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IN PERSPECTIVE REVIEW

p53 and Its Downstream Proteins as Molecular Targets of Cancer

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The *p53* tumor suppressor gene plays a key role in prevention of tumor formation through transcriptional dependent and independent mechanisms. Transcriptional-dependent mechanisms are mainly mediated by p53 regulation of downstream targets, leading to growth arrest and apoptosis. Mutational inactivation of the *p53* gene is detected in more than 50% of human cancers. Mutation of p53 renders cancer cells more resistant to current cancer therapies due to lack of p53-mediated apoptosis. Extensive studies have been conducted to identify small molecules that manipulate p53, including restoration of mutant p53 conformation to wild-type, disruption of murine double minute-2 (Mdm2)-p53 binding to increase p53 level and inhibition of Mdm2 E3 ubiquitin ligase activity to prevent p53 degradation. Another approach was to identify and validate ''drugable'' target(s) in p53 signaling pathways that modulate p53-induced apoptosis. We profiled a p53 temperature-sensitive lung cancer cell model with the Affymetrix human HG-U133 GeneChip, covering the entire human transcriptome. We identified thousands of unique genes that were either induced or repressed in response to p53-induced apoptosis. A follow-up study characterized a p53-repressed gene, *SAK*, a polo-like kinase (PLK) family member, as an appealing cancer drug target. Snk/Plk-akin kinase (SAK) silencing via small interfering RNA (siRNA) induced apoptosis, whereas SAK overexpression attenuated p53-induced apoptosis. Thus, SAK repression by p53 contributes to p53-induced apoptosis. Future work is directed at determining the normal cell response to SAK silencing. If a therapeutic window is obtained, a SAK inhibitor identified from high throughput screening (HTS) could serve as a lead compound for development of a novel class of apoptosis-inducing anticancer drugs.

Key words: chip profiling; SAK; cancer target identification and validation; anticancer drug discovery

INTRODUCTION

Apoptosis is a genetically programmed process of cell death required for maintaining homeostasis under physiological conditions and for responding to various internal and external stimuli. Cells committed to apoptosis are characterized by membrane blebbing, cytoplasmic shrinkage, nuclear chromatin condensation, and DNA fragmentation [1]. Cancer is associated with decreased apoptosis, and development of apoptosis resistance in cancer cells is a significant contributing factor to the failure of cancer therapies. Thus, induction of apoptosis in apoptosis-resistant cancer cells through a variety of approaches would be an ideal strategy for effective cancer therapy [2,3]. One promising approach to achieve this end is through the modulation of p53 or the components of p53 signaling pathways.

The p53 protein is known to act biochemically as a transcription factor and biologically as a tumor suppressor. p53 specifically binds to a consensus DNA binding sequence, consisting of repeats of the 10 bp motif 5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, located in the promoter or introns of its downstream target genes and thus transactivates the expression of these genes. The biological functions of p53 are mainly mediated through transcriptional regulation of its downstream target genes. For example, p53-induced growth arrest is achieved mainly by transactivation of p21 (for G_1 arrest), of 14-3-3σ (for G_2 arrest), and of placenta transforming growth factor-beta (PTGF-β). p53-induced apoptosis, on the other hand, is mediated by activation of genes involved in two major apoptotic pathways: (1) the mitochondrial pathway, with genes such as *BAX*, *NOXA*, *PUMA*, *p53AIP1*, *PIGs*, and *APAF-1*; and (2) the death receptor pathway, with genes such as *KILLER/DR5*, *FAS*, and *PIDD*. p53 also transcriptionally represses cell survival genes, such as *IGFR, BCL-2,* or *survivin*,

Abbreviations: Mdm2, murine double minute-2; HDAC, histone deacetylase; IGFR, insulin-like growth factor receptor; PIGs, p53-induced genes; SAK, Snk/Plk-akin kinase; PLK, polo-like kinase; SiRNA, small interfering RNA.

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leading to apoptosis induction [4,5]. Furthermore, a number of recent studies have shown that cytoplasmic p53 can regulate apoptosis in a transcriptionally independent manner through binding to mitochondria and modulating the Bcl-2 homolog region-3 (BH3) family of proapoptotic proteins such as BAX [6,7]. Figure 1 illustrates the p53 signaling pathways in the regulation of growth arrest and apoptosis.

As a key regulator of cell growth and cell death, p53 is activated by many environmental stimuli, including DNA-damaging agents. Activated p53 acts as a guardian of the genome by inducing growth arrest to allow cells to repair the damage or apoptosis, if the damage is too severe and irreparable [8]. Thus, not surprisingly, p53 is frequently inactivated via multiple mechanisms during human carcinogenesis. The most common mechanism of p53 inactivation is mutation at the *p53* gene, which occurs in more than 50% of all human cancers. Moreover, in some cancer cells that bear a wild-type p53, p53 is nonfunctional, either being targeted for degradation by overexpression of murine double minute-2 (Mdm2), an E3 ubiquitin ligase that binds to p53, or by being excluded from the nucleus where p53 acts as a transcription factor [4].

p53 AS A DIRECT DRUG TARGET

Due to its pivotal role in controlling abnormal cell growth and its frequent inactivation in a majority of human cancers, p53 has been a central cancer target for mechanism-driven novel cancer drug discovery. Several different approaches aiming at p53 reactivation or mutant p53 elimination have been attempted. One approach is to restore wild-type p53 conformation in mutant p53-containing cancer cells by small molecule compounds. At least two classes of compounds have been identified so far, CP-31398 and PRIMA-1 [9,10]. CP-31398 was identified through a screening based upon an in vitro biochemical assay that detects wild-type or mutant p53 conformation via two specific antibodies [9], whereas PRIMA-1 was identified through a cell-based assay by monitoring cell growth inhibition [10]. Indeed, at a high drug concentration, both compounds were found to restore p53 conformation and to induce growth arrest or apoptosis in a subset of cancer cells or to sensitize cancer cells to radio- and chemotherapies [9,10]. However, the selectivity, specificity, and mechanism of action of these compounds are still unclear. The second approach is to disrupt Mdm2-p53 binding by small molecules, thus activating wild-type p53. Three such compounds, Nutlin, RITA, and a nonpeptide Mdm2 inhibitor, with distinct chemical structures, were reported [11–13]. These compounds bind to Mdm2 or p53 in the p53-binding pocket and release p53 from Mdm2-mediated p53 degradation. Mechanistically, these compounds will only work in human cancers harboring a wild-type p53 and preferentially

