Molecular Cancer

Research

CHK2 kinase expression is down-regulated due to promoter methylation in non-small cell lung cancer

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Abstract

Background: CHK2 kinase is a tumor suppressor that plays important role in DNA damage signaling, cell cycle regulation and DNA damage induced apoptosis. CHK2 kinase expression was known to be ubiquitous in mammalian cells. CHK2-/- cells were remarkably resistant to DNA damage induced apoptosis, mimicking the clinical behavior of non-small cell lung cancer to conventional chemo and radiation therapy.

Result: We reported that the CHK2 expression is diminished or absent in both non-small cell lung cancer (NSCLC) cell lines and clinical lung cancer tumor specimens. The absent CHK2 expression in NSCLC was due to hypermethylation of the CHK2 gene promoter, preventing from binding of a transcriptional factor, leading to silence of the CHK2 gene transcription.

Conclusion: Since the CHK2 null mice showed a remarkable radioresistance, which bear significant similarity to clinical behavior of NSCLC, down-regulation of CHK2 kinase expression by CHK2 gene silencing and methylation in non-small cell lung cancer suggest a critical role of CHK2 kinase in DNA damage induced apoptosis and a novel mechanism of the resistance of NSCLC to DNA damage based therapy.

Introduction

CHK2 kinase is an important cell cycle regulator in DNA damage response pathway. In response to exogenous or endogenous DNA damage agents, CHK2 is phosphorylated and activated by ATM kinase at the Thr68, and the activated CHK2 phosphorylates a number of downstream targets including CDC25, BRCA1, p53, E2F and others that are important in cell cycle checkpoint control, DNA damage repair and DNA-damage-induced apoptosis [1-5].

CHK2 germline mutation was found to associate with a subset of Li-Fraumeni syndrome (LFS), a cancer predisposing familiar syndrome with a majority of patients carrying p53 mutations [6], although this notion was challenged by some recent studies [7,8]. Somatic mutations of CHK2 gene have been found in subsets of diverse types of human cancers including breast, lung, vulva, colon, ovary, osteosarcoma, and lymphomas (see review in [9]). The majority of these mutations were missense mutation, base deletion or conversion resulting truncated

proteins, some of which have been characterized at the protein levels [10].

Normal CHK2 gene is located at chromosome 22 [7]. There are multiple copies of CHK2 homologous sequences within the genome, particularly at the 3' end of the coding sequence. Totally six homologous sequence fragments at the chromosome 5, 7, 10, 15, 16, 22 and X were found encompassing the exon 10 to 14 of the CHK2 genes and share 95–98% sequence similarity. These homologous sequences within the genomic DNA complicate the mutation analysis of the CHK2 gene [6,7]. It is also reported that some of these CHK2 homologous sequences are not transcribed, and therefore nonfunctional [6]. The effect of multiple copies of CHK2 homologous sequences on normal CHK2 gene transcription and possibly translation is yet to be elucidated.

Lung cancer remains to be the leading killer in cancer death. Lung cancer is generally classified as small cell lung cancer (20% of total patients) and non-small cell lung cancer (80%). The major problem for lung cancer treatment is the natural resistance of cancer cells to conventional therapy. The burning question in lung cancer treatment is how to overcome the resistance of cancer cells to DNA damage agents such as cisplatin or ionizing radiation (IR) [11]. Increasing evidence suggests that CHK2 kinase plays a critical role in DNA damage induced apoptosis. CHK2 knockout mice showed that the CHK2 null cells were remarkably resistant to IR induced apoptosis [4,12]. The radioresistant phenotypes of the CHK2 knockout mice bear significant similarity to the clinical behavior of human non-small cell lung cancer to chemoradiation therapy. We report here that the CHK2 protein expression is down-regulated through hypermethylation of the CHK2 gene promoter in both the non-small cell lung cancer cell lines and the human non-small cell lung cancer tumor tissues. The lung cancer cell lines were known to be resistant to cisplatin, a leading chemotherapy drug. These results suggest an important role of CHK2 kinase in cisplatin-induced apoptosis of the tumor cells, and potentially a novel molecular mechanism for the natural resistance of human non-small cell cancer to DNA-damage induced therapy.

