

The Biology Behind

Gadd45a: An Elusive Yet Attractive Candidate Gene in Pancreatic Cancer

Commentary re: K. Yamasawa *et al.*, Clinicopathological Significance of Abnormalities in Gadd45 Expression and Its Relationship to p53 in Human Pancreatic Cancer. Clin. Cancer Res., 8: 2563–2569, 2002.

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Introduction

To date, pancreatic cancer remains one of the most lethal and least understood tumors known and is characterized as being resistant to most conventional chemotherapeutic regimens available (1). The fact that the etiology of this malignant gastrointestinal tumor is unknown, along with its invasive and metastatic properties, places a high priority on elucidating the molecular mechanism(s) underlying this disease with the aim of ultimately developing novel and effective therapeutic strategies to target this malignancy. Whereas effective therapies remain to be established, several molecular markers have been identified in the last decade, all of which are potential targets for future drug and/or genetic therapies. The vast majority of genes identified to date that have altered expression and/or mutations in pancreatic cancer are primarily oncogenes (such as *K-ras*), tumor suppressor genes (*p16*, *p53*, *DPC*, *BRCA2*, *p300*, *MKK4*, *TGF- β 1*, *TGF- β 2*, and so forth), or DNA repair genes [*hMLH1* and *hMSH2* (2)]. Some of these factors are directly involved in cell cycle regulation and apoptosis, whereas others are part of more complex signaling pathways mediating the conversion of cell surface signals into the activation of downstream cytoplasmic and nuclear factors (2, 3). In this issue of *Clinical Cancer Research*, Yamasawa *et al.* (4) identified *Gadd45a* (a growth arrest and DNA damage-inducible gene) as a new factor in the development of pancreatic cancer. This preliminary study identified point mutations in over 13% of tumors from 59 patients with invasive ductal carcinomas of the pancreas (Fig. 1). Moreover, overexpression of *Gadd45a* protein, along with possible p53 loss of function, significantly contributes to poor prognosis, compared with patients with undetectable *Gadd45a*. The relatively high mutation frequency in exon 4 of *Gadd45a* creates the need for a better understanding of this gene and to consider it as a novel target for future therapeutic measures. The purpose of this review is to present mechanistic insight into the potential roles of *Gadd45a* in protecting against tumorigenesis. The fact that mutations in either *Tp53* or *ATM* genes in cancer-prone ataxia-telangiectasia patients result in decreased *Gadd45a* ex-

pression after IR² draws an interesting correlation between *Gadd45a* and cancer. Cells derived from these patients demonstrate not only reduced p53 expression but also reduced *Gadd45a* expression (5). Additionally, *Gadd45a* has been found to associate with several cytoplasmic and nuclear factors and has been implicated in several cellular functions, including MAPK signaling (6), cell cycle regulation (7–9), DNA repair and genomic stability (10, 11), apoptosis (6), and immune responses (12). Defects in any one (or combination) of these processes may contribute to cancer.

Gadd45a and Other Gadd45 Family Members

Gadd45a is a small acidic globular protein (M_r 18,000; Ref. 11). *Gadd45a* was originally cloned by subtractive hybridization screening of UVR Chinese hamster cells (13), and the human homologue was subsequently cloned and localized to the short arm of chromosome 1 at 1p31.1-31.2 (14). *Gadd45a* (also known as *Gadd45/Gadd45 α*) belongs to a family of three genes, including *Gadd45b* (*Myd118/Gadd45 β*) and *Gadd45g* (*CR6/OIG37/Gadd45 γ*). Whereas all three *Gadd45* genes share approximately 57% homology and are stress inducible, *Gadd45a* is the only member to be activated by p53. Concordantly, strong p53 response elements have been identified within intron 3 (14). Although *Gadd45a* is a p53 effector gene, p53-independent induction may also be achieved, depending on the insult (15). Moreover, both genotoxic stresses (*i.e.*, UVR, IR, cisplatin, and Adriamycin) and nongenotoxic stresses (*i.e.*, apoptotic and/or growth-inhibitory cytokines, serum starvation, endoplasmic reticulum stress, and antimicrotubule agents such as vincristine) will induce *Gadd45a* activation (16; Fig. 2). It has recently been shown by a combination of protein chemistry and immunochromatological methodologies such as gel-exclusion chromatography, Fergusson's analysis, and ELISA that recombinant *Gadd45a* self-associates. Whereas *Gadd45a* will form monomers, homotrimers, and homotetramers, the predominant conformation is homodimeric. Even though *Gadd45a* is normally found in very low abundance under normal circumstances, when overexpressed, nuclear foci of high protein concentrations are detected by immunohistochemistry, which could conceivably create an environment conducive for oligomerization. Deletion analysis of the *Gadd45a* protein defined the self-oligomerization regions to be within two regions: (a) NH₂-terminal amino acid residues

