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p53, *CHK2*, and *CHK1* Genes in Finnish Families with Li-Fraumeni Syndrome: Further Evidence of *CHK2* in Inherited Cancer Predisposition[1]

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Abstract

Germ-line mutations in the *p53* gene predispose individuals to Li-Fraumeni syndrome (LFS). The cell cycle checkpoint kinases CHK1 and CHK2 act upstream of p53 in DNA damage responses, and recently rare germ-line mutations in *CHK2* were reported in LFS families. We have analyzed *CHK1*, *CHK2*, and *p53* genes for mutations in 44 Finnish families with LFS, Li-Fraumeni-like syndrome, or families phenotypically suggestive of LFS with conformation-sensitive gel electrophoresis. Five different disease-causing mutations were observed in 7 families (7 of 44 families; 15.9%): 4 in the *p53* gene (5 of 44 families; 11.4%) and 1 in the *CHK2* gene (2 of 44 families; 4.5%). Interestingly, the other *CHK2*-mutation carrier also has a mutation in the *MSH6* gene. The cancer phenotype in the *CHK2*-families was not characteristic of LFS, and may indicate variable phenotypic expression in the rare families with *CHK2* mutations. No mutations in the *CHK1* gene were identified. Additional work is necessary to completely unravel the molecular background of LFS.

Introduction

LFS[3] is a familial cancer syndrome characterized by multiple primary neoplasms in children and young adults, with a predominance of sarcomas and breast cancer and an increased incidence of brain tumors, leukemia, and tumors of the adrenal cortex. The criteria for classical LFS and extended LFL were established by Li *et al.* (1) and Birch *et al.* (2), respectively. Germ-line mutations in the *p53* gene, a tumor suppressor most frequently mutated in human cancer (3), have been observed in 63% of LFS and 27% of LFL families, respectively (4), indicating heterogeneous genetic background for the disease. p53 has an important role in DNA repair and cell cycle control in both G_1 and G_2 checkpoints (5). The cell cycle checkpoint kinases CHK1 and CHK2 act upstream of p53 in DNA damage responses (6). *CHK2* is a human homologue of *Cds1* in *Schizosaccharomyces pombe* and *Rad53* in *Saccharomyces cerevisiae*, and *CHK1* is a human homologue of the *S. pombe* checkpoint kinase *Chk1*. In the presence of damaged DNA, Cds1/Rad53 as well as Chk1 phosphorylate and inactivate Cdc25C, resulting in G_2 arrest and prevention of the initiation of mitosis (7). Furthermore, both CHK1 and CHK2 have been shown to phosphorylate p53 at multiple DNA damage-inducible sites (6). p53 also reciprocally down-regulates CHK1 (8) and CHK2 (9), indicating that p53 may play interdependent and complementary roles with CHK1 and CHK2 in cell cycle regulation after DNA damage. CHK2 is activated by ATM in response to γ-radiation (10), whereas ATR activates CHK1 in response to UV-induced DNA damage (11). It is not yet clear whether these two pathways cross-regulate each other, although coregulation between these two kinases have been reported in *S. pombe* (12). A model has also been proposed in which Chk1 and Rad53 function in parallel to prevent anaphase entry and mitotic exit after DNA damage (13). Failure of these checkpoint functions results in genomic instability, a mutagenic condition that predisposes cells to neoplastic transformation and tumor progression (14). Recently, Bell *et al.* (15) reported rare germ-line mutations in the *CHK2* gene in Li-Fraumeni families, whereas no mutations in *CHK1* were found. Here we have analyzed the *CHK1*, *CHK2*, and *p53* genes for mutations in 44 Finnish families with either LFS or LFL, or phenotypically suggestive of LFS.

Materials and Methods

Patients. The study cohort consisted of 44 families that fulfilled the criterion for either LFS (3 families), LFL (7 families), or that were phenotypically suggestive of LFS (34 families). Detailed description of the families is given in Table 1. Families were recruited through a systematic interview of breast cancer patients at the Department of Oncology, or through the Department of Clinical Genetics, Helsinki University Central Hospital, Helsinki, Finland. Nine of the families had been identified previously as breast cancer families, also fulfilling the criterion of three or more breast or ovarian cancer cases, diagnosed at any age, in first- or second-degree relatives (16–18). Family history was initially based on the patient reports, with subsequent confirmation of the diagnoses reported by the index of patients through hospital records or the Finnish Cancer Registry. Previously, the accuracy of diagnostic information of breast and ovarian cancer in first- and second-degree relatives as reported by the patient has been shown to be 95% (19).

All patients participating in the study signed an informed consent. The study has been approved by the Ethical Committees of the Departments of Obstetrics and Gynecology and Oncology, Helsinki University Central Hospital, and appropriate research permissions were obtained from the Ministry of Social Affairs and Health in Finland.

Mutation Analyses. DNA from blood lymphocytes was extracted using the standard phenol-chloroform protocol or QIAamp DNA blood maxi-kit (Qiagen, Hilden, Germany). In six of the kindreds originally identified as breast cancer families, involvement of *BRCA1* and *BRCA2*, the two major genes predisposing to breast cancer, had previously been excluded by mutation analysis of the entire coding regions (16, 17). In all other families, *BRCA1* and *BRCA2* involvement was excluded by protein truncation test of *BRCA1* exon 11 and *BRCA2* exons 10 and 11, covering about 60% of the genes, respectively, and by screening all 21 known Finnish mutations with allele-specific oligonucleotide hybridization or RFLP analysis. In two independent studies with a total of 188 Finnish breast cancer families, where the whole coding regions of *BRCA1* and *BRCA2* were screened, 11 founder mutations were shown to account for >80% of all *BRCA1*- and *BRCA2*-positive cases (17, 20). Fourteen families also had been screened previously for *p53* mutations in another study (21).

In the analysis of *CHK1*, *CHK2*, and *p53* genes, the entire coding regions

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[3] The abbreviations used are: LFS, Li-Fraumeni syndrome; LFL, Li-Fraumeni-like syndrome; CSGE, conformation-sensitive gel electrophoresis.

Table 1 *Description of the families studied*

Family type	No. of families
LFS families[a]	3
LFL families[b]	7
Families phenotypically suggestive of LFS	
Breast cancer diagnosed under the age of 30 years	7
Breast cancer and sarcoma in the same patient	8
Breast cancer and family history of LFS-related[c] cancer	17
Three primary tumors in the same patient	2
Total	44

[a] According to Li *et al.* (1).
[b] According to Birch *et al.* (2).
[c] Sarcoma, brain tumor, leukemia, tumors of adrenal cortex, and breast cancer.

and exon-intron boundaries were screened for germ-line mutations by CSGE (22, 23). Special attention was paid to the design of the PCR primers for *CHK2* exons 10–14, which have multiple homologous sequences around the genome (24). All homologous sequences obtained from database searches using the National Center for Biotechnology Information BLAST server (25) were aligned using Clustal X alignment algorithm, whereafter the primers were designed to include at least a few nucleotide differences between the homologous copies and the *CHK2* sequence in chromosome 22. In the CSGE analysis, the detection was done by autoradiography by end-labeling one of the PCR primers with [γ-^{33}P]dATP (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using T4-polynucleotide kinase (New England Biolabs, Beverly, MA). Samples with a bandshift were reamplified and sequenced using ABI 310 automated DNA sequencer (Applied Biosystems, Foster City, CA).

RNA from the *CHK2* mutation carriers was extracted using the RNeasy mini-kit (Qiagen). Confirmation of the presence of mutations in the expressed, *CHK2*-specific sequence and not in homologous copies was done using exonic primers in exons 8 (forward) and 10 (reverse).

To estimate the mutational status of the novel exonic variants, these alterations were screened in DNA samples from 95 healthy Finnish control individuals (190 chromosomes) using the CSGE method.

Haplotype Analysis. To assess whether the same mutations found in different families originated from a common ancestor, a haplotype analysis was performed. The polymorphic microsatellite markers used for *p53* were: D17S1810, D17S1832, D17S938, TP53, D17S1353, and D17S786; and for *CHK2* were: D22S1167, D22S1144, D22S275, and D22S280. The analysis was done by end-labeling one of the primers with [γ-^{33}P]dATP (Amersham Pharmacia Biotech AB) and separating the denatured PCR products in a 10% mildly denaturing polyacrylamide gel.

Results and Discussion

p53 is a tumor suppressor gene most frequently mutated in human cancer, and germ-line mutations in its very conserved coding region are frequently identified in the multicancer LFS. However, *p53* mutations are not found in all Li-Fraumeni families, and other genes are expected to play a role in the etiology of this hereditary cancer syndrome. Previously, the search for mutations in the *CHK2* gene revealed three mutations in LFS/LFL families (15). Later, however, one of the variants was shown to reside in a homologous fragment in chromosome 15 (24, 26). Another candidate susceptibility gene for LFS could be the *CHK1* gene, although no mutations in the first analysis of 22 families by Bell *et al.* (15) were observed. Both CHK1 and CHK2 are involved in the stabilization of p53 after DNA damage and controlling the G_2 checkpoint by inactivating Cdc25C (7).

The mutation analysis of *p53*, *CHK1* and *CHK2* genes in 44 Finnish families with LFS or LFL or that were phenotypically suggestive of LFS revealed several alterations in all three genes (Tables 2 and 3). Most of the variants were intronic, but eight different changes in the exons were also observed. Altogether, five different disease-associated mutations were found in 7 families (7 of 44; 15.9%): 4 in the *p53* gene (5 of 44 families; 11.4%) and 1 in the *CHK2* gene (2 of 44 families; 4.5%). No mutations in the *CHK1* gene were identified.

In the *p53* gene, five different exonic changes were found. Of these, the protein truncating mutations Arg196Stop and Arg 213Stop in exon 6, and the missense change Arg248Gln in exon 7, are all located in the conserved, mutation-prone region of the gene and have been reported as disease-associated mutations (3). The codon 248 is a mutational hot spot in the *p53* gene, and the Arg248Gln mutation is the most frequently found *p53* germ-line mutation in LFS families. It is also a very common somatic mutation in breast as well as in other tumors (3). In this study, the mutation was found in two families that originate from different parts of the country and are not known to be related. Haplotype analysis also showed different marker haplotypes in these families around the *p53* gene (data not shown). In one of these families, the index patient carrying the mutation was diagnosed with breast cancer at 34 years of age and was diagnosed previously with osteosarcoma at age 11. Her healthy parents had deceased at the ages of 71 and 81 years, and none of her six siblings have been diagnosed with cancer. Thus, this may be a *de novo* mutation. In the other family, the index case was diagnosed with breast cancer at the age of 22 and had a LFL-like family history with sarcoma, bilateral breast cancer, brain tumor, and liver cancer.

The fourth *p53* germ-line change (Pro151Thr) has been identified as a somatic mutation in several different tumor types, *e.g.*, breast, brain, bladder, and colon, but not previously as a germ-line mutation in Li-Fraumeni families (3). However, other germ-line changes in the same codon have been found to associate with the disease (3), indicating this site as important for the protein function. The mutation was also found in all five cancer patients in the family, and in one case where tumor DNA was available, a patient diagnosed with leiomyosarcoma at the age of 29 years, loss of the wild-type allele was

Table 2 *Mutations found in screening of p53, CHK1, and CHK2*

In some kindreds there is a family history of cancer on both sides, and the likeliest for inherited cancer predisposition is included.

Gene	Family	Exon	Nucleotide change	Effect on protein	Family type	Index patient (cancer and age at diagnosis)	Other cancers in the family
p53	7154	5	451 C→A	Pro151Thr	LFS	Breast ca[a] 29y	Osteosarcoma 24y, leiomyosarcoma 29y, pancreatic adenoca 59y, adrenocortical ca 1y
	7106	6	586 C→T	Arg196Stop	LFS	Liposarcoma 32y, breast ca 35y and 37y	Osteosarcoma 25y, breast ca 42y[b]
	1084	6	637 C→T	Arg213Stop	Phenotypically suggestive of LFS	Breast ca 28y	Sarcoma (histiocytoma) 64y, melanoma 67y and lung ca 75y
	2204	7	743 G→A	Arg248Gln	Phenotypically suggestive of LFS	Osteosarcoma 11y, breast ca 34y	Throat ca 65y
	7138	7	743 G→A	Arg248Gln	LFL	Breast ca 22y	Brain ca 11y, liver ca 29y and bilateral breast ca 32y, leiomyosarcoma 54y
CHK2	7116	10	1100delC	Frameshift, stop at codon 381	Phenotypically suggestive of LFS	Breast ca 40y	Vaginal melanoma 55y, endometrial ca 46y, stomach ca 57y,[b] ovarian ca 30y
	5130	10	1100delC	Frameshift, stop at codon 381	Phenotypically suggestive of LFS	Breast ca 34y, colorectal ca 34y	Breast ca 38y, meningioma 50y, prostate ca 64y,[b] endometrial ca 52y,[b] kidney ca 51y[b]

[a] ca, carcinoma.
[b] Diagnosis could not be verified from medical documents; the age at death.

Table 3 *Polymorphisms found in screening of p53, CHK1, and CHK2*

Gene	Exon/Intron	Nucleotide change	Effect on protein	No. of families with a variant
p53	4	215 G→C	Arg72Pro	12
	Int2	74(+38) G→C		18 (3 homozygotes)
	Int2	75(−29) C→A		1
	Int3	96(+41) ins16		6 (1 homozygote)
CHK1	13	1411 G→A	Val471Ile	5[a]
	Int2	66(−36) G→T		24 (3 homozygotes)
	Int10	1101(+62) G→A		3
	Int11	1233(+35) G→A		25
CHK2	3	470 T→C	Ile157Thr	4[b]
	Int1	319(+43) insA		1
	Int3	593(−14) C→T		2 (1 homozygote)

[a] Present also in 5 of 95 population controls.
[b] Present also in 2 of 95 population controls.

observed. This missense variant was not found in 95 healthy controls, further supporting the pathogenic nature of the change.

The fifth change in exon 4 (Arg72Pro) was present in 12 patients and considered as a neutral polymorphism, as previously reported (3). Altogether, *p53* mutations were found in two of three LFS families, in one of seven LFL families (14.3%), in one family with LFS-suggestive phenotype, and as an apparent *de novo* mutation in one patient without family history of cancer but with both childhood sarcoma and early onset breast cancer, a phenotype highly indicative of the LFS.

No mutations were found in the *CHK1* gene. Four different relatively common bandshifts were observed in CSGE, and sequencing revealed that three of these were far in the introns (Table 3). One exonic variant (Val471Ile) was present in 5 patients and also in 5 of 95 population controls, suggesting that it is a polymorphism. Furthermore, it is located at the very end of the gene (total 476 amino acids), outside the most conserved functional domains (27). Previously, somatic mutations in *CHK1* have been observed in colorectal and endometrial tumors (28). A shorter somatic isoform of *CHK1* mRNA, as well as a rare polymorphism or possible germ-line mutation in a patient with small cell lung cancer, have also been reported (29).These findings suggest that the loss of CHK1 function may be important in tumor formation in different cancer types. According to our data, *CHK1* is not a major gene for LFS.

In the *CHK2* gene, two exonic variants were found. Interestingly, both of these were the same as in the original report by Bell *et al.* (15). A frameshift mutation in exon 10 (1100delC), which leads to a premature stop at codon 381 as compared with the 543 amino acids in a full-length protein, was found in two families. These families originate from different parts of the country, are not known to be related, and segregate different chromosome 22 haplotypes. This mutation was not observed in 95 healthy control individuals, and functional analyses have shown that this mutation results in loss of kinase activity of the Chk2 protein (30). Sequencing of the *CHK2*-specific cDNA from the index patients of both families confirmed the presence of the mutations in the expressed *CHK2* sequence. It thus appears to be a true disease-causing mutation and a mutational hot spot in the *CHK2* gene.

The phenotypes of the two families (5130 and 7116) with a *CHK2* mutation are not typical for LFS or LFL, with no sarcomas or childhood cancers (Fig. 1). This is in contrast with the work of Bell *et al.* (15), who found their *CHK2* mutation in a classical LFS family. In family 7116, the index patient had breast cancer diag-

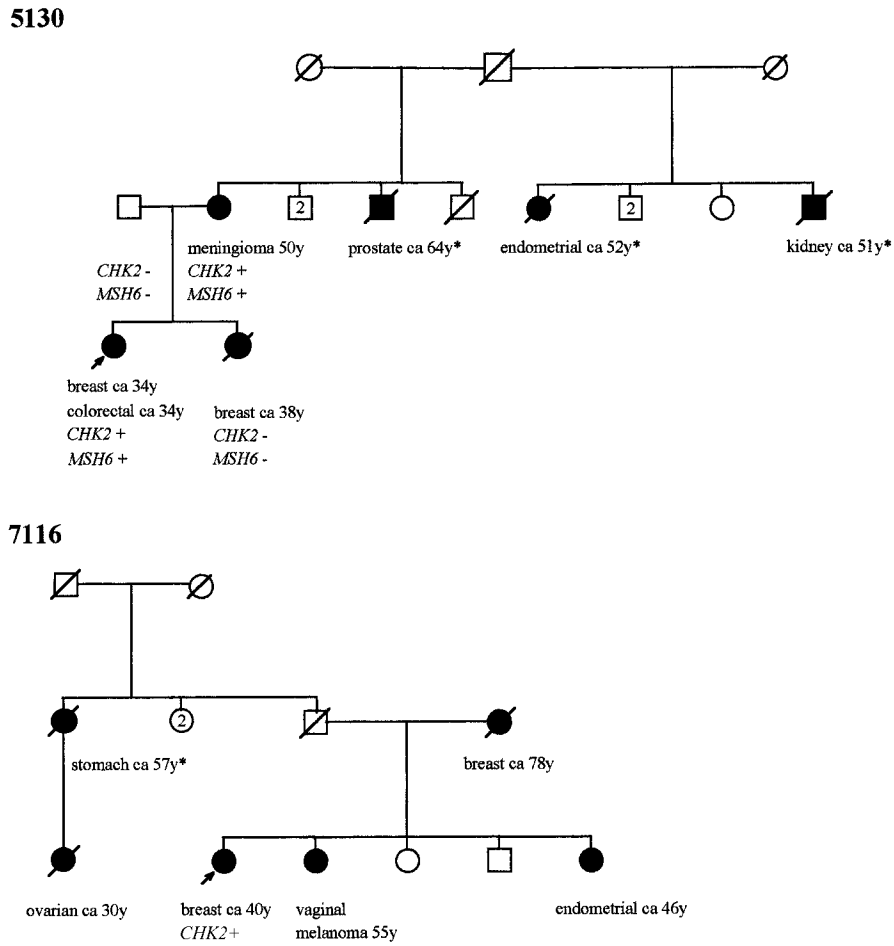

Fig. 1. Pedigrees of the families with a *CHK2*-mutation. *, diagnosis could not be verified from medical documents; the age at death; ca, carcinoma

nosed at the age of 40 years, and although there were several other cancer cases in the family, none of them were typical for LFS (Table 2; Fig. 1). Interestingly, and independently of this study, also an *MSH6* mutation was found in family 5130. Immunohistochemical analysis of MSH6 first indicated the possible involvement of this gene, and direct genomic sequencing revealed a novel truncating germ-line mutation in exon 4 (2983 G to T; Glu995Stop). The index patient is affected both with breast and colorectal cancer at the age of 34 years. The *MSH6* mutation was also found in the patient's mother who is affected with benign meningioma, and who also has the *CHK2* mutation. However, a sister with a breast cancer diagnosed at the age of 38 years is negative for both of these mutations, and her breast tumor is most likely sporadic. In the other *CHK2*-positive family, immunohistochemical analysis of MLH1, MSH2, and MSH6 appeared normal, both in the breast tumor of the index patient and in the endometrial tumor of her sister. Previously, germ-line mutations in the *MSH6* gene have been identified in patients from atypical hereditary nonpolyposis colorectal carcinoma families with a later-age of onset of carcinomas and a high frequency of extracolonic malignancies, especially endometrial carcinoma (31). *MSH6* has not been connected with an increased risk for breast cancer, but in a recent study by Wang *et al.* (32), a possible interaction between BRCA1 and MSH6 through a so-called BRCA1-associated genome surveillance complex was reported. Whether germ-line mutations of *CHK2* and *MSH6* genes cooperate to influence the disease phenotype in such double heterozygous patients is not known, and additional studies are required to address this issue.

The other exonic *CHK2* change observed in both our and Bell *et al.*'s (15) study was a missense alteration in exon 3, changing isoleucine to threonine at codon 157. This site is located within the forkhead homology-associated domain of the gene, a conserved protein-interaction domain essential for the activation of the yeast homologue Rad53 in response to DNA damage (33). The Ile157Val was also suggested as a disease-causing mutation by Bell *et al.* (15). However, this particular amino acid is not well conserved among different organisms (10). Furthermore, this variant was relatively common in our study cohort (4 of 44 cases), and was also seen in healthy population controls (2 of 95). Additionally, in biochemical characterization, this variant behaved like the wild-type Chk2, although the alteration may affect associations with other proteins (30). However, these findings suggest that this alteration may not be a disease-causing mutation, but a polymorphism. In another recent study, no *CHK2* mutations were found among 17 French LFS- or LFL families (34). Taken together, one germ-line *CHK2* mutation (1100delC) has now been found in three families in two independent studies.

In this study cohort, 18 families also fulfilled the criterion for hereditary breast cancer (at least three cases of breast or ovarian cancer in first- or second-degree relatives), but none of these families had mutations in any of the genes studied. CHK2 has been shown to regulate BRCA1 function after DNA damage (35). Additional analyses are needed to assess the possible role of CHK2 in hereditary breast cancer indicated by the association with BRCA1.

Of the three LFS families in this study, two had a germ-line *p53* mutation. Overall, the *p53* mutations were more clearly associated with the classical LFS and LFL phenotypes, whereas mutations in the *CHK2* gene were found in families only suggestive of LFS. This may indicate variable phenotypic expression in the rare families with *CHK2* mutations. Yet other genes may account for the remaining Li-Fraumeni and Li-Fraumeni-like families.

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p53, *CHK2*, and *CHK1* Genes in Finnish Families with Li-Fraumeni Syndrome: Further Evidence of CHK2 in Inherited Cancer Predisposition

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