Results
CHK2 expression is markedly diminished in non-small cell lung cancer cell lines
We have determined the expression level of CHK2 kinase in the lung cancer cell line A549 in response to cisplatin (Fig 1). We used the ovarian cancer cell A2780 as a control. The level of CHK2 kinase expression was detectable by immunoblot analyses in the control A2780 ovarian cancer cells, and not detected in A549 cells. Additionally, CHK2 expression was detected in H69 cells, a small cell
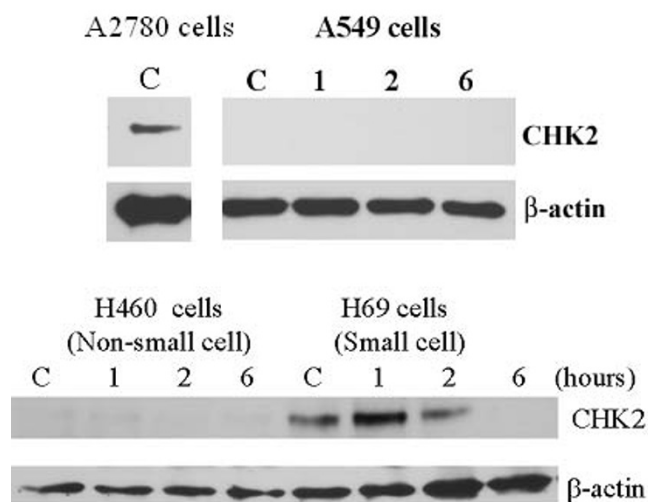

Figure 1
CHK2 expression is markedly diminished or absent in non-small cell lung cancer cell lines by immunoblotting analysis: The cells were treated with cisplatin (33 μM) for the time listed above, and the total cell lysates were harvested and analyzed by immunoblotting with anti-CHK2 polyclonal antibody. β-actin was used to normalize the loading in the each lane.

lung cancer cell line, but not in another non-small cell lung cancer cell line H460 cells. The level of CHK2 expression in H69 cells was decreased in response to cisplatin. These results indicate that the CHK2 expression levels vary in different cancer cell lines, and appear to be markedly diminished in the non-small cell lung cancer cells A549 and H460 cells. Since CHK2 kinase appears to be critical in regulation of DNA-damage induced apoptosis in the knockout mice models, we attempted to correlate the tumor cell sensitivity to cisplatin in four different cancer cell lines. IC50 of cisplatin for these four different cancer cell lines have been previously documented (Table 1). It at least appears that CHK2 expression levels correlate with cisplatin sensitivity, although more definitive studies are required to reach the conclusion.

Expression of the CHK2 mRNA is significantly decreased in non-small cell lung cancer cell lines
Based upon the above results, we sought to determine the level of CHK2 mRNA in the cancer cell lines. It is reported that there are multiple CHK2 homologous DNA sequences within the genome located at the various chromosomes [6,7]. There was only one species of CHK2 mRNA detected within both the ovarian cancer and lung cancer cell lines by Northern blot analyses using full-length CHK2 cDNA as a probe (Fig 2A). In contrast to A2780 and H69 cells where abundant CHK2 mRNA was

Table 1: IC50 concentration of Cisplatin

Cell type	A2780	H69	A549	H460
Cisplatin	3 μM	0.17 μM	64 μM	80 μM
CHK2	+++	+++	---	±
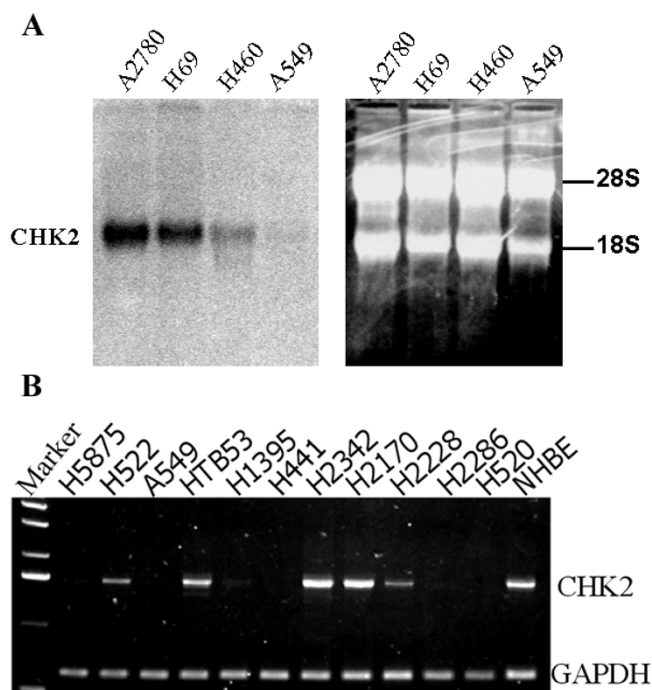
Figure 2
A: Expression of the CHK2 mRNA is significantly decreased in A549 and H460 cells, but not in H69 and A2780 cells: Totally 20 μg RNA was used in each lane. The 18S and 28S rRNAs were used to normalize the loading of RNA sample in each lane. The probe was full-length CHK2 cDNA. B: Semi-quantitative RT-PCR analyses of the CHK2 mRNA expression in 11 non-small cell lung cancer cell lines. NHBE is a normal bronchial epithelial cell line. GAPDH was used to normalize the experimental conditions.

cell line. We used semi-quantitative PCR analyses to determine the level of the CHK2 kinase mRNA in these cell lines (Fig 2B). The PCR primers for CHK2 were as described in the material and method section. The GAPDH primers were as described previously [13]. Totally 5 of the 11 cell lines showed expression of CHK2 mRNA, and 6 cell lines were negative by PCR analyses (Fig 2B). Normal bronchial epithelia cells readily expressed normal CHK2 mRNA. These results suggest that the diminished expression of CHK2 protein in non-small cell lung cancer cell lines is at least in part due to decreased expression of CHK2 mRNA in these cells. Furthermore, abnormal CHK2 mRNA expression in non-small cell lung cancer cell lines is a frequent, rather than rare observation.

The promoter of the CHK2 gene is methylated in non-small cell lung cancers
Since A549 and H460 cells are both non-small cell lung cancer cells, and hypermethylation of genes is well-documented in lung cancer, we determined to see if the decreased CHK2 mRNA expression is due to methylation of the CHK2 gene promoter. Using a web-based software CpG island searcher, we analyzed the CHK2 gene promoter sequence (Genbank accession number AL 117330) to see if there is a CpG rich regions. Between -615 and -411 of the CHK2 gene promoter (refers to the start of translation as +1), there is a region of DNA sequence that is rich in CpG islands, the potential hypermethylation sites (Fig 3A). We used methylation specific PCR techniques (MSP) to determine the methylation status of the CHK2 promoter in both the lung cancer cell lines and the patient's tumor samples using the oligonucleotide primers as shown in Table 2. The PCR products were cloned into TOPO T-A cloning vector and sequenced by commercial sequencing services (Certigen Inc., Lubbock, TX). There was methylation of CHK2 promoter present in the A549 and H460 cells, but no methylation detected within A2780 cells (Fig 3B).

We tested to see if demethylating agents, such as 5-aza-cytidine, can re-activate the CHK2 gene expression in both A549 and H460 cells by using immunoblot analyses. We treated the A549 and H460 cells for up to 5 days with 5 μM 5-aza-cytidine, and no reactivation of the CHK2 gene expression was detected (data not shown).

present, there was significantly decreased expression of CHK2 mRNA in H460 cells, and non-detectable in A549 cells.

To further confirm the absence or the decrease of the CHK2 kinase mRNA in the non-small cell lung cancer cell lines, we obtained a panel of non-small cell lung cancer cell lines from ATCC (H5875, H522, A549, HTB53, H1395, H441, H2342, H2170, H2228, H2286, H520 and NHBE). NHBE is a normal human bronchial epithelial

A

(-615) CCTCC C̲GGGCTCAAG C̲GATTCTCCT GCCTCAGCCT CCC̲GAGTAGC
TGAGAATACA GG**TGCCCGCC** ACCAC̲GCCTG GCTAATTTTT TGTATTTTTA
　　　　　　　Sp1
GTAGAGAC̲GG AGTTTCACTA TGTT**GGCCAG GC**TGGTCTT̲G **AACTCCTGAC**
　　　　　　　　　　　　　　　　AP-2　　　　　　　　　　　　　RARα
CTCATGATC̲T **GCCCGT**CTTG TCCTCCCAAA GTGC̲GGGACT ACAGGAGTGA
　　　　　　　Sp1
ACCCCC̲GC̲GC (-411)

B

C

Figure 3
The promoter of the CHK2 gene is methylated in non-small cell lung cancers: A: The CHK2 gene promoter sequence from -615/-411 from the Genbank (AL117330). The putative transcriptional factor binding sites were underlined and potential CpG islands were bold and highlighted. B: Methylation specific PCR analyses of the genomic DNA isolated from the cancer cell lines: W- the wild type primers, M- methylation specific primers, U-unmethylation specific primers. The expected PCR product was 205 bps, and confimed by DNA sequencing analyses. C: Methylation specific PCR analyses of the genomic DNA isolated from the primary non-small cell lung cancer tumor specimens. Pt 7 was the normal match lung control from the patient 6 (tumor).

