantly different among the several groups of samples tested (1.21% for the sporadic prostate cancer groups, 2.34% for familial prostate cancer, 1.18% for unaffected control groups) (table 1). Whether this functionally related *CHEK2* variant confers susceptibility to prostate cancer, or even to other cancers, remains to be clarified.

The presence of *CHEK2* mutations in prostate cancer highlights the importance of the integrity of the DNA-damage–signaling pathway in prostate cancer development. The fact that mutations in BRCA1 and BRCA2, two other proteins in this pathway, confer an increased risk of prostate cancer further supports this notion (Gayther et al. 2000). Moreover, the recently developed genomic instability-based transgenic mouse model for prostate cancer demonstrated the presence of a similar phenotype of early stages of human prostate cancer and that the genomic instability could be an early event in this disease (Voelkel-Johnson et al. 2000). Overall, our data provide new genetic evidence for the involvement of the DNA-damage–signaling pathway in prostate cancer development. Although the mechanism by which *CHEK2* mutations contribute to the development of prostate cancer remains unclear, future studies will add to the observations in the present report. The finding of germline mutations in *CHEK2* in both sporadic and familial prostate cancer may facilitate early diagnosis of this cancer and may provide additional insights into the biology of this malignancy, for future therapeutic applications.

Acknowledgments

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *CHEK2* [accession number XM_009898])

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for prostate cancer [MIM 300200], HPC1 [MIM 601518], HPC2/ELAC2 [MIM 605367], BRCA1 [MIM 113705], TP53 [MIM 191170], LFS [MIM 151623], ataxia telangiectasia and ATM [MIM 208900], androgen receptor [MIM 313700], BRCA2 [MIM 600185], CHEK2 [MIM 604373], and Cdc25A [MIM 116974])

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Characterization of Chk2 mutations in prostate cancer

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ABSTRACT.

The checkpoint kinase 2 (Chk2) is a tumor suppressor that participates in the DNA damage-signaling pathway. It is phosphorylated and activated following DNA damage, resulting cell cycle arrest and apoptosis. Previously, we identified germline mutations of Chk2 in patients with prostate cancer. In this study, we have identified additional two somatic mutations of Chk2 in prostate cancer patients and investigated the functions of these mutants *in vivo*. We have shown that most of the germline mutations of Chk2 and one somatic mutation (R117G) have modest reduced Chk2 kinase activity in comparison with wild type Chk2. In addition, one somatic mutation (E321K) totally abolishes Chk2 kinase activity. Several clinical Chk2 mutations reside in the FHA domain of Chk2. To understand the role of FHA domain in the regulation of Chk2, we generated a series of missense mutations within this domain. Here, we demonstrated the requirement of an intact FHA domain for the full activation of Chk2. Taken together, these results provide evidence that Chk2 mutations identified in prostate cancer may contribute to the development of prostate cancer in patients.

The abbreviations used are: Chk2, checkpoint kinase 2; SQ, SQ-rich; IR, ionizing radiation; FHA, forkhead-associated; HA-tag, hemagglutinin-tag

Introduction

Maintenance of genomic integrity depends on the coordination of cell cycle checkpoints, repair systems and apoptosis (zhou BB 2000). DNA damage checkpoints play important roles in maintaining genomic integrity following stresses (Zglinicki et al. 2005). Mutations in genes involved in these checkpoint pathways, such as p53 and ATM, result in genomic instability and cancer predisposition (Blasina et al. 1999).

Checkpoint kinase 2 (Chk2) is a major downstream effector of ATM. As a protein kinase involved in the DNA damage response, Chk2 is rapidly phosphorylated at Thr68 and activated in an ATM-dependent manner following IR (Matsuoka et al. 2000). TTK/hMps and RPA are also required for Chk2 phosphorylation at Thr 68 (Wei et al. 2004; Araya et al. 2005). DNA-dependent protein kinase (DNA-PK) also regulates Chk2 activation by phosphorylating Chk2 (Li and Stern 2005). Therefore, multiple upstream regulators including ATM control the activation of Chk2 following DNA damage.

The activated Chk2 can in turn mediates p53 responses (Tominaga et al. 1999). Chk2 directly phosphorylates Ser-20 site of p53 *in vitro*, and possibly mediates p53 stabilization following DNA damage (Chehab et al. 2000). Chk2 can also phosphorylates p53 at its C-terminus and participates in the regulation of DNA damage-induced acetylation of p53 at this region (Ou et al. 2005). Besides p53, Chk2 also regulate several additional substrates. For example, Chk2 phosphorylates breast cancer tumor suppressor 1 (BRCA1) and regulates BRCA1 function in promoting cell survival after DNA damage (Lee et al. 2000). More recently, several studies suggest that Chk2 also phosphorylates PML (Yang et al. 2002), E2F-1 (Stevens et al. 2003) and p73 (Urist et al. 2004) in response to DNA damage. These phosphorylation events may contribute to p53-independent apoptosis pathway or other cellular processes following DNA damage.

Several lines of evidence support a role of Chk2 as a tumor suppressor. Most, if not all, of above-mentioned proteins functioning upstream or downstream of Chk2 are involved in tumorigensis. Heterozygous germline mutations in Chk2 were identified in a subset of Li-Fraumeni syndrome patients that have wild-type p53 alleles (Bell et al. 1999), raising the possibility that mutation of either p53 or Chk2 is sufficient for the development of Li-Fraumeni syndrome. Similarly, while p53 mutations are rare in myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML) (Fenaux et al. 1990), a recent study identified a Chk2 mutation in FHA domain and another mutation in Chk2 kinase domain in same patient (Tavor et al. 2001). In 41 bone marrow samples from individuals with myelodysplastic syndrome (MDS), Hofmann et al. found one patient with a Chk2 mutation at codon 507 of Chk2 (Hofmann et al. 2001). Moreover, several Chk2 mutations were also identified in colon, lung, bladder, ovary, vulva, and breast cancer patients (Bartek and Lukas 2003). Furthermore, we previously reported that Chk2 may also be mutated in a number of prostate cancer patients (Dong et al. 2003). Taken together, these observations suggest that Chk2 is a tumor suppressor.

Although many Chk2 mutations have been found in patients, only a few of these mutations has been further characterized. Chk2 functions through its kinase activity. To further understand the contribution of Chk2 mutations in prostate cancer development, we examined all of the known Chk2 mutations identified in prostate cancer patients (Dong et al. 2003). These studies suggest that most of these mutations affect Chk2 kinase activity, supporting a role for Chk2 in the development of prostate cancer.

Materials and Methods

Tissues and cell lines for mutation screen

Eighty-four primary prostate tumor tissues were analyzed in this study. Tumors in this set were unselected and collected between 1997 and 1998 at Mayo Clinic. Surgical specimens were collected from patients between 48 and 75 years of age. Matched normal adjacent prostate tissues were used to determine whether the mutations identified are somatic or germline. Three commonly used prostate cancer cell lines (LnCaP, PC-3, and DU-145) were also included in this study.

Genomic PCR and Mutation Analyses

DNA isolation from tumor tissues and cell lines was performed following the manufacturer's protocol (QIAGEN). Thirteen pairs of intronic primers covering 14 exons of the Chk2 gene (Genbank No. XM_009898) were designed (available upon request). Primers used for amplification of exons 10-14 were particularly designed so that either one or both primers for each set of primers had a base mismatch in the most 3' nucleotide compared with sequences from nonfunctional copies of Chk2. The primers thus preferentially amplified the functional Chk2 on chromosome 22 rather than nonfunctional copies elsewhere in the genome. PCR amplification was done in a volume of 12.5 μl containing 25 ng of genomic DNA, each primer at 0.2 μM, each dNTP at 0.2 mM, 2.0 mM $MgCl_2$, 0.5 U of *Taq* polymerase (AmpliTag Gold, Perkin Elmer), and 1 X buffer provided by the manufacturer. Denaturing high-performance liquid chromatography (DHPLC) analyses and direct sequenceing of the PCR products were performed as previously described (Dong et al. 2003).

Constructs

Mammalian expression plasmid encoding HA-tagged Chk2 was described earlier (Wu et al. 2001). QuikChange site-directed mutagenesis kit (Strategene) was used to introduce point mutations in the Chk2 coding sequence for the generation of expression constructs encoding Glu64Lys, Arg117Gly, Arg 145Pro, Ile157Thr, Arg181Cys, Glu239Lys, Thr323Pro, Thr321Lys, and Thr383Ala mutants. GST-Cdc25C, containing the C-terminal fragment (residues 200-256) of Cdc25C, was used as a Chk2 substrate (Ward et al. 2001).

Cell lines and culture conditions

All cell lines were obtained from American Tissue Culture Collection and cultivated in RPMI 1640 supplemented with 10% fetal bovine serum. To establish stable cell lines expressing HA-tagged wild-type or mutant Chk2, HCT15 cells were cotranfected with plasmids encoding the indicated HA-tagged wild-type or mutant Chk2 and pcDNA3. G418-resistant clones were isolated and exogenous Chk2 expression was confirmed by Western blotting using anti-HA or anti-Chk2 antibody. Clones with similar expression levels of Chk2 were selected for this study. The stable cell lines were cultivated in RPMI 1640 supplemented with 10% fetal bovine serum plus 100 microgram/ml G418.

Antibodies

The generation of anti-Chk2 monoclonal antibody, rabbit-derived anti-Chk2 polyclonal antisera (Wu et al. 2001) and anti-Chk2pT68 antibody were reported previously(Ward et al. 2001).

In vitro Chk2 kinase assay

HA-tagged wild-type and mutant Chk2 were immunoprecipitated with anti-HA antibodies from extracts of cell lines stably expressing wild-type or various mutants Chk2. For Chk2 kinase assay,

immunoprecipitated Chk2 was incubated with substrates for 30 min at 30°C in 30 μl of kinase buffer (50 mM Tris pH 7.5, 10 mM $MgCl_2$, with 10 μM ATP and 10 μCi of ^{32}P-ATP). The reactions were stopped by the addition of 30 μl of 2× laemmli's SDS sample buffer. Proteins were separated by 12.5% SDS-PAGE and transferred onto PVDF membrane. ^{32}P incorporation in substrates was visualized by autoradiography.

Results

Chk2 mutations were identified in patients with prostate cancer.

To understand Chk2 is involved in the development of prostate cancer, we screened mutations in Chk2 gene in patients with prostate cancer. In 84 unselected primary prostate tumor samples, we identified a total of 9 mutations. Seven were considered to be germline or early somatic mutations since they were also present in their matched normal prostate tissues (Dong et al. 2003) (table 1). However, the remaining 2 mutations (A349G and G961A) were only present in tumors but not in matched normal prostate tissues, and were considered to be somatic Chk2 mutations (table 1). In addition, these two mutations were not present in 95 normal control DNA samples (data not shown) nor in a total of 1,000 of genomic DNA samples isolated from affected or unaffected men (Dong et al. 2003). These two somatic mutations changed the conserved amino acids in the Chk2 protein, Arg117Gly (A349G) and Glu321Lys (G961A), raising the possibility that these mutants may affect Chk2 function in the cell.

Chk2 mutants identified in prostate cancer affect Chk2 kinase activity.

To examine whether these Chk2 mutations identified in prostate cancer affect Chk2 function, we introduced these mutations into Chk2 expression vector using QuikChange site-directed mutagenesis. To facilitate the study of these Chk2 mutants, we generated cell lines stably expressing HA-tagged wild-type and mutant Chk2 in Chk2-deficient HCT15 cells (Bell et al. 1999). Only cell lines expressing mutant Chk2 at levels similar to that of wild-type Chk2 were used for further analyses.

To study how these mutations affect Chk2 activation, we examined Chk2 kinase activation before and after DNA damage. Briefly, these cells were irradiated and collected one hour later. Chk2 was immunoprecipitated from whole cell extract with anti-HA antibody and Chk2 kinase assays were

performed using GST-Cdc25C fragment as substrate. Western blots with anti-Chk2 and anti-phospho-Chk2 T68 antibodies were also performed to examine, respectively, Chk2 protein level and phosphorylation of Chk2 at Thr-68 site following DNA damage.

Compared to wild-type Chk2, the Glu321Lys mutant did not have any detectable Chk2 kinase activity (Figure 1A). Interestingly, this mutant still underwent the ATM-dependent phosphorylation of Thr-68 site following DNA damage. In addition, mutants of Arg117Gly, Arg181Cys, Thr476Lys, Glu64Lys, Glu239Lys, Thr323Pro partially reduced Chk2 kinase activity (Figure 1). Except for the Glu64Lys mutant, none of these Chk2 mutants had a reduction of Thr-68 phosphorylation of Chk2 following DNA damage (Figure 1). These observations are quite different from those observed for Arg145Pro mutant. The Arg145Pro mutant abolishes both Chk2 kinase activity and Thr-68 phosphorylation of Chk2 following DNA damage (Wu et al. 2001). Thus, it is likely that these mutations of Chk2 identified in prostate cancer affect some steps in Chk2 activation after the initial phosphorylation of Chk2 at Thr-68 site by ATM.

Generate Chk2 mutations within the FHA domain of Chk2.

The multiple-step regulation of Chk2 activation following IR has been well studied. In response to irradiation, Chk2 is first phosphorylated at Thr-68 by ATM (Ahn et al. 2000). The phosphorylated Thr-68 site of Chk2 interacts with the FHA domain of another Chk2 molecule, and thus leads to the formation of Chk2 oligomers (Ahn and Prives 2002). It is speculated that Chk2 oligomerization may further regulate Chk2 activation. One way to further activate Chk2 is through Chk2 autophosphorylation. Two of the Chk2 autophosphorylation sites are Thr-383 and Thr-387, located within the activation loop of the Chk2 kinase domain (Lee et al. 2001). Mutation of these two residues (Thr to Ala) abolishes Chk2 activation (Ahn and Prives 2002), indicating that autophosphorylation of

Chk2 directly affect Chk2 kinase activity. In addition, we and others have identified Ser-516 of Chk2 as an additional Chk2 autophosphorylation site (Schwarz et al. 2003). Mutation of this residue (Ser-516-Ala) reduces Chk2 kinase activity and impairs Chk2-dependent apoptosis following DNA damage (Wu and Chen 2003). Thus, there seems to be an elaborate regulation of Chk2 activation following DNA damage, and the FHA domain of Chk2 is involved in this complex regulation.

Recent studies suggest that the FHA domain is a phospho-protein binding domain (Durocher et al. 2000). Because of this biochemical activity, it is speculated that the FHA domains may play critical roles in signal transduction. The FHA domain of Chk2 is conserved from yeast to mammals. To further study the role of FHA domain in Chk2 activation, we generated several additional missense mutations in Chk2 FHA domain (Table 2; G116A+R117A, S140A+H143A, N171A). Based on the structure of the Chk2 FHA domain (Li et al. 2002), all these residues are presented on the conserved phospho-protein binding surface. Residues R117 and S140 are proposed to be directly involved in the interaction between the FHA domain and phospho-peptides (Li et al. 2002). Another set of mutations within the Chk2 FHA domain are CEYCFD-to-NAAIRS and GPKNSY-to-NAAIRS (Table 2). They localize at the variable-loop surface of the Chk2 FHA domain (Durocher et al. 2000). The functional significance of these variable loops is not yet clear. We chose the sequence NAAIRS because it is thought to be a flexible linker based on its appearance in both alpha-helical and beta-sheet structures (Wilson et al. 1985).

Most but one FHA domain mutants of Chk2 have normal Chk2 activation following DNA damage.

Similar to that described above for the analysis of Chk2 mutations identified in prostate cancer, we generated HCT15 stable cell lines expressing HA-tagged Chk2 containing various mutations within the FHA domain. While wild-type Chk2 was activated following DNA damage, the G116A+R117A mutant completely abolished Chk2 kinase activity, as shown by the mutant protein's inability to

autophosphorylate or phosphorylate its substrate Cdc25C (Figure 2A). It is interesting to point out that the two mutations identified in prostate cancer (R117G and R127C) seems to target at the same region and have reduced kinase activity in vivo.

Surprisingly, none of the other mutants in the FHA domain, S140A+H143A, N171A, CEYCFD-NAAIRS and GPKNSY-NAAIRS, affected Chk2 kinase activity (Figure 2). These mutants also did not affect the phosphorylation of Thr-68 site following DNA damage (Figure 2).

Discussion

Chk2 is a tumor suppressor involved in the development of several human cancers. We have previously identified Chk2 mutations in prostate cancer patients. Here, we examined whether these Chk2 mutations affect Chk2 activation in vivo. In this report, we have shown that most of these Chk2 mutations reduce or abolish Chk2 kinase activity, suggesting that these Chk2 mutations may contribute to the prostate cancer development.

Chk2 contains three domains, the SQ/TQ, FHA, and kinase domains. SQ/TQ consensus sites are sites phosphorylated by ATM/ATR (Matsuoka et al. 2000). One of the critical phosphorylation sites is the Thr-68 site. Phosphorylation of Thr-68 is important for Chk2 activation and oligomerization. So far, four Chk2 mutations have been identified in the SQ/TQ region (Bartek and Lukas 2003). It has not been determined how these mutations affect Chk2 activity. We identified a Chk2 mutation, Glu64Lys, in the SQ/TQ region in a patient prostate cancer. This mutation reduces both phosphorylation of Chk2 at the Thr-68 site and Chk2 kinase activity following IR (Figure 1B). It is likely that the Glu64Lys mutation might change the conformation of the Chk2 SQ/TQ domain and thus reduce the ability of ATM to phosphorylate and activate Chk2.

We also identified 4 mutations of Chk2 within the kinase domain of Chk2. While the Glu321Lys mutant completely abolishes Chk2 kinase activity following IR, the other three mutants, Glu239Lys, Thr323Pro, Thr476Lys, partially reduce Chk2 kinase activity. This is similar to an early report that the D311V mutant of Chk2 identified in lung cancer only reduces Chk2 kinase activity by 50% (Matsuoka et al. 2001). These findings suggest that impairment of full activation of Chk2 might contribute to tumorgenesis.

The FHA domain of Chk2 is highly conserved from yeast to mammals. Rad53, a yeast homology of Chk2, associates with checkpoint protein Rad9 through its FHA domain following DNA damage (Sun et al. 1998). This FHA domain-mediated interaction is important for Rad53 activation (Sun et al. 1998). In mammals, Chk2 forms oligomers through interaction of the FHA domain of one Chk2 molecule and the pT68 site of another Chk2 molecule (Xu et al. 2002). Therefore, it appears that the FHA domain is also important for the tumor suppressor function of Chk2. Arg145Pro and Ile157Thr mutations in the Chk2 FHA domain have been previously identified (Bell et al. 1999). The Arg145Pro mutation destabilizes the mutant Chk2 protein, reducing its half-life from >120 min to 30 min (Lee et al. 2001). In addition, the Arg145Pro mutant also abolishes Chk2 kinase activity and Thr-68 phosphorylation following DNA damage (Wu et al. 2001). In contrast to the Arg145Pro mutant, we did not detect any defects in the Ile157Thr mutant (Wu et al. 2001). However, other groups have reported that the Ile157Thr mutant may have defects in the regulation of BRCA1 (Li et al. 2002), CDC25A (Falck et al. 2001b) and p53 (Falck et al. 2001a). In this study, we characterized an additional FHA domain mutation identified in prostate cancer patients, Arg117Gly. Arg117 is a conserved residue in the FHA domain and is proposed to be required for the phospho-peptide binding activity of the Chk2 FHA domain (Li et al. 2002). An early study suggested that the Arg117Ala mutation abolishes Chk2 oligomerization (Ahn et al. 2002). Indeed, we have shown that the clinical Arg117Gly mutation has greatly reduced Chk2 kinase activity following DNA damage. In agreement with this observation, the Gly116Ala/Arg117Ala double mutation completely abolishes Chk2 kinase activity, suggesting that these residues are critical for Chk2 function. Surprisingly, several other mutants in the conserved or variable regions of the FHA domain did not affect Chk2 kinase activity. It is possible that these mutants may affect the interaction of Chk2 with other cellular proteins and thus influence the DNA damage responses. Future experiments are needed to further understand the physiological function of the FHA domain of Chk2.

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Figure legends

Figure 1. Chk2 mutants in prostate cancer patients reduce Chk2 kinase activity. A). HCT15 cell lines stably expressing wild-type, T467K, E321K, R181C, and R117G mutant Chk2 were treated with 10 Gy of IR. Cells were collected one hour later and lysed. Chk2 was immunoprecipitated with anti-HA antibody and Chk2 kinase assays were performed (upper panel). Western blots with anti-pT68 and anti-Chk2 antibodies were included in the lower two panels. B). HCT15 cell lines stably expressing wild-type Chk2, E64K, E239K, T323P and T387A mutant Chk2 were analyzed similar to that described in A.

Figure 2. Characterization of Chk2 FHA domain mutants. HCT15 cell lines stably expressing wild-type, N171A, and G116AR117A mutant Chk2 (A) or VGPKNS-NAAIRS, S140AH143A, and CEYCFD-NAAIRS mutant Chk2 were analyzed similarly to that described in Figure 1.

Figure 3. Diagram of Chk2 and Chk2 mutations identified in prostate cancer patients. The positions of tumor-associated mutations are indicated (top). The additional mutations in the Chk2 FHA domain are also indicated (bottom). The bold letters indicate mutations that completely abolish Chk2 activity.

Table 1

Chk2 mutations in prostate cancer

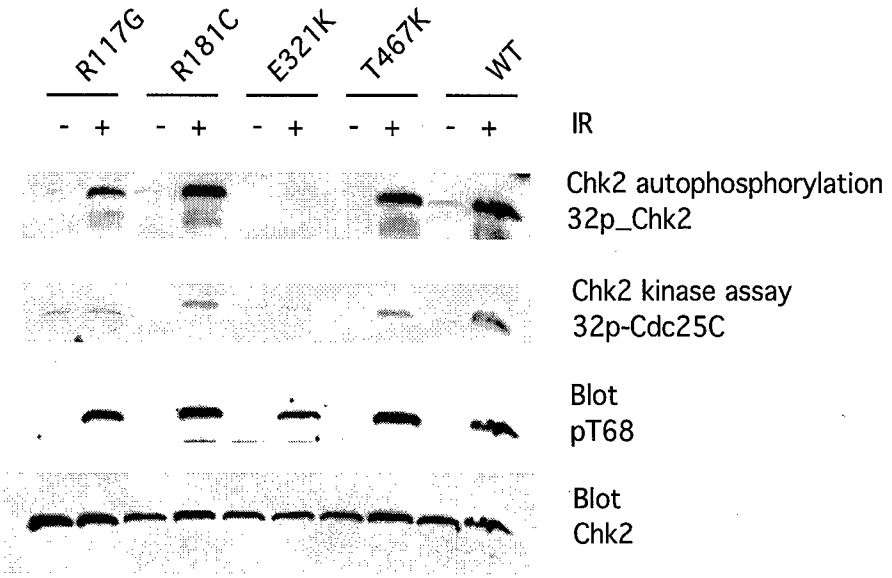
mutants	Amino acid change	Domain	
1	Glu64Lys	S/TQ-rich	germline
2	Arg117Gly	FHA	somatic
3	Arg145Pro	FHA	germline
4	Ile157Thr	FHA	germline
5	Arg181Cys	Unknown	germline
6	Glu239Lys	Kinase	germline
7	Glu321Lys	kinase	somatic
8	Thr323Pro	Kinase	germline
9	Thr476Lys	Kinase	germline

Table 2

Chk2 mutations within the FHA domain of Chk2

mutants	Amino acid change	Domain
1	N171A	FHA
2	S140AH143A	FHA
3	G116AR117A	FHA
4	CEYCFD-NAAIRS	FHA
5	VGPKNS-NAAIRE	FHA

1A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R181C
E321K
T467K
WT
- + - + - + - + - +
IR
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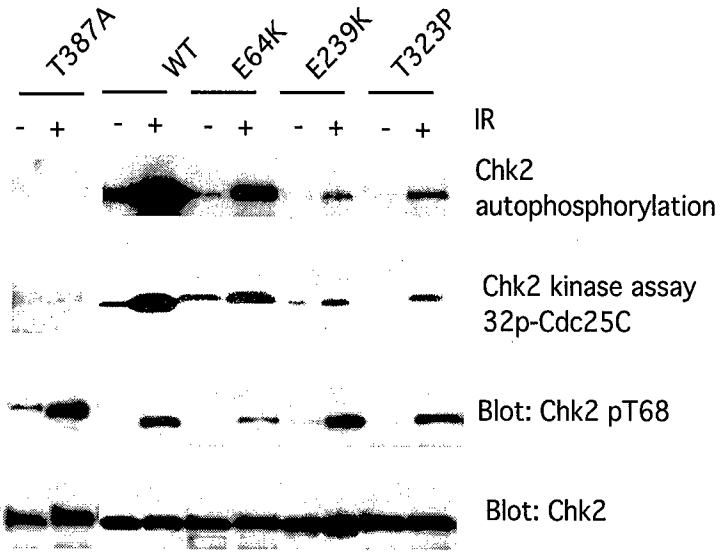
32p_Chk2
Chk2 kinase assay
32p-Cdc25C
Blot
pT68
Blot
Chk2

1B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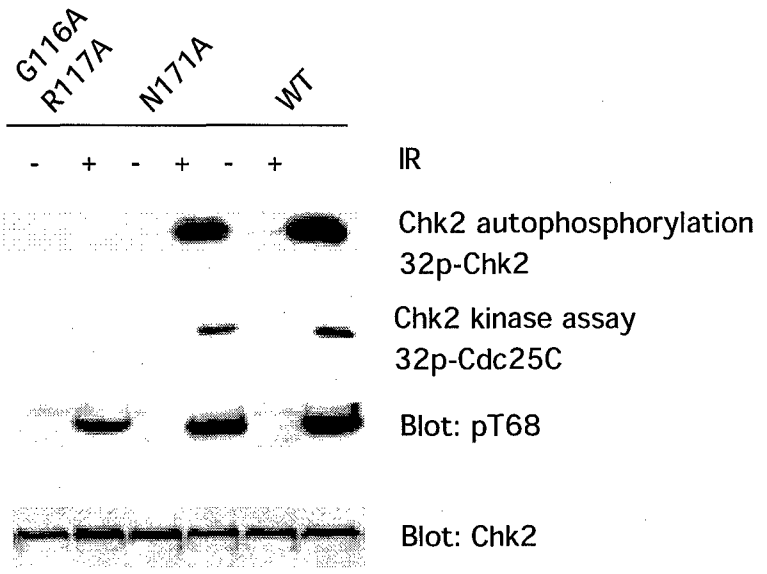
WT
E64K
E239K
T323P
- + - + - + - + - +
IR
Chk2
autophosphorylation
Chk2 kinase assay
32p-Cdc25C
Blot: Chk2 pT68
Blot: Chk2

2A


G116A
R117A
N171A
WT
- + - + - +
IR
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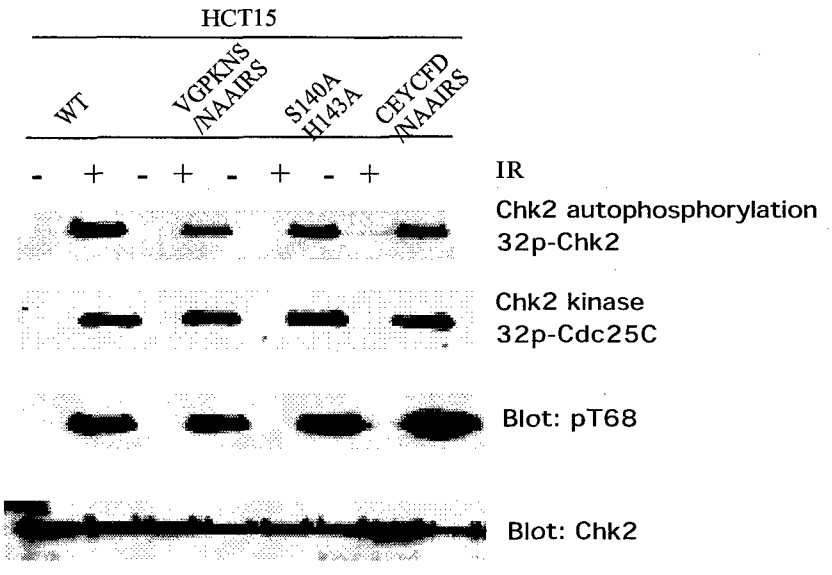
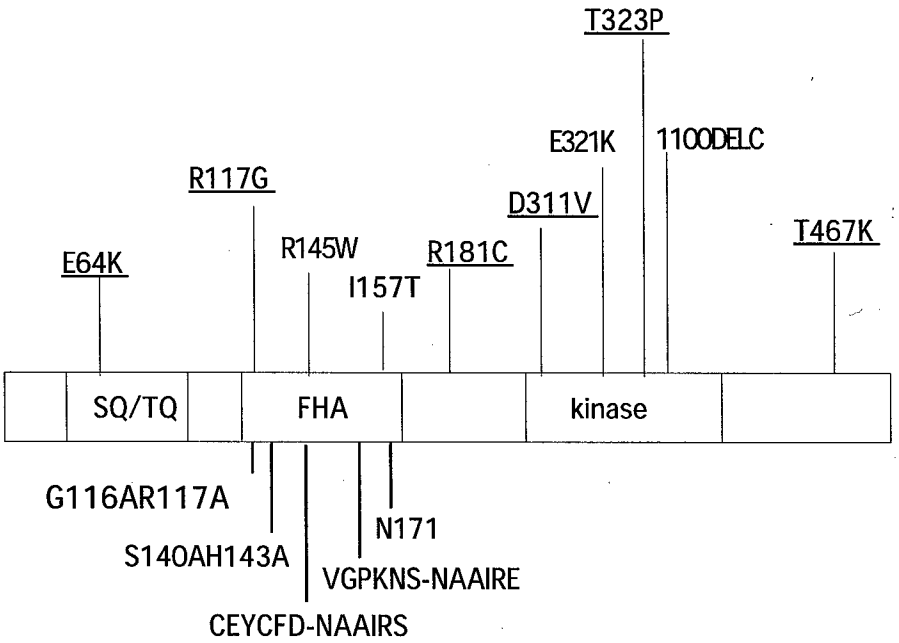
Chk2 kinase assay
32p-Cdc25C
Blot: pT68
Blot: Chk2

2B


HCT15
WT
VGPKNS
/NAAIRS
S140A
H143A
CEYCFD
/NAAIRS
- + - + - + - +
IR
Chk2 autophosphorylation
32p-Chk2
Chk2 kinase
32p-Cdc25C
Blot: pT68
Blot: Chk2


T323P
E321K
1100DELC
R117G
D311V
T467K
E64K
R145W
R181C
I157T
SQ/TQ
FHA
kinase
G116AR117A
N171
S140AH143A
VGPKNS-NAAIRE
CEYCFD-NAAIRS