Inactivating Mutations Targeting the chfr Mitotic Checkpoint Gene in Human Lung Cancer

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ABSTRACT

A hallmark of cancer is inactivation of cell cycle checkpoints. However, very few mutations targeting mitotic checkpoint genes have been described, and in those instances, a wild-type copy of the gene was retained. chfr is a mitotic checkpoint gene that functions in early prophase delaying chromosome condensation in response to microtubule poisons. In a panel of 53 lung carcinomas for which matched normal tissue was available, we identified three missense mutations in the chfr gene, at least one of which was associated with loss of heterozygosity. In tissue culture checkpoint assays, the tumor-associated missense mutants had reduced activity or were inactive. Together with recent data suggesting that the chfr gene is frequently silenced in various tumors because of methylation of its promoter, these findings suggest that chfr is inactivated by multiple mechanisms in human cancer.

INTRODUCTION

Mitosis, the process by which cells segregate their genetic material, involves many biochemically distinct processes, whose ordered execution is controlled by checkpoints (1–4). The best characterized mitotic checkpoint is the spindle checkpoint, which inhibits sister chromatid separation until all chromosomes have become aligned on the mitotic spindle (2–4). The spindle checkpoint times the metaphase to anaphase transition and functions in every mitosis even in the absence of microtubule poisons (5). Complete inactivation of this checkpoint leads to embryonic lethality and has not been described in cells that retain the capacity to divide (6–8). However, partial inactivation of the spindle checkpoint, induced mostly by heterozygous mutations of spindle checkpoint genes, has been documented in cancer cells, albeit infrequently, and may contribute to the genetic instability that is characteristic of human cancer (9–16).

Another checkpoint that functions in mitosis involves the chfr gene (17–19). This checkpoint functions very early in mitosis before chromosomes condense and is evident only in cells exposed to microtubule poisons; in such cells, chromosome condensation and entry into prometaphase are delayed (19).

Previous analysis of human cancer cell lines and tumors suggests that the chfr gene is frequently inactivated in human cancer. In cancer cell lines of colon, brain, bone, and esophageal origin and in freshly isolated lung, colon, and esophageal carcinomas, the chfr promoter is methylated with frequencies ranging between 20% and 40% (19–23).

Nevertheless, chfr mutations have not yet been identified in either cancer cell lines or primary tumors. The one reported mutation in the chfr gene in the U2OS osteosarcoma cell line turns out to be a polymorphism (19, 20).

Guided by the frequent methylation of the chfr promoter in human cancer, we examined whether mutations in the chfr coding sequence could be identified in human tumors by analyzing a previously described panel of 53 NSCLCs (24). Our findings suggest that chfr is mutated in human cancer. Furthermore, at least in one case, the mutation was associated with LOH, implying that chfr is the only mitotic checkpoint gene known thus far to have both its alleles inactivated in human cancer.

MATERIALS AND METHODS

Tissue Specimens, Microdissection, and Nucleic Acid Extraction. The 53 NSCLCs and matched normal specimens were part of a previously published collection of specimens from 68 NSCLC patients (24). All samples were obtained less than 15 min after surgical resection from patients who had not undergone any prior chemotherapy or radiotherapy. Part of the tumor specimen was snap frozen in liquid nitrogen and stored at −70°C, whereas another part was formalin fixed and paraffin embedded. Adjacent normal tissue was also collected. The frozen specimens were used to prepare contiguous 5-μm sections. The first of these was stained with H&E to visualize the tumor cells and guide the removal of excess normal tissue from the remaining sections using a surgical blade. DNA and RNA were prepared from these sections as described previously (24). Tumors were classified according to the WHO criteria and tumor-node-metastasis (TNM) system, and the diagnosis for each patient has been published previously (24).

SSCP and DNA Sequencing. Three different partially overlapping fragments of chfr cDNA, each about 800-nts long, were amplified by PCR from both normal and tumor tissue. Each of the three long PCR fragments was then used to generate shorter overlapping PCR fragments about 250-nts long using the primers shown in Table 1. The short PCR products from each normal-tumor pair were initially screened for mutations by SSCP analysis. Samples bearing putative mutations were then sequenced. For each specimen, SSCP analysis was performed twice using independently amplified DNA products. For those specimens in which mutations were identified at the cDNA level, the corresponding exon of the genomic DNA was amplified using primers listed in Table 1 and sequenced.

PCR reactions were performed using a modified Taq polymerase (Advanced Biotechnologies). For the SSCP analysis, PCR products were resuspended in 95% formamide solution, denatured by heating to 95°C for 8 min, subjected to electrophoresis on nondenaturing 10% acrylamide/bis-acrylamide (29:1) gels, and stained with silver nitrate, as described previously (24). Sequencing was performed using BigDye Terminator chemistry on an ABI PRISM 377 DNA Sequencer (PE-Applied Biosystems).

LOH Analysis. Genomic DNA fragments from pairs of normal and tumor specimens containing a polymorphic CA-repeat adjacent to the chfr gene (nts 88761–88786 of the DNA sequence with GenBank accession number AC023047; March 5, 2001) were amplified using forward (GGT-TGC-AGT-GAG-CCG-AGA-TAG) and reverse (GCA-CTT-GTT-TTT-CTA-GTT-TCG) PCR primers to generate polymorphic PCR products 196- or 204-bp long. After electrophoresis on nondenaturing 10% acrylamide/bis-acrylamide (19:1) gels and staining with ethidium bromide, the intensities of the bands

4 The abbreviations used are: NSCLC, non-small cell lung carcinoma; LOH, loss of heterozygosity; RT-PCR, reverse transcription-PCR; SSCP, single-strand conformational polymorphism; nt, nucleotide; HA, hemagglutinin; SQ, squamous cell carcinoma.
were determined using the Kodak Digital Science Image Analysis System (Kodak), as described previously (25).

**mRNA Expression.** Expression of *chfr* at the mRNA level was examined by RT-PCR, as described previously (25). Briefly, a *chfr* cDNA fragment was amplified in the same reaction with a *gapdh* cDNA fragment; the PCR products were electrophoresed on nondenaturing 10% polyacrylamide gels, and, after staining with ethidium bromide, band intensities were determined using the Kodak image analysis system.

**Mitotic Checkpoint Assay.** DLD-1 colon carcinoma cells, which have a defective early prophase checkpoint (19), were cotransfected with 2.5 μg of a pcDNA3.1 plasmid (Invitrogen) with no insert or with inserts encoding various Chfr proteins and 0.5 μg of a plasmid expressing green fluorescent protein fused to histone H2B (26) using FuGENE 6 (Roche) according to vendor protocol. Thirty h later, the cells were treated with 1 μM nocodazole (Calbiochem) for 16 h, harvested with trypsin, and fixed in cold 70% ethanol. The mitotic index was determined by inspecting the cells with a fluorescence microscope.

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**Table 1** List of primers used to amplify *chfr* cDNA and genomic DNA fragments

<table>
<thead>
<tr>
<th>nt positions</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>69–853</td>
<td>CGG-GGC-GGG-GAT-GTG-AAT</td>
<td>CTC-CAA-ATC-CTC-CTG-ATC-CT</td>
</tr>
<tr>
<td>1564–2406</td>
<td>CGG-GGC-GAT-GTG-AAT</td>
<td>AAA-ATG-TAC-AAA-AGA-GCA-AAA-CTG</td>
</tr>
<tr>
<td>257–479</td>
<td>TCT-CTG-GAG-ATC-ATG-GAA-TTG</td>
<td>CAC-CTG-ACC-TGA-CTC-CTG-ATC-CA</td>
</tr>
<tr>
<td>418–650</td>
<td>AAT-GAA-CGC-GAA-CAC-AAC-GT</td>
<td>TCA-TGC-CTT-CTT-CAC-TTA-A</td>
</tr>
<tr>
<td>610–853</td>
<td>GAA-CCA-CAG-CGA-TCA-ACT-CT</td>
<td>TGC-CAA-ATC-CTC-CTG-ATC-CT</td>
</tr>
<tr>
<td>809–1070</td>
<td>CGT-CCT-TTT-CGT-TGG-AA</td>
<td>CCG-CGC-AGA-ACG-GTC-GC</td>
</tr>
<tr>
<td>1015–1257</td>
<td>GAC-CTG-CTG-CAC-GAC-TG</td>
<td>ATG-TCT-TGA-ATG-TTA-TTC-CTG</td>
</tr>
<tr>
<td>1198–1407</td>
<td>AGT-GGC-AGT-GTC-CTG-AG</td>
<td>GCC-GGC-TGC-CTG-ATC-CA</td>
</tr>
<tr>
<td>1373–1617</td>
<td>GCC-GGC-AGT-GTC-CTG-AG</td>
<td>TGC-TGA-GGG-GCG-ACA-AG</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>ACC-ATG-CAG-CAC-GCA-CCC-AG</td>
<td>ACC-ATG-CAG-CAC-GCA-CCC-AG</td>
</tr>
<tr>
<td>14024–13735</td>
<td>GTC-TGC-TGC-TTC-TGC-CTC-TT</td>
<td>ACC-ATG-CAG-CAC-GCA-CCC-AG</td>
</tr>
</tbody>
</table>

* The nt positions for cDNA refer to GenBank sequence file AF170724, and nt positions for genomic DNA refer to GenBank file AC023047 dated March 5, 2001.

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Fig. 1. Mutation and LOH analysis of the *chfr* gene in NSCLC. A, SSCP analysis for patient 28. B, DNA sequencing chromatograms for the same patient. The mutated nucleotide is underlined. C, LOH analysis based on the polymorphic CA-repeat marker adjacent to the *chfr* gene. The two polymorphic alleles with 13 and 17 CA-repeats are indicated. The numbers below the gel indicate for each specimen the ratio of the two alleles; these ratios were adjusted so that for each patient, the ratio for the alleles in the normal tissue would be 1. T, tumor; N, normal tumor-adjacent tissue. D, list of mutations and polymorphisms (PL) mapped on the Chfr protein. Amino acid substitutions and the corresponding nucleotide changes refer to the GenBank *chfr* cDNA sequence file AF170724. For the tumor-associated mutations, patient numbers are also indicated. Chfr protein domains are in red. FHA, FHA domain; RD, RING domain; CR, cysteine-rich region.
PCR-amplified fragments of the chfr and F536S mutant Chfr in cells treated with the proteasome inhibitor epoxomycin (EpxC), mutants in transiently transfected DLD-1 cells. NT, nontransfected cells; normal tissues for each sample. mRNA and genomic DNA were then guided by analysis of frozen sections separated the tumor and adjacent samples were promptly snap frozen after collection, and a pathologist forms of therapy, including chemotherapy or radiation therapy. The tissue was available (24). All tissue samples were obtained during previously described panel of 53 NSCLCs, for which normal adjacent for mutations targeting the chfr gene.

**RESULTS**

Identification of chfr Mutations and LOH in NSCLC. To search for mutations targeting the chfr gene in human cancer, we analyzed a previously described panel of 53 NSCLCs, for which normal adjacent tissue was available (24). All tissue samples were obtained during surgical therapy of patients who had not previously received other forms of therapy, including chemotherapy or radiation therapy. The samples were promptly snap frozen after collection, and a pathologist guided by analysis of frozen sections separated the tumor and adjacent normal tissues for each sample. mRNA and genomic DNA were then isolated and used for molecular analysis of the chfr locus.

Putative mutations were initially identified by SSCP analysis using PCR-amplified fragments of the chfr cDNA. For those samples that scored positive by SSCP, the PCR products were sequenced in both orientations. Sequence variations present in the tumor but not in the normal specimen were further verified at the genomic DNA level by amplifying and sequencing the corresponding exon of the chfr gene.

Several tumors scored positive by SSCP analysis, and after sequencing, three of these were shown to harbor chfr missense mutations. Qualitatively, the SSCP and DNA sequencing chromatograms were similar in all three tumors with mutations; novel tumor-specific bands were observed in the SSCP chromatograms (Fig. 1A), and sequencing revealed mutations in the cDNA and genomic DNA of the tumor, but not in the normal tissue sample from the same patient (Fig. 1B). The chfr mutations did not correlate with a specific diagnosis or tumor stage. The diagnosis for the three patients with chfr mutations were as follows: patient 28, SQ stage I; patient 48, SQ stage III; and patient 56, SQ stage II. These three patients were all male smokers ranging in age from 58 to 73 years.

The identification of tumor-associated mutations in the chfr gene begs the question of LOH. To address this question, a CA-repeat located within the bacterial artificial chromosome clone that contains the chfr gene was identified (nts 88761–88786 of the genomic DNA sequence with GenBank accession number AC023047; March 5, 2001). This repeat was polymorphic with two different alleles identified; one had 13 CA repeats, and the other had 17. Two of the three patients with tumor-associated mutations were analyzed for LOH (by this time, we had used up all of the sample from the third patient). Both patients were heterozygous for the CA-repeat marker; one exhibited LOH (patient 48), whereas the other patient (patient 56) did not have LOH (Fig. 1C). In regard to the latter patient, our conclusion is based on the assumption that there was little contamination of the tumor tissue by normal cells.

At the nucleotide level, two of the three mutations were transitions, and one was a G to C transversion (patient 56; Fig. 1D). Transversions are uncommon in cancer, except, however, for cancer of the lung, for which a high frequency of transversions is attributed to the carcinogens present in cigarette smoke (27).

In addition to the mutations, we also identified eight polymorphisms (Fig. 1D), some of which had been described previously (20). Five polymorphisms mapped to the coding sequence, and of these, three were silent, whereas the other two resulted in conservative substitutions of hydrophobic residues within the cysteine-rich region of the protein (A497V and V580M; Fig. 1D). Three polymorphisms mapped to the noncoding region and substituted nucleotides G2156 with A, G2319 with A, or deleted both nucleotides 2240 and 2241. We also noted two alternative splicing events that led to deletion of.

**Fig. 2.** Functional analysis of tumor-associated Chfr mutant proteins. A, mitotic index of DLD-1 cells expressing wild-type Chfr or tumor-derived Chfr mutants after exposure to nocodazole for 16 h. The data are presented as means and SDs of three independent experiments. B, protein levels of HA-tagged wild-type Chfr or tumor-derived Chfr mutants in transiently transfected DLD-1 cells. C, protein levels of HA-tagged wild-type and F536S mutant Chfr in cells treated with the proteasome inhibitor epoxomycin (Epx). NT, nontransfected cells; Vec, cells transfected with empty vector.

**Fig. 3.** chfr expression at the mRNA level is decreased in a subset of NSCLCs. RT-PCR analysis for expression of chfr and gapdh in tumor (T) and normal tumor-adjacent (N) specimens. Patients 27 and 65 have a 5-fold reduction in chfr expression in the tumor specimens. Patient 48 is one of the patients described above with a mutation in the chfr gene.
residues 135–146 in the region between the FHA and RING domains or deletion of Ala170 in the COOH-terminal cysteine-rich region, respectively.

In terms of the domains of Chfr targeted by the mutations, two of the three mutations targeted the region between the FHA and RING domains, whereas the third targeted the COOH-terminal cysteine-rich region of the protein (Fig. 1D). In all cases, the resulting amino acid substitutions were nonconservative; two mutations replaced or introduced proline residues (P166L and R202P), whereas the third substituted a phenylalanine with a serine (F536S).

**Effects of chfr Mutations on Checkpoint Function and Expression Levels.** If the identified mutations in the chfr gene were associated with cancer development, then the proteins encoded by the mutant genes should be inactive in mitotic checkpoint assays. We therefore examined the activity of the tumor-associated missense mutants in a checkpoint assay that involves transiently transfecting DLD-1 cells with a chfr gene, exposing the transfected cells to the microtubule poison nocodazole, and scoring the fraction of cells in mitosis 16 h later. DLD-1 cells have undetectable levels of endogenous Chfr protein, and in this assay, transient expression of wild-type chfr leads to a reduction in the mitotic index by about 50%, reflecting a cell cycle delay in early prophase. All three tumor-associated chfr mutants were defective in the checkpoint assay (Fig. 2A).

To explore the basis for loss of function, we examined whether the missense mutations affected Chfr protein levels. In the transiently transfected cells, two mutants were expressed at the same level as wild-type Chfr, whereas expression of the mutant with the Phe536 to Ser substitution (F536S) was virtually undetectable (Fig. 2B). This substitution may destabilize Chfr by preventing the protein from adopting its native state in a manner reminiscent of the DNA damage signaling kinase ATM, which is destabilized by missense and truncating mutations in patients with ataxia-telangiectasia (28). Indeed, treatment of cells with the proteasome inhibitor epoxomicin resulted in increased levels of the Chfr F536S mutant (Fig. 2C).

The identification of inactivating mutations in the chfr gene in NSCLC complements four recent studies showing that in colon, esophageal, and lung cancer, the promoter of the chfr gene is frequently methylated, and the chfr gene is not expressed (20–23). In our panel of 53 NSCLCs, chfr mRNA levels were examined in 22 pairs of normal and tumor tissue by semiquantitative RT-PCR. Expression of chfr was reduced by >5-fold in 10 of the 22 tumors studied using gapdh expression as a standard (Fig. 3). Although the molecular basis for the decreased expression of chfr was not explored, the most likely cause is promoter methylation.

**DISCUSSION**

Defects in cell cycle checkpoints are clearly associated with human cancer. However, very few tumors with mutations targeting mitotic checkpoint genes have been described previously (9–15). In two well-studied patients, the bab1 spindle checkpoint gene was targeted by mutations, but there was no LOH, and the wild-type copy of the gene was retained (9). The paucity of reported mutations most likely reflects the fact that the previously studied mitotic checkpoint genes all participate in the spindle checkpoint pathway (2–4), a pathway that is essential for cell proliferation (5–8). In contrast, chfr is a component of a novel checkpoint pathway that functions in early prophase only when cells are exposed to microtubule poisons (19). The increased frequency of homozygous genetic alterations found in this novel mitotic checkpoint pathway suggests that its inactivation is not lethal for the cell but instead may confer selective advantage for evolvinng tumor clones. Inactivation of this pathway in NSCLC may also explain the efficacy of Taxol-based chemotherapy for this cancer (29, 30) because inactivation of chfr sensitizes tissue culture cancer cell lines to microtubule poisons (19).

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**REFERENCES**


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