Table 2: Oligonucleotide primers used for MSP

Wild Type forward	(-615) CCTCC **C**GGGCTCAAG **C**GATTCTCCT GCCT (-587)
Methylated forward	(-615) TTTTT **C**GGGTTTAAG **C**GATTTTTTT GTTT (-587)
Unmethylated forward:	(-615) TTTTT **T**GGGTTTAAG **T**GATTTTTTT GTTT (-587)
Wild type reverse	(-411) GC**G**C**G**GGGGT TCACTCCTGT AGTCCC**G**CA (-439)
Methylated reverse:	(-411) AC**G**C**G**AAAAT TCACTCCTAT AATCCC**G**CA (-439)
Unmethylated reverse:	(-411) AC**A**C**A**AAAAT TCACTCCTAT AATCCC**A**CA (-439)

Table 3: Expression of CHK2 in Lung Tumors

Tumor Type	Nuclear % Positive	Nuclear Intensity	Total score
Non-small cell carcinoma (n = 46)	3	1	3 (n = 38) (83%)
	3	2	6 (n = 7) (15%)
	3	3	9 (n = 1) (2%)
Normal Lung (n = 41)	3	1	3 (n = 4) (10%)
	3	2	6 (n = 19) (46%)
	3	3	9 (n = 18) (44%)

We further tested 10 primary lung cancer tumor samples and one matched normal lung control tissues. The lung tumor tissues were obtained from The Tumor Tissue Bank at the West Virginia University with appropriate internal review board approval (IRB). The genomic DNA was extracted from the fresh frozen tumor samples using Qiagen Tissue extraction kit. The genomic DNA was subjected to MSP analyses (Fig 3C). Patient 7 was the matched normal control lung tissue from the patient 6. Methylation of the CHK2 gene promoter was detected in all the lung tumor samples, but not in the normal control lung tissue (pt 7). These results demonstrated that the CHK2 gene promoter is preferentially methylated within both the lung cancer cell lines and the lung cancer tumor tissues, but not in the ovarian cancer cells or the matched normal control lung tissue.

CHK2 kinase expression is decreased in lung cancer tumor specimens by immunohistochemistry

We used the primary lung cancer tumor tissue microarray in combination with conventional immunohistochemical staining and automated imaging analyses to evaluate the expression level of CHK2 kinase in human primary lung cancer tumor samples. High-density human lung cancer tumor tissue array slides were obtained from Clinomics Biosciences Inc. (Pittsfield, MA) and stained with the anti-CHK2 antibody as described. The staining results were digitally acquired and analyzed using ACIS system (Chroma Vision, San Juan Capistrano, CA). Table 3 summarized the results of 46 non-small cell lung cancer tumor samples and 41 normal matched lung control tissues. In

contrast to the matched normal lung control tissues in which moderate or strong stains for CHK2 kinase were observed (total score of 6 or 9), 83% non-small cell lung cancer tumors stained weakly to non-detectable (total score less than 3) (Fig 4A). The immunostaining signals were only observed within the nuclei, not in cytoplasm. Representative staining of both negative and positive signals were shown in Fig 4B. We performed the same immunohistochemical staining for addition 46 non-small cell lung cancer tumor specimens individually, and we have found essentially identical results as described for the tumor tissue microarray study (Data not shown). These results demonstrated that the CHK2 expression levels in non-small cell human lung cancer tumors are significantly decreased by immunohistochemical staining method in totally 92 lung cancer tumors.

Methylation of the CHK2 promoter silenced the transcription and prevented from binding of the transcriptional factor(s)

We cloned the DNA sequence between -615/-411 of the wild type CHK2 proximal promoter amplified by the wild type PCR primers as described above into a luciferase reporter gene vector pGL3-promoter, and used the chimeric construct in transient transfection assays to determine if the proximal promoter -615/-411 of the CHK2 gene is functionally important for the CHK2 gene transcription (Fig 5A). The positive control plasmid reporter was from Promega Corp. and the promoter and enhancer were derived from SV40 virus (Promega Corp. Madison, WI). The CHK2 promoter sequence was tested to be active
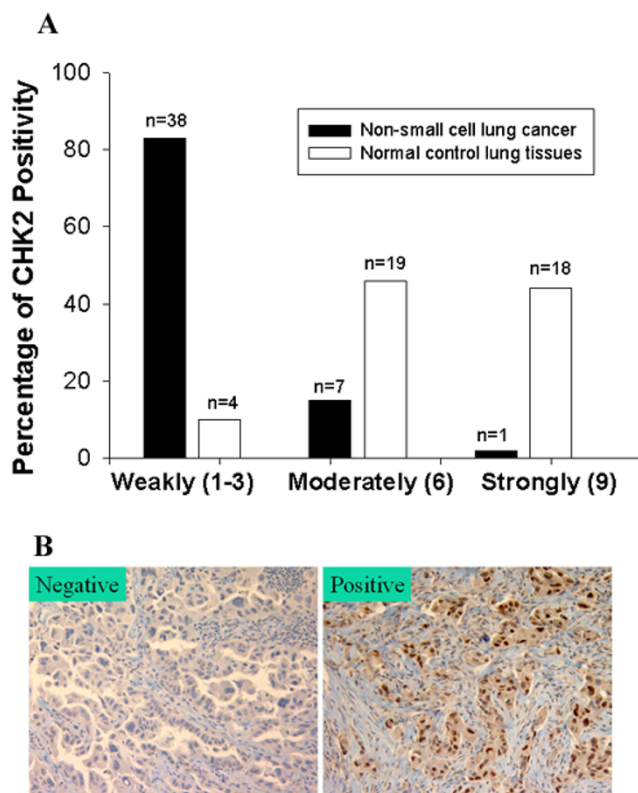
Figure 4
CHK2 kinase expression is decreased in lung cancer tumor specimens by immunohistochemistry. A: Graphic representation of the data summarized in Table 3. B: Representative immunostaining of the lung cancer tissue microarray with anti-CHK2 antibody.

in all cell lines (data not shown). Furthermore, in vitro methylation of the chimeric construct using SSS I CpG methylase and S-adenosylmethionine significantly abolished the transcriptional activity of the CHK2 promoter nearly 90–95%. These results indicate that the CHK2 gene promoter is highly sensitive to methylation, and the -615/-411 region of the CHK2 promoter is critically important for CHK2 gene activation.

We used the same fragment of the promoter DNA labeled radioactively as a probe for gel mobility shift assays to determine if there is one or more transcriptional factor(s) binding to the DNA sequence, and if methylation of the DNA sequence will influence the binding of the transcriptional factor (s) (Fig 5B). As expected, there was one prominent protein-DNA complex with the nuclear extract from the A549 cells that can be effectively competed with the unlabeled probe DNA (50X). We methylated the probe DNA in vitro using SSS I CpG methylase and S-ade-

nosylmethionine, and used the in vitro methylated probe DNA as the unlabeled competitor (50X) for the DNA-protein binding assays. In vitro methylation of the probe DNA abolished the binding activity of the probe as demonstrated by failure to compete with the wild type DNA probe for binding to the transcriptional factor.

Discussion and conclusion
CHK2 kinase is an important component of the DNA damage-signaling pathway. The critical role of CHK2 kinase in mediating the DNA damage signal was demonstrated by using the knockout mice model in which G1/S checkpoint defect and a remarkable radioresistance were observed [12,14]. Furthermore, the function of CHK2 kinase appears to relate to p53 stabilization/accumulation and the regulation of the p53 regulated target genes, such as Bax, Noxa and Puma that are important for DNA damage-induced apoptosis [12,15,16].

We have demonstrated that the CHK2 kinase expression is regulated at the transcriptional level in non-small cell lung cancer cell lines and the patients' tumor samples. The protein expression level of the CHK2 kinase in lung cancer was significantly diminished to non-detectable levels. This decreased expression of CHK2 kinase is due to the hypermethylation of the CHK2 promoter sequence. In lung cancer, hypermethylation of the promoter sequences leading to gene silence is common, and many genes critical for cell cycle regulation and cell proliferation, such as p16, p21, p14, and RARβ, were reported to be silenced by promoter methylation [17-19].

It is a well-known clinical fact that the non-small cell lung cancer is resistant to conventional chemoradiation therapy, and the current therapeutic regimens are far from optimal [11,20]. The molecular mechanism of the resistance of the lung cancer cells to cisplatin and radiation is unclear. The conventional cisplatin based chemotherapy and radiation therapy are, in principle, DNA damage based therapies, and result in DNA-damage induced tumor cell apoptosis. Many genes, such as p53, BCL-2 gene family proteins are implied in the apoptotic processes [21]. Recently, the p53-regulated BH3-only proteins such as Noxa and Puma were found to be critical in induction of apoptosis by chemotherapeutic agents [15,16]. Genetically, CHK2 kinase functions as an activator of the p53 tumor suppressor [4,12,14]. It is attempting to speculate that the lack of the CHK2 kinase expression in the non-small cell lung cancer mimics the CHK2 knockout mice in which a radioresistance of the CHK2-/- cells were noted, since the clinical behavior of the non-small cell lung cancer is remarkably similar to the feature of the CHK2-/- cells. Preliminary attempt to restore the CHK2 function in the non-small cell lung cancer cell lines A549 and H460 failed, since the CHK2 kinase alone appears to
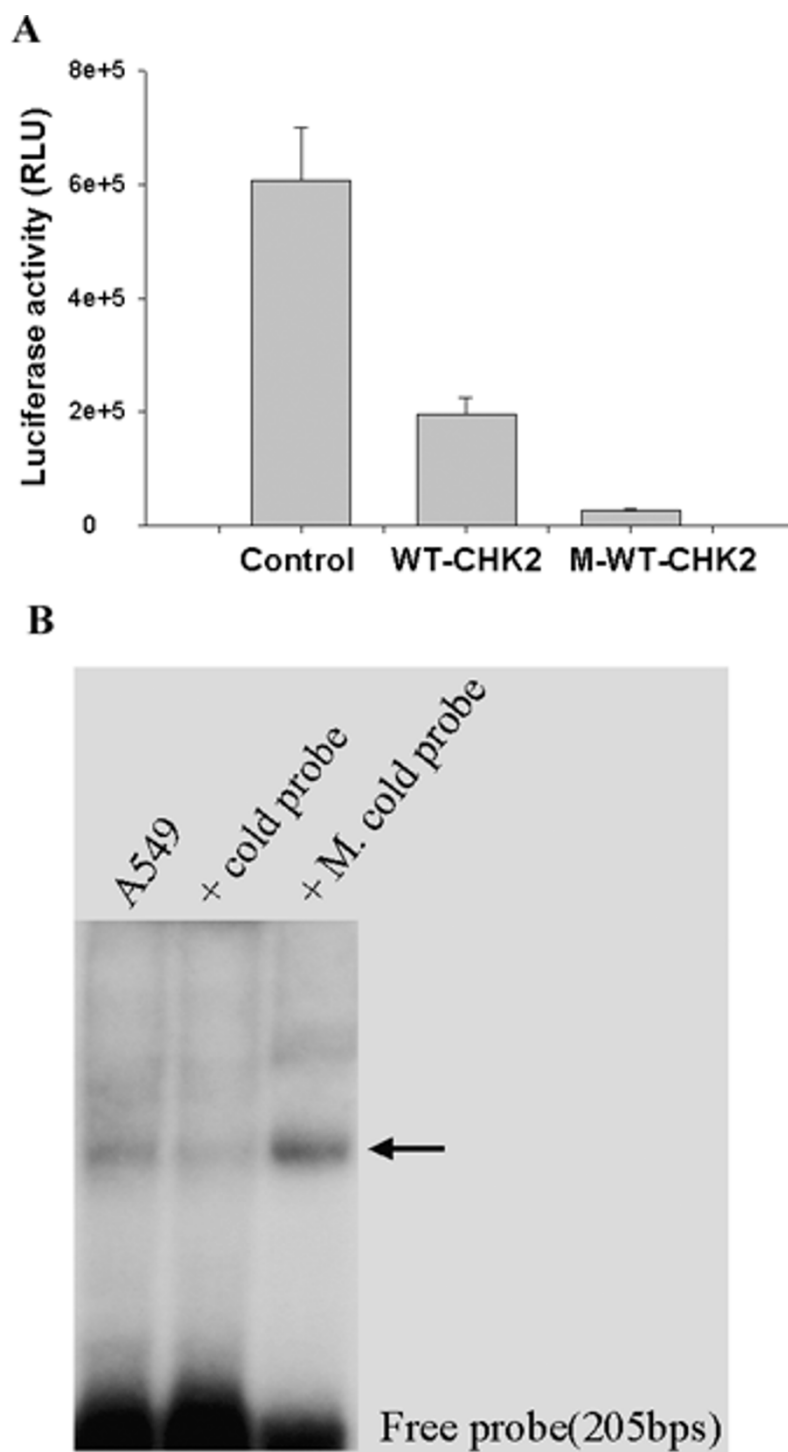
Figure 5
Methylation of the CHK2 gene promoter inhibited the CHK2 gene transcription and prevented from binding of the transcriptional factor: A: CHK2 promoter sequence -615/-411 was cloned into pGL3 luciferase reporter gene, and transfected into A549 cells, and the luciferase activity was assessed to determine the function of the CHK2 promoter sequence. WT-CHK2 represented a wild type sequence of the CHK2 promoter. M-WT-CHK2 represented in vitro methylated wild type CHK2 promoter by SSS1 CpG DNA methylase. B: gel mobility shift assays using the wild type -615/-411 sequence of the CHK2 promoter as a probe. The unlabeled probe was 50X in excess, and the M-cold probe (50X in excess) was in vitro methylated wild type -615/-411 DNA as unlabeled competitor for binding.

induce apoptosis in these cells without addition of cisplatin (Zhang, P. unpublished). Such examples have been described for p53 tumor suppressor in leukemic cells [22]. The ATM-CHK2-p53-BCL-2 family members pathway seems to be one of the important mechanisms leading to resistance of lung cancer.

We have demonstrated the down-regulation of the CHK2 kinase expression by methylation of the CHK2 gene promoter in non-small cell lung cancer. We have identified a region of -615/-411 of the CHK2 gene promoter that is important for transcriptional activation. The transcriptional factor binding to this region of DNA sequence is unclear at this time. It has been reported that Sp1 gene family members bind to the similar CG-rich sequences, and the binding can be prevented by methylation [23-25]. It is likely that the transcriptional factor binding to the -615/-411 sequence of the CHK2 gene promoter is of Sp1 gene family members, such as Sp1 and Sp3. We are actively pursuing this direction with a hope to identify a positive regulator of the CHK2 gene transcription so that we could potentially increase the CHK2 expression in the lung cancer cells.

We have also noted that the CHK2 kinase is degraded in response to cisplatin treatment in the ovarian cancer A2780 cells and the small cell lung cancer H69 cells (Fig 1, Zhang, P., unpublished result). The degradation of CHK2 kinase in response to cisplatin treatment in these cells appears at the protein level (unpublished). These results suggest that the CHK2 kinase expression is regulated at both the transcriptional level and post-translational (the protein) level. The detailed mechanism of the CHK2 degradation is under investigation.

Finally, although the expression of CHK2 kinase was reportedly ubiquitous in the normal cells and tissues [9], the expression of CHK2 kinase in cancer cells are not. Many of the cancer cells from the patients, such as colon cancer, testicular embryonal carcinoma, do not express CHK2 kinase, whereas the testicular seminoma express high level of CHK2 kinase (data not shown). Whether the absence of CHK2 kinase expression in these tumors correlates closely with resistance of these tumors to DNA damage inducing therapy, such as chemo and radiation therapies, requires further study.

Recent work by Matsui, et al identified the transcription start site of the CHK2 gene [26]. Based upon the start site of transcription in their work, the region in this manuscript identified to be hypermethylated is in fact in the intron 1, rather than a promoter region.

Materials and methods
Immunoblot analyses
The immunoblot analysis of various proteins were performed as described previously [27]. Briefly, the total cellular proteins were separated on 10% polyacrylamide gels under SDS denaturing conditions and electrophoretically transferred to nitrocellulose membrane (Biorad Inc, CA). The membranes were blocked with 5% non-fat milk solution and incubated for 1 h with a 1:200 dilution of primary antibodies (all from Santa Cruz Biotechnologies, CA). After washing off the unbound antibodies several times with 1 × TBS-Tween 20, the membranes were incubated for 1 hour with a 1:2,000 dilution of sheep anti-rabbit horseradish peroxidase-conjugated IgG (Santa Cruz Biotechnologies, CA) and washed several times. Immunodetection was performed with a chemiluminescence Western blotting kit according to the supplier (Pierce Biotechnology, Inc. Rockford, IL).

Northern blot analyses
Total RNA was isolated from the cultured cells or the tumor tissues from the patients using TriZol reagent (Invitrogen, San Diego CA). Totally 20 μg of RNA was used for each lane for Northern blot analyses. The RNA was loaded on to 1.2% formaldehyde agarose gel. The RNA was blotted onto nylon membrane (Hybond N, Amersham Biosciences, Inc.), and the membrane was UV crosslinked according to standard procedures. Full length CHK2 cDNA was amplified by PCR based on the sequence from the expression vector kindly provided by Dr. Junjie Chen at Mayo Clinic. The cDNA probe was labeled by random priming using a Prime-A-Gene labeling kit (Promega Corp., Madison, WI) as instructed by the manufacturer. Hybridization overnight at 65°C was followed by washes (2 × 15 min at room temperature in 2 × SSC, 1% SDS and then 2 × 30 min at 68°C in 0.1 × SSC, 1% SDS). The results were analyzed by using Phosphoimager (Molecular Dynamics Inc.).

Semiquantitative PCR analyses of CHK2 expression in cell lines
A panel of non-small cell lung cancer cell lines were obtained from ATCC, and cultured under the conditions following the ATCC instruction. The total RNA was isolated from these cells using TriZol reagent (Invitrogen, San Diego CA) as previously described [13]. Reverse transcription and PCR analyses were performed using the First Stranded cDNA synthesis kit from Invitrogen, CA. The PCR primers for CHK2 kinase were the following: Forward primer: 5'-ATGTCTCGGGAGTCGGATGT-3', Reverse: 5'-TCACAACACAGCAGCACACA-3'. The PCR product is the full-length coding region of the human CHK2 kinase based upon the cDNA sequence under the Genbank accession #AF174135. The relative expression

level of the CHK2 mRNA was normalized with the GAPDH levels within the same cells.

Methylation specific PCR (MSP) analyses of the CHK2 promoter

Methylation-specific PCR analyses of the genomic DNA isolated from the cell lines and the tumor specimens were essentially identical as described [28,29]. Methylation and unmethylation specific primers were selected based upon the sequences identified by the computer software CpG Island Searcher http://www.uscnorris.com/cpgislands/cpg.cgi. The primers are centered within the proximal promoter region at -615 to -411 of the CHK2 promoter. These primers listed in Table 2 were used for MSP studies of the genomic DNA isolated from the non-small cell lung cancer A549 and H460 cells, ovarian cancer A2780 cells and 11 tumor specimens from primary non-small cell lung cancer patients. The genomic DNA was isolated using Qiagen Tissue DNA extraction kit following the manufacturer's instruction. Totally 1 µg DNA was denatured by NaOH and treated with 3 M sodium bisulfite, 10 mM hydroquinone for 16 hours at 50°C as described [28]. The modified DNA was purified using Wizard DNA purification system as described [28,29] and used for MSP assays. The expected PCR product of 205 bp- fragment was visualized by 2.0% agarose gel and ethidium bromide staining. The PCR products from each pair of primers was cloned into TA-cloning vector and sequenced to confirm the methylation status of each amplified fragments as described [28,29].

Luciferase reporter assays

We cloned the wild type promoter fragment amplified from MSP into luciferase reporter vector to test the function of the promoter fragment in A2780, A549 and H460 cells to ensure the promoter that is methylated is functionally important in these cells. One microgram of the chimeric reporter construct was transfected into the lung cancer cell lines A549 and H460 cells and the ovarian cancer A2780 cells in the 6-well plates for 24 hours using Lipofectamine 2000 (Invitrogen, CA). After 24 hours, the total cell lysates were harvested for luciferase assays using Luciferase assay system (Promega Corp. WI). The total proteins from the cell lysates were quantified using Bradford protein assay system (Biorad Inc. CA) and used to normalize the luciferase activity.

To assess the effect of methylation of the CHK2 gene promoter sequences on its function, we used SSS I CpG methylase (New England Biolabs, MA) to methylate the wild type CHK2-luciferase reporter construct in vitro and use the methylated reporter plasmid for transfection and luciferase assays. In vitro methylation was performed as instructed by the manufacturer. Briefly, 5 microgram of the reporter plasmid was incubated with 10 units of SSS I

CpG DNA methylase overnight in the presence of 160 µM S-adenosylmethionine. The methylated plasmid was purified using Qiagen micro-column before being used for transfection.

Gel mobility shift assays

We also used the WT PCR product of the CHK2 promoter between -615/-411 amplified in the MSP assays as a probe for mobility gel shift assays to see if the promoter sequence is bound to a transcriptional factor. The 205-bp-fragment was amplified by PCR using genomic DNA and labeled with T4 polynucleotide kinase and γ-32P-ATP. The labeled probe was purified using Micro-spin column to remove the un-incorporated radioactive material. The gel mobility shift assays were performed as previously described [30]. The nuclear extracts were prepared from A549, H460 and A2780 cells as described [30]. Unlabeled probe (50X in excess) was used for competition.

We also used SSS I CpG methylase (New England Biolabs, MA) to methylate the wild type -615/-411 fragment in vitro as described above and used the methylated probe (50X in excess) to compete with the wild type probe for nuclear protein binding.

Immunohistochemical staining and the automated imaging analyses

We used immunohistochemical staining in combination with automated imaging analyses to detect the presence of CHK2 protein in the primary lung cancer tumor specimens. We have obtained the high-density lung cancer tumor tissue array slides from Clinomics Biosciences Inc. (Pittsfield, MA). We used classic immunohistochemical staining method to detect the CHK2 protein expression using anti-CHK2 antibody (Santa Cruz Biotechnologies Inc. CA). The high-density lung cancer tissue micro-array slides consist of 46 non-small cell lung cancer tumors, 4 small cell lung cancer tumors, more than 200 normal tissues including lung, liver, brain etc. The slides were sectioned at 5 micron in thickness, de-paraffinized in xylene, dehydrated, rehydrated in a series of 100%, 90% and 70% ethanol, and stained with the CHK2 antibody in the Ventana Benchmark II Autostainer (Ventana Medical International Inc. AZ).

After staining, the slides were digitized, acquired, and the densitometric quantitation was analyzed by using Automated Cellular Imaging System (ACIS, Chroma Vision, San Juan Capistrano, CA). The scoring system we have used for the result analysis was essentially as described by others [31,32]. Briefly, the total area of the micro-core was scanned and acquired by the ACIS software of the imaging/microscopy system, and the stained tumor cells for CHK2 expression were quantified as follows. Scores of 1–3 were assigned according to the percentage of positive

tumor cells (1, <25%; 2, 25–50%; 3, >50%) and the staining intensity of the tumor cells (score 1 for 0–25%, score 2 for 25–50%, score 3 for >50%). The two scores were multiplied to give an overall score of 1–9, where 1–3 is considered weak, 4–6 is moderate, and 9 is strong staining [32]. The imaging analysis has been previously described and used for Her-2/Neu expression in the breast cancer patients and the prostate stem cell antigen expression in the prostate cancer patients [31,32].

Authors' contributions

PZ designed and interpreted the experiments and drafted the manuscript. JW and GW performed the experiments. BY helped in Northern blot analyses and RT-PCR analyses of CHK2 kinase mRNA. JR and ER provided guidance and insightful discussion throughout the work. All authors proved the final draft of the manuscript.

Acknowledgment

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