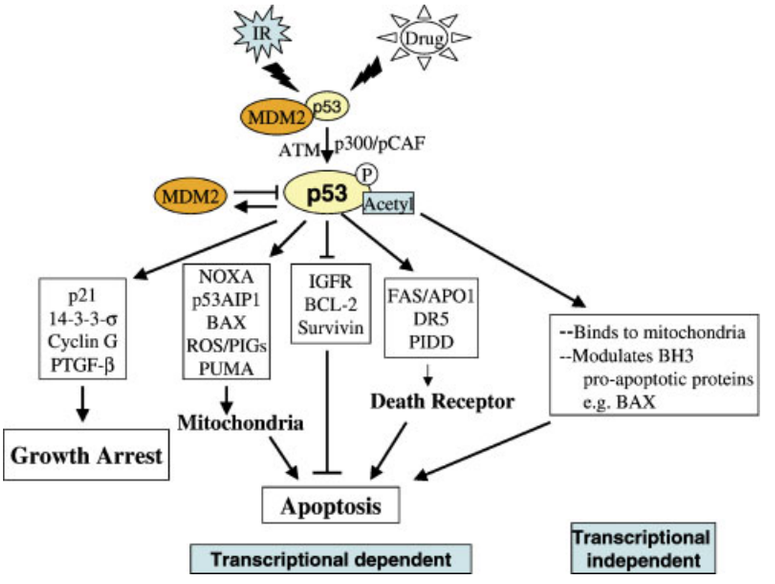

Figure 1. p53 signaling pathways for growth arrest and apoptosis: Under unstressed conditions, the p53 level is very low in cells due to MDM2 binding and degradation. Upon DNA damage by ionizing radiation or anticancer drugs, p53-MDM2 binding is dissociated as a result of p53 phosphorylation by ATM and acetylation by p300/pCAF, leading to p53 activation. Activated p53 acts as a transcription factor to transactivate growth regulatory genes such as *p21* and *14-3-3σ* to induce growth arrest. p53 regulates apoptosis in transcriptional-dependent and -independent manners. Under a transcriptional-dependent mechanism, p53 induces apoptosis by transactivating the genes in both mitochondrial and death receptor pathways as well as trans repressing cellular survival genes such as IGFR and BCL-2. Under a transcriptional-independent mechanism, p53 binds to mitochondria and modulates activity of BH3-containing proapoptotic proteins.

with Mdm2 overexpression. The third approach is to identify inhibitors of the Mdm2 E3 ubiquitin ligase, aiming at inhibiting Mdm2-mediated p53 degradation. One such compound, HLI98, was recently reported [14]. The compound stabilized p53 and Mdm2 and activated p53-dependent transcription and apoptosis with some p53-independent cytotoxicity. Again, the compound worked much better in wild-type p53-containing cancer cells than those with a mutant p53 [14]. Nevertheless, the compounds that activate wild-type p53 through disrupting or inhibiting Mdm2 have an intrinsic risk to be toxic to wild-type p53-containing normal cells. The fourth approach is to eliminate mutant p53 via Hsp90-active agents such as geldanamycin [15] and via induced degradation by histone deacetylase (HDAC) inhibitors, such as trichostatin [16]. However, depletion of mutant p53 cannot and does not restore p53 functions, although it may abolish the gain-of-function effect of mutant p53.

CANCER DRUG TARGETS IN p53 SIGNALING PATHWAYS

An ideal cancer target will meet the following criteria: (1) it plays an essential role in carcinogenesis, and/or is required for the maintenance of cancer cell phenotype, and/or is apoptosis protective and renders cancer cells resistant to apoptosis; (2) it is activated or overexpressed in cancer cells and its activation or overexpression is associated with a poor prognosis of patient survival; (3) inhibition of its expression or activity induces growth suppression and/or apoptosis in cancer cells; (4) it is preferably ''drugable;'' that is, it is an enzyme or G protein-coupled receptor (GPCR) or a cell surface molecule, which can be easily screened with a high throughput for small molecular inhibitors or targeted by specific antibodies; and (5) importantly, it is either not expressed or is only expressed at a very low level in normal cells and its inhibition has a minimal effect on normal cell growth and function. Inhibition of such a target would achieve a maximal therapeutic index with a minimal toxicity.

Based upon the above criteria, p53 is not an ideal direct cancer drug target, because it is a tumor suppressor and a transcription factor. An alternative approach will be to identify ''drugable'' p53 downstream targets that regulate p53-induced apoptosis, followed by drug screening of their chemical inhibitors for anticancer drug discovery. Two categories of genes that are responsive to p53-induced apoptosis are expected to be identified. The first category will be those that are transactivated by p53. These genes can be further subgrouped into apoptosis-inducing genes such as BAX, p53-upregulated modulator of apoptosis (PUMA), or NOXA (see Figure 1) or apoptosis-inhibiting genes, such as heparin-binding EGF-like growth factor [17], Cox-2 [18], or discoidin domain receptor 1 [19]. Induction of these genes by p53 is believed to counter-act p53-induced apoptosis as a cellular defensive mechanism. The genes that are transrepressed by p53 fall into the second category, which can also be subgrouped into apoptosis-inducing genes such as endothelial cell nitric-oxide synthase [20] or p53-interacting protein p53BP2 [21] and apoptosis-inhibiting genes, such as insulin-like growth factor receptor (IGFR), BCL-2, or survivin (Figure 1). Although apoptosis-inducing genes can be used as anticancer agents through a gene therapy approach or to elucidate apoptosis signaling pathways, the direct pharmaceutical targets for small molecule intervention have to come from the apoptosis-inhibiting genes. Indeed, the inhibitors of BCL-2 and IGFR as cancer therapeutic agents are under extensive discovery and development [22,23].

CHIP-PROFILING TO IDENTIFY NOVEL CANCER DRUG TARGETS IN p53 SIGNALING PATHWAYS

To further identify novel ''drugable'' cancer drug targets in p53 signaling pathways, we used a p53-temperature sensitive lung cancer cell model [24] after a detailed characterization. H1299 lung carcinoma cells (p53-null) were transfected with a mutant p53A138V, which is temperature sensitive. The p53 in these cells adopts a mutant conformation at 39°C and a wild-type conformation at 32°C. Shifting the culture temperature from 39 to 32°C restores the wild-type p53 conformation, as confirmed by induction of p53 target genes, p21 and Mdm2, induction of p53 transactivation, and induction of growth arrest. Induction of apoptosis only occurs in the presence of DNA damaging agents [25]. The advantage of this model is the ability to use the same cancer line with differences only in p53 status, dependent upon culture temperature. Changes due to temperature shift can be easily eliminated by including a control line that undergoes the same temperature shift in the experiment. Using this model we profiled the Affymatrix gene chip HG-U133, consisting of 39 000 genes that cover the entire human transcriptome, in an attempt to identify genes that respond to p53-induced growth arrest or apoptosis. As shown in Table 1, which summarizes the detailed experimental design, cells were grown at 39°C and switched to 32°C for 6 h (early), 16 h (intermediate), or 24 h (late) in the absence (to induce growth arrest) or in the presence of etoposide (to induce apoptosis). As a temperature control, H1299-Neo control cells were similarly treated. A total of 24 sets of chips were used for profiling. Comparisons among samples 1–6 and 13–18 were made in order to identify specific genes that respond to p53-induced growth arrest with elimination of false positives associated with temperature shifting. Similarly, the comparison among samples 7–12 and 19–24 was made to identify specific genes that respond to p53-induced apoptosis with elimination of those that respond to

Table 1. Chip Experimental Design

Group no.	Cell line	Treatment	Growth phenotype
1	H1299-p53ts	6 h DMSO at 39°C	Mutant p53, normal growth
2	H1299-p53ts	16 h DMSO at 39°C	Mutant p53, normal growth
3	H1299-p53ts	24 h DMSO at 39°C	Mutant p53, normal growth
4	H1299-p53ts	6 h DMSO at 32°C	Wild-type p53, growth arrest
5	H1299-p53ts	16 h DMSO at 32°C	Wild-type p53, growth arrest
6	H1299-p53ts	24 h DMSO at 32°C	Wild-type p53, growth arrest
7	H1299-p53ts	6 h Etoposide at 39°C	Mutant p53, normal growth
8	H1299-p53ts	16 h Etoposide at 39°C	Mutant p53, normal growth
9	H1299-p53ts	24 h Etoposide at 39°C	Mutant p53, normal growth
10	H1299-p53ts	6 h Etoposide at 32°C	Wild-type p53, apoptosis
11	H1299-p53ts	16 h Etoposide at 32°C	Wild-type p53, apoptosis
12	H1299-p53ts	24 h Etoposide at 32°C	Wild-type p53, apoptosis
13	H1299-Neo	6 h DMSO at 39°C	p53-null, normal growth
14	H1299-Neo	16 DMSO at 39°C	p53-null, normal growth
15	H1299-Neo	24 DMSO at 39°C	p53-null, normal growth
16	H1299-Neo	6 h DMSO at 32°C	p53-null, normal growth
17	H1299-Neo	16 h DMSO at 32°C	p53-null, normal growth
18	H1299-Neo	24 h DMSO at 32°C	p53-null, normal growth
19	H1299-Neo	6 h Etoposide at 39°C	p53-null, normal growth
20	H1299-Neo	16 h Etoposide at 39°C	p53-null, normal growth
21	H1299-Neo	24 h Etoposide at 39°C	p53-null, normal growth
22	H1299-Neo	6 h Etoposide at 32°C	p53-null, normal growth
23	H1299-Neo	16 h Etoposide at 32°C	p53-null, normal growth
24	H1299-Neo	24 h Etoposide at 32°C	p53-null, normal growth

etoposide, regardless of p53 status. After subtraction from controls for temperature shifting or etoposide treatment, a total of 2977 genes were identified as being either induced or repressed by p53-induced growth arrest, 3033 genes by p53-induced apoptosis, and 1057 genes by both growth arrest and apoptosis by greater than twofold in at least one given time point. Approximately, one half of the responsive genes were induced, whereas the other half was repressed. Among 113 common genes that respond to both arrest and apoptosis at all three time points, 10 are well-known p53 targets, including p21, MDM2, growth arrest and DNA damage-45 (GADD45), cyclin G_1, NOXA, BTG, DDB48, activating transcription factor-3 (ATF3), and two p53-induced genes (PIG) genes (ferredoxin reductase and quinone oxidoreductase). This indicated that our cell model and chip profiling method produce the expected response for known p53-related genes and thus are reliable for identifying genes not known to be regulated by p53 [25].

Previously some p53 target genes were noticed as being preferentially responsive to growth arrest, whereas others were responsive to apoptosis. To compare the expression patterns of genes that are responsive to growth arrest and apoptosis, we performed hierarchical clustering analysis based upon similar patterns of gene expression (Figure 2). The genes in the analysis were selected if they were differentially expressed with at least a twofold change at all three time points under growth arrest conditions and at all three time points under apoptosis conditions. A total of 113 genes were identified and all 89 known transcripts, representing 66 unique genes were analyzed. The red color indicates increased expression and blue color represents decreased expression. The magnitude of the change is depicted by the intensity of color. This analysis gave rise to five clusters of genes. Cluster 1 consists of a group of genes that showed a higher induction under growth arrest conditions, particularly at the intermediate or later time points and included genes such as connective tissue growth factor (*CTGF*). Cluster 2 contains a group of genes that again showed higher induction under growth arrest conditions, but at the early time point, and include genes such as ethanolamine kinase (*EKI1*). Cluster 3 consists of genes that showed higher expression under apoptosis conditions at all time points, and include genes such as quinone oxidoreductase (*PIG3*) and ferredoxin reductase (*FDXR*), two well-known apoptosis-associated genes, whereas cluster 4 consists of genes that showed a similar induction level between the two growth conditions, and included genes such as *BTG2* and *Gadd45*. Cluster 5 consists of genes whose expression was downregulated in both growth conditions, and included *ID1* and *ID3* (inhibitor of DNA binding). Thus, cluster analysis of the chip profiling data revealed a group of genes that is preferably induced during growth arrest conditions, whereas others are preferably induced by apoptosis.

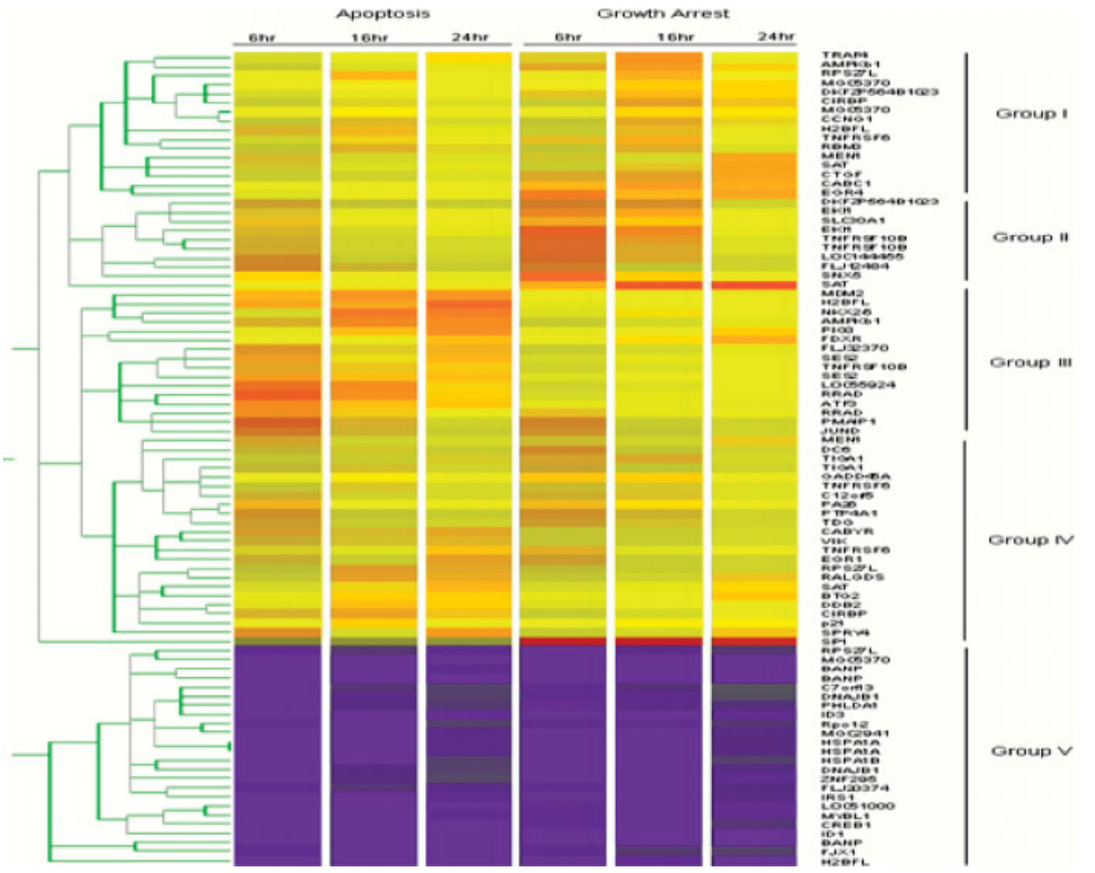

Figure 2. Hierarchical clustering analysis of the expression data of p53 responsive genes performed in Gene Spring 4.2.1 (Silicon Genetics). The fold change data of 89 transcripts representing 65 unique genes were used. These genes were differentially expressed with at least a twofold change at all three time points under growth arrest and apoptosis conditions. Each column represents a treatment condition and each row represents a transcript of the gene. The degree of fold change in gene expression is represented by different colors. The red color indicates increased expression and the blue color represents decreased expression.

CHARACTERIZATION OF SAK AS A p53-REPRESSED GENE: MECHANISM OF ACTION

Among many p53-repressed genes, we selected Snk/Plk-akin kinase (SAK) for further detailed characterization, because SAK appears to meet some of criteria defined as an ideal cancer drug target. First of all, SAK, also called Plk4, encodes a new member of the polo-like kinases (PLKs) with other members including PLK-1, PLK-2/Snk, and PLK-3/Fnk/Prk, known to play pivotal roles in cell cycle regulation, specifically in the control of entry and exit of mitosis [26]. Secondly, SAK is a kinase with a Ser/Thr kinase domain at the N-terminus and is thus ''drugable'' [27,28]. Thirdly, SAK is highly expressed in colon cancers, compared to adjacent normal intestinal mucosa [29]. Lastly, SAK knock-out studies showed that SAK expression appears to be required for the cellular exit from mitosis through the APC-dependent inactivation of the Cdc2/cyclinB complex [27] and SAK haploinsufficiency causes mitotic infidelity and carcinogenesis [30]. Thus, manipulation of SAK level is likely to disturb well-coordinated cell cycle progression, leading to cell death.

After confirming the chip data in H1299 p53 temperature sensitive model using Northern analysis to show a SAK repression by wild-type p53, we went on to demonstrate that SAK expression was also downregulated by wild-type p53 in several cancer cell models, indicating a wild-type p53-dependent, rather than cell line-dependent downregulation. Gene repression induced by p53 is known to fall mechanistically into three general categories. First, p53 may directly bind to a promoter through either its consensus binding site or other sites that overlap with some cis-elements such as E2F, SP-1, or HNF3. Secondly, p53 may interact with a transcription factor in the promoter and interfere with its normal function of transactivation. Third, p53 may directly interact with some factors of the basal transcription machinery such as TBP. The binding of p53 to these factors could recruit a HDAC complex to the

minimal promoter region and cause repression of transcription. Such repression may be reversed in the presence of HDAC inhibitors.

To further characterize the molecular basis of SAK p53 repression, we cloned a 1.7 kb SAK promoter sequence and identified three putative p53 binding sites. However, both the in vitro gel retardation assay and the in vivo chromatin immunoprecipitation assay revealed that p53 does not bind to any of these sites, indicating SAK repression by p53 did not occur through a direct p53 binding to the promoter. Transcriptional analysis with luciferase reporters driven by a series of SAK promoter deletion fragments identified SP-1 and CREB binding sites, which together conferred a twofold SAK repression by p53. However, the repression was not reversed by cotransfection of SP-1 or CREB, suggesting a lack of interference between p53 and SP-1 or CREB, which excluded the second possibility. Significantly, p53-mediated SAK repression was largely reversed in a dose-dependent manner by Trichostatin A, a potent HDAC inhibitor, suggesting an involvement of HDAC transcription repressors in SAK repression by p53 [31].

SAK, AN APPEALING CANCER DRUG TARGET?

To understand the biological significance of SAK repression by p53 during apoptosis, we knocked down SAK expression using small interfering RNA (siRNA) and looked for phenotype changes. HeLa cells were transfected with two SAK RNAi (K1 and K2) and a semiquantitative RT-PCR study revealed that K1, but not K2, caused a significant reduction of endogenous SAK expression. Accompanying the SAK reduction, morphological signs of apoptosis appeared in K1-, but not K2-transfected cells. A quantitative DNA fragmentation assay showed that indeed K1siRNA, but not K2siRNA, induced apoptosis in a dose-dependent manner. Thus, SAK repression probably contributes to p53-induced apoptosis [31].

If SAK repression is critical for p53-mediated apoptosis induction, one would expect that overexpression of SAK should block or attenuate the effect of p53. To test this hypothesis, we went on to establish SAK stable lines by transfecting a SAK-expressing plasmid or vector control into wt p53-containing U2-OS cells. Two independent SAK overexpressing clones, along with a vector control clone, were then subjected to etoposide treatment to induce p53, followed by a gel assay for DNA fragmentation, a hallmark of apoptosis. Etoposide treatment significantly induced DNA fragmentation in U2-OS vector control cells. The degree of DNA fragmentation was significantly reduced in both SAK overexpressing clones, indicating that SAK overexpression attenuates p53-induced apoptosis. Thus, SAK repression by p53 contributes to p53-induced apoptosis [31].

SAK has been previously shown to be overexpressed in colon cancer tissues. Our work presented here showed that silencing of SAK level could induce apoptosis in cancer cells. Further validation of SAK as a cancer target requires determination of the normal cellular response to SAK elimination via siRNA silencing. If a differential apoptotic response between normal and cancer cells can be established, which is the basis for therapeutic index, SAK could be an appealing cancer drug target.

SUMMARY AND CONCLUSION

Overall, chip profiling in combination with knowledge-based computer analysis provided us with a wealth of information on genes or signaling pathways possibly involved in p53-induced growth arrest and apoptosis. This will lead to a better understanding of p53 biological functions and its mechanism of action. The gene list derived from this genome-wide expression profiling and analysis provides a solid basis for further characterization and functional assays of each individual gene or several genes in a particular pathway as potential p53 targets. SAK, as an example presented here, appears to be a promising target if its manipulation in normal cells shows a minimal effect. Thus, we hope our study can provide a battery of potential targets for further characterization as novel gene-targeted cancer therapies with an ultimate goal to discover and develop a novel class of apoptosis-inducing anticancer drugs.

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