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² The abbreviations used are: IR, ionizing radiation; MAPK, mitogen-activated protein kinase; UVR, UV-irradiated; PCNA, proliferating cell nuclear antigen; Cdk, cyclin-dependent kinase; JNK, c-Jun NH₂-terminal kinase.

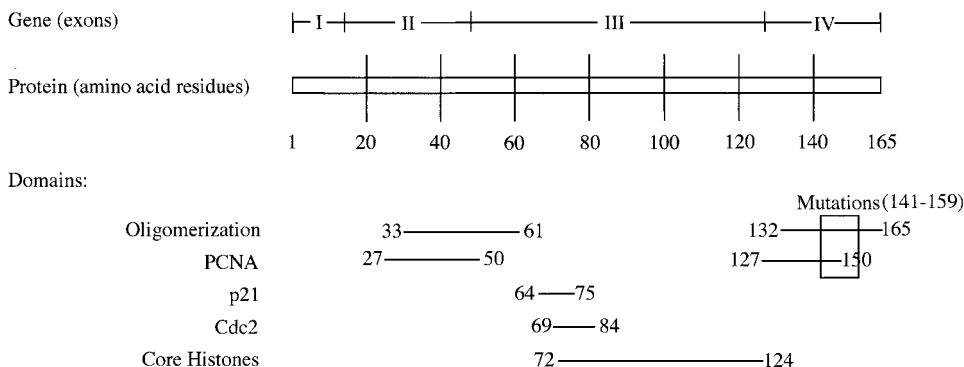
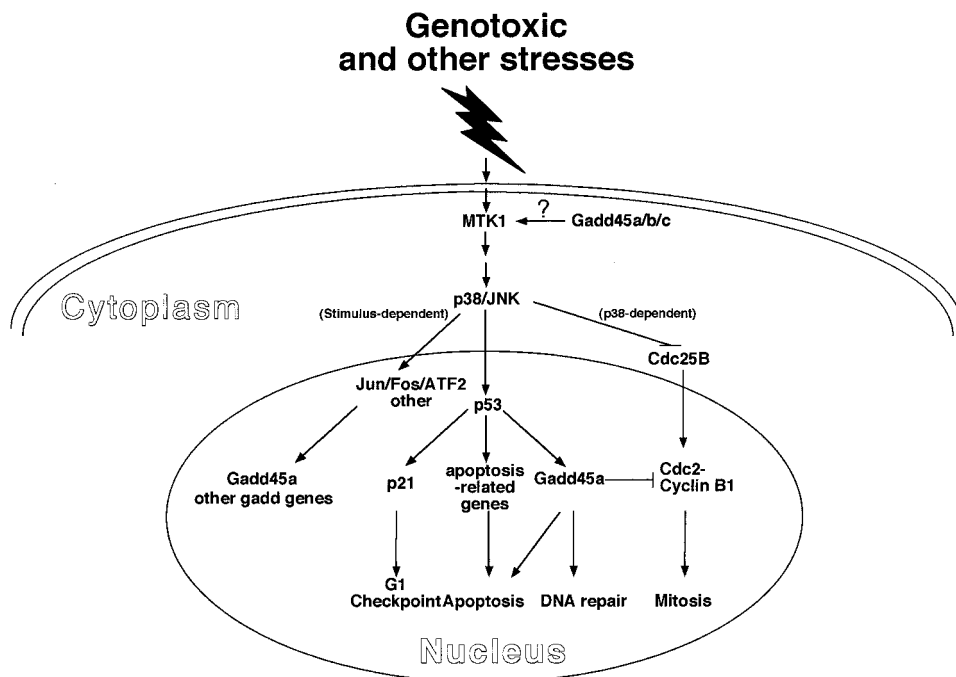


Fig. 1 Schematic diagram of the *Gadd45a* gene. *Gadd45a* has four exons (I-IV), which code for 165 amino acids. Serial deletion analysis of the *Gadd45a* