Genetic and Epigenetic Analysis of CHEK2 in Sporadic Breast, Colon, and Ovarian Cancers
Louise H. Williams, David Choong, Sandra A. Johnson, and Ian G. Campbell

Abstract

Purpose: Germ-line variants in CHEK2 have been associated with increased breast, thyroid, prostate, kidney, and colorectal cancer risk; however, the prevalence of somatic inactivation of CHEK2 in common cancer types is less clear. The aim of this study was to determine if somatic mutation and/or epigenetic modification play a role in development of sporadic breast, colon, or ovarian cancers.

Experimental Design: We undertook combined genetic and epigenetic analysis of CHEK2 in sporadic primary breast, ovarian, and colon tumors [all exhibiting chromosome 22q loss of heterozygosity (LOH)] and cancer cell lines. Expression of Chk2 was assessed by immunohistochemistry in 119 ovarian tumors.

Results: Two novel germ-line variants were identified; however, none of the primary tumors harbored somatic mutations. Two CpG clusters previously implicated in CHEK2 silencing were investigated for evidence of hypermethylation. No methylation was detected at the distal CpG island. The proximal CpG cluster was methylated in all tumor and normal DNA, suggesting that this might not represent a true CpG island and is not relevant in the control of CHEK2 expression. Twenty-three percent of ovarian tumors were negative for Chk2 protein by immunohistochemistry, but there was no significant correlation between LOH across the CHEK2 locus and intensity of Chk2 staining (P = 0.12).

Conclusions: LOH across the CHEK2 locus is common in sporadic breast, ovarian, and colorectal cancers, but point mutation or epigenetic inactivation of the retained allele is uncommon. Loss of Chk2 protein in ovarian cancer was not associated with allelic status, suggesting that inactivation does not occur as a consequence of haploinsufficiency.

The CHEK2 gene has recently been identified as a breast cancer susceptibility gene (1–3), and some germ-line variants may increase the risk of colorectal, thyroid, prostate, and kidney cancer in specific populations (4, 5). CHEK2 encodes the human homologue of the Cds1 and RAD53 checkpoint kinases and plays a central role in the DNA damage checkpoint pathway. In response to ionizing radiation–induced DNA damage, Chk2 is activated by the ataxia-telangiectasia mutated (ATM) protein and subsequently phosphorylates several substrates, including p53, BRCA1, Mdm2, Cdc25A, and Cdc25C, leading to cell cycle arrest, activation of DNA repair mechanisms, or apoptosis. Activated Chk2 has been identified in early precursor lesions of urinary bladder, breast, lung, and colon carcinomas (but not in normal tissues) before the occurrence of genomic instability and malignant conversion (6), suggesting that DNA damage checkpoints are activated in the early stages of tumorigenesis. Therefore, mutations in CHEK2, or other genes involved in the ATM-Chk2-p53 pathway, may allow tumorigenic cells to evade normal cell cycle checkpoints, leading to aberrant cell proliferation and survival, increased genomic instability, and ultimately tumor progression.

Although numerous studies have investigated for CHEK2 germ-line mutations in high-risk cancer families, the frequency of somatic genetic mutation in sporadic cancers has not been investigated extensively. Furthermore, the possibility that CHEK2 might be silenced through promoter methylation has only been investigated in lung (7), lymphoid (8, 9), vulval (10), and breast cancers (11), with only two of these studies (8, 11) doing combined genetic and epigenetic analyses. Consequently, the aim of this study was to determine the frequency of genetic or epigenetic alterations of CHEK2 in sporadic breast, ovarian, and colon cancers. To enrich for samples that had potentially undergone biallelic inactivation of CHEK2, we analyzed tumors with confirmed loss of heterozygosity (LOH) across the CHEK2 locus.

Materials and Methods

Tumor and normal DNA. Tumor and matching normal lymphocyte DNA from 114 ovarian and 43 colorectal cancers were obtained from...
the Tissue Bank at Peter MacCallum Cancer Centre (East Melbourne, Victoria, Australia). Tumor and matching normal DNA from 45 breast tumors were obtained from Dr. Nick Hayward (Queensland Institute of Medical Research, Brisbane, Queensland, Australia). Cancer cell lines used in this study included five breast (BT-20, MDA-MB-468, MCF-7, T47D, and BT-482), five colon (LOVO, HCT-116, HT-29, CaCO2, and LIM2099), and five ovarian (HEY, JAM, MDA-MB-468, MCF-7, T47D, and BT-482), five colon (LOVO, HCT-116, HT-29, CaCO2, and LIM2099), and five ovarian (HEY, JAM, MDA-MB-468, MCF-7, T47D, and BT-482) cell lines.

**Table 1. PCR primers used for SSCP analysis**

<table>
<thead>
<tr>
<th>Exon</th>
<th>CHEK2 primer sequence (5′–3′)</th>
<th>Annealing temperature (°C)</th>
</tr>
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<tr>
<td>Exon 1a</td>
<td>ACAAACAGGGGCTTTCAACAGATT AGGGCCATATCCAGGCTTCTAC</td>
<td>58</td>
</tr>
<tr>
<td>Exon 1b</td>
<td>ATGGAGGGAGCTGCTGAGGAG GGGCCCATATTCTTTCCTTTTTTT</td>
<td>60</td>
</tr>
<tr>
<td>Exon 2</td>
<td>TGACCAATTTACGACTCTCTA TACATGAATTTCAACAGGCCCTT</td>
<td>62</td>
</tr>
<tr>
<td>Exon 3</td>
<td>TCTGCCATGAGGAGCTGAAA AATCCATCTATATCTCCTATT</td>
<td>58</td>
</tr>
<tr>
<td>Exon 4</td>
<td>AATGACGAAATGAGAAGCAAC AAGGGGAATCCGCTTTGGAATAAA</td>
<td>57</td>
</tr>
<tr>
<td>Exon 5</td>
<td>AGGTGATGACGCTCTTATTGGTA ACCCGCAAGGTGAGTCTCATA</td>
<td>60</td>
</tr>
<tr>
<td>Exon 6</td>
<td>ACTGAAAGCTCTATCTCTTCTT CATTGCAACGTTATGGAATCTG</td>
<td>58</td>
</tr>
<tr>
<td>Exon 7</td>
<td>AAAGACTTGGTGAAAGCCAGAG CGCTTGGGACAGTTCCTAAGCTC</td>
<td>58</td>
</tr>
<tr>
<td>Exon 8</td>
<td>ACTAAAGAGAGCGACTGTCGA AAGTTCGCCCCAGAAGTGACAC</td>
<td>57</td>
</tr>
<tr>
<td>Exon 9</td>
<td>TTTCGCAAAGAATCTACAGGAG TAGTATCTACCTGCAATTGCTGGA</td>
<td>58</td>
</tr>
<tr>
<td>Exon 10</td>
<td>ATACCTCTCTACCAAGTCTGCG GCAAGTGTCAACATTACCTTCTTT</td>
<td>62</td>
</tr>
<tr>
<td>Exon 11</td>
<td>GCACATAACATTTAACTCACACA TGGAAAGAATCGACCTTACTTCTTTC</td>
<td>58</td>
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<tr>
<td>Exon 12</td>
<td>CATGCTCTCCAGCAGCAG TTATCTCTTCTACAGTGGTTTGC</td>
<td>58</td>
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<tr>
<td>Exon 13</td>
<td>AGCTCTTTAAGCAGACTAAT GGATTTTTATTCTCCAGACACGC</td>
<td>58</td>
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<tr>
<td>Exon 14</td>
<td>CATCGAGCTGTGAAAGAAC AAATTTGTTTGAACATTTCCCTATTTTCC</td>
<td>57</td>
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**Denaturing high-performance liquid chromatography analysis of normal DNA samples.** The frequency of novel germ-line CHEK2 sequence variants among 258 noncancer controls was estimated using denaturing high-performance liquid chromatography (DHPLC; ref. 13). The optimal melting temperatures for each PCR fragment [using single-strand conformational polymorphism (SSCP) primers] were calculated using the DHPLC Melt Program algorithm provided by Stanford Genome Technology Center.2

**Methylation-sensitive SSCP.** Two CpG islands are located upstream of the CHEK2 gene (Fig. 1). Four sets of methylation-independent primers (MIP2, MIP3, MIP4, and MIP5) were designed, covering a total of 53 CpGs in the distal CpG island (located 6,000–8,000 bp upstream of the translation initiation site), and one set of primers (MIP1) targeting the proximal CpG island (300–600 bp upstream of the translation initiation site). DNA samples were bisulfite treated using the MethyEasy kit (Human Genetic Signatures, Sydney, New South Wales, Australia). Amplification was done (see Table 2 for conditions used), and the PCR products were analyzed by SSCP as described above. Normal lymphocyte DNA treated with SssI methylase before bisulfite conversion was used as a positive control. The methylation status of representative methylation-sensitive SSCP products was confirmed by bisulfite sequencing (BigDye Terminator v3.1).

**Immunohistochemical detection of Chk2.** Chk2 protein expression in ovarian cancer was assessed by immunohistochemistry using a tissue microarray comprising 2-mm-diameter tumor cores (100 of which were also analyzed for LOH). Tissue array sections (4 μm) were affixed to glass slides, dehydrated, and rehydrated. Antigen retrieval was done by boiling (using a microwave) in 10 mmol/L citrate buffer for 30 minutes. Sections were incubated in 3% hydrogen peroxide to quench endogenous peroxidase activity, washed in PBS, blocked in 5% bovine serum albumin, washed in PBS, and incubated with Chk2 primary antibody (1:1,000; Lab Vision, Fremont, CA) at 4°C overnight. A universal linker and horseradish peroxidase label (Supersensitive Link-Label Immunohistochemistry Detection System, BioGenex, San Ramon, CA) followed by incubation with 3,3′-diaminobenzidine chromogen (Biocare Medical, Concord, CA) were used to visualize Chk2 localization. Sections were then counterstained with hematoxylin. Negative controls minus the primary antibody were used. Scoring of Chk2 staining was done using the following criteria: 0, negative staining; 1, weak staining in ≤5% of tumor cells; 2, moderate staining in >5–25% of tumor cells; 3, strong staining in >25% of tumor cells. Differences between categorical variables were evaluated by Fisher’s exact test.
Results

**LOH analysis across the CHEK2 locus.** A total of 114 ovarian cancers, 45 breast cancers, and 43 colorectal cancers was analyzed for LOH across the CHEK2 locus using up to 18 microsatellite markers spanning chromosome 22. LOH across the CHEK2 locus was observed in 54% (62 of 114), 44% (20 of 45), and 30% (13 of 43) of the ovarian, breast, and colorectal cancers, respectively. Among the cases showing LOH, the majority seemed to have lost the entire chromosome, particularly for the ovarian and colon tumors. In the small percentage of cases where only partial loss occurred, none involved exclusively the CHEK2 locus (data not shown).

**Mutation analysis.** Seventy-one primary cancers (including 38 ovarian, 20 breast, and 13 colon tumors), all exhibiting LOH across the CHEK2 locus, as well as 15 cancer cell lines, were assessed for CHEK2 mutations by SSCP analysis. A previously described silent polymorphism in exon 1, codon 84 (A252G; 8, 10, 11, 14–19) was detected in one ovarian tumor, one breast tumor, and three cell lines (2774, LIM2099, and HT-29). This polymorphism was also detected in the matching normal DNA.

**Methylation analysis of the CHEK2 promoter.** Methylation-sensitive SSCP analysis was performed by bisulfite sequencing, thereby confirming the fully methylated sequence indicated by the methylation-sensitive SSCP analysis.

**Immunohistochemical detection of Chk2.** One hundred and nineteen ovarian cancers were evaluated for Chk2 protein expression by immunohistochemistry using a tissue array containing 2-mm tumor cores. Chk2 staining was observed to be predominately nuclear with cytoplasmic staining observed in conjunction with, but never independent of, nuclear staining. Moderate or strong nuclear staining (scored 2 or 3; Fig. 4C and D) was observed in the majority of ovarian tumors (92 of 119, 77%), but a small proportion of tumors showed negative (17 of 119, 14%; Fig. 4A) or very weak (10 of 119, 8%; Fig. 4B) Chk2 immunoreactivity. There was no significant correlation between LOH across the CHEK2 locus and intensity of Chk2 staining (P = 0.12). Additionally, there was no significant correlation of Chk2 immunoreactivity with tumor histologic subtype. We also observed that the ovarian tumor harboring the −4 bp C>T variant described above showed weak Chk2 immunoreactivity (Fig. 4B).

Discussion

Despite being implicated in familial cancer syndromes, such as Li-Fraumeni (14), and being identified as a low-penetration breast cancer susceptibility gene (1), relatively few studies have investigated sporadic cancers for somatic CHEK2 mutations (10, 11, 16, 18–21). There have been only a handful of studies examining CHEK2 for promoter methylation (7–11) and even fewer studies using a combined genetic and epigenetic approach as reported here (8, 10, 11). The evidence to date suggests that CHEK2 behaves as a classic tumor suppressor gene and requires both copies to be inactivated (10, 11, 18, 20). Therefore, to enhance the likelihood of identifying somatic mutations, we focused our study on tumors with verified LOH across the CHEK2 locus.

Table 2. PCR primers used for methylation-sensitive SSCP analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>PCR conditions (annealing temperature, cycles, MgCl2 concentration)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP1</td>
<td>TTTTGTATTGAGTTGGAAGTGTAGTG AAAAAGTCATAAAAACCTACTATTATCCA</td>
<td>56 °C, 40 cycles, 1.5 mmol/L MgCl2</td>
</tr>
<tr>
<td>MIP2</td>
<td>GAGTGGGGTTAGGTGTTTGTAG CCAACCAATCACAACATTATCCTA</td>
<td>59 °C, 35 cycles, 1.5 mmol/L MgCl2</td>
</tr>
<tr>
<td>MIP3</td>
<td>GGGTITTAAAAATGTTTITTAG AAAAAATTTCTTCTACCAACAT</td>
<td>52 °C, 40 cycles, 1.5 mmol/L MgCl2</td>
</tr>
<tr>
<td>MIP4</td>
<td>TTTTTTGAAGATATATTTTTT CAAAGATTCACTTCTACCAACAT</td>
<td>50 °C, 45 cycles, 2 mmol/L MgCl2</td>
</tr>
<tr>
<td>MIP5</td>
<td>TTTTTTTTTTTTTTTTTTTTTTTT CAAAGATTCACTTCTACCAACAT</td>
<td>50 °C, 40 cycles, 1.5 mmol/L MgCl2</td>
</tr>
</tbody>
</table>
This study is the first to investigate CHEK2 somatic mutation and methylation status in sporadic colon cancers. We did not detect any somatic mutations or methylation in primary colon tumors; however, we did identify a novel variant in the colon cancer cell line HCT-116. This variant, T1064C, which results in a leucine to proline substitution (codon 355), was not detected in 258 normal controls nor in the expressed sequence tag or nonredundant databases at the National Center for Biotechnology Information BLAST site, suggesting that it is not a common sequence variant. As matching normal DNA is not available for this cell line, we cannot exclude the possibility that the T1064C substitution may represent a somatic mutation. Although the T1064C variant may be a benign polymorphism, it is located within the kinase domain of Chk2, and the possibility that it alters kinase activity cannot be excluded. Furthermore, the leucine residue is conserved in mouse and Drosophila, providing further evidence for a functional role. It should be noted that this variant was not reported by Bell et al. (14) who analyzed HCT-116 and other cell lines for CHEK2 mutations using DHPLC. This discrepancy could either have resulted from differences in the sensitivity of the two techniques used (SSCP versus DHPLC) or reflect the accumulation of genetic alterations in vitro in this cell line. Recent work by Kilpivaara et al. (22) suggests that the I157T CHEK2 variant may be associated with an increased risk of both familial and sporadic colorectal cancer (germ-line mutation), and although we failed to detect this variant, a larger sample size would be needed to assess if this variant appears as a somatic mutation in colorectal cancer.

In ovarian cancer, there have been no studies investigating methylation and only one study looking for somatic mutations (19). This group identified one somatic mutation among 20 tumors; however, these tumors were not selected for based on LOH. We identified two germ-line variants among our 38 ovarian tumors (with 22q LOH): one novel variant, a C>T base substitution at −4 bp, which may disrupt the Kozak consensus sequence, and a second previously reported polymorphism.
A cytosine at the −4 position of the Kozak consensus sequence is conserved in ~50% of vertebrate translation initiation consensus sequence, with thymine being the most infrequent base to be found at this site, suggesting that the C>T substitution observed may disrupt translation initiation (23). Although microsatellite analysis of the tumor harboring this variant indicated LOH across chromosome 22, the sequence trace shown in Fig. 2B suggests that the variant may be heterozygous. As the tumor showed faint Chk2 expression by immunohistochemistry (scored 1; Fig. 4B), it is possible that this variant results in reduced protein expression.

The A252G polymorphism was also detected in three cell lines (2774, LIM2099, and HT-29) and in one primary breast tumor. A reasonably large cohort of sporadic breast cancers has previously been analyzed for CHEK2 mutations (~300 tumors in four separate studies; refs. 11, 16, 18, 19). In total, these studies revealed only four somatic mutations, and as we failed to identify any somatic mutations in our panel of 20 sporadic cancers, it seems that CHEK2 somatic mutations are rare in sporadic breast cancer.

The two CpG islands located upstream of the CHEK2 gene have both been previously reported; Zhang et al. (7) investigated methylation of the proximal CpG island in non–small cell lung cancer, whereas methylation of the distal CpG island has been studied in breast cancer (24 tumors) as well as in vulval and lymphoid malignancies (8–11). Consistent with previous findings, we did not detect methylation of the distal CpG island. Although Kato et al. also failed to find methylation of this distal CpG island in four lymphoid cell lines, they showed that treatment with 5Aza-dC restored expression of Chk2 in Hodgkin’s lymphoma cell lines, suggesting that there may be an as yet unidentified CpG island at which methylation is playing a role in regulating Chk2 expression in these cells. Our study of the proximal CpG island, previously identified by Zhang et al. (7), revealed methylation in all primary tumor samples, cell lines, and normal lymphocyte DNA controls, suggesting that methylation of this site is not significant in regulating gene transcription and consequently in tumorigenesis. As methylation of this site has been previously reported (7) and suggested to be pathogenic, a closer investigation of the role of this methylation may be required. Our analysis of this site suggests that, in fact, this region is not highly CpG rich; it therefore may not act as a regulatory CpG island. Although CpGs within CpG islands are normally unmethylated, those that are outside of CpG islands are typically methylated (24), which would be consistent with our finding that all DNA analyzed, including normal lymphocyte DNA, was methylated.

In view of the absence of point mutations and promoter hypermethylation of CHEK2, we investigated whether LOH might influence the expression of Chk2 protein. Although, typically, tumor suppressor genes are inactivated by “two hits,” some tumor suppressor genes may require only one hit if inactivation of one allele leads to haploinsufficiency of the protein (see ref. 25 for review). To test this, we used a tissue microarray that was available for the ovarian cancers to

![Fig. 4. Immunohistochemical detection of Chk2. Representative negative (A, score 0), weak (B, score 1), moderate (C, score 2), and positive (D, score 3) staining in serous ovarian tumors. B, ovarian tumor harboring −4 bp C>T variant. Bar, 100 μm.](www.aacjournals.org)
correlate Chk2 protein expression with allelic status across the CHEK2 locus. The majority of tumors showed moderate or strong nuclear staining, and there was no significant association between LOH across the CHEK2 locus and decreased Chk2 staining as would be expected if haploinsufficiency of Chk2 was playing a role in tumor development. The percentage of samples negative for Chk2 staining observed in our study (23%) are comparable with previously reported levels of Chk2 staining in ovarian tumors (26), where 13.1% showed altered (negative) Chk2 staining, and also in breast tumors [23.5% negative (11)]. Interestingly, in non–small cell lung cancers, Zhang et al. (7) reported very high frequency (83%) of weak or negative Chk2 staining, yet no somatic mutations were identified in 35 tumors (19), suggesting that Chk2 may be inactivated by an alternate mechanism.

Although adding to the growing list of germ-line variants identified in the CHEK2 gene, our findings suggest that somatic mutations and/or methylation of CHEK2 are not common in sporadic breast, ovarian, or colon cancers.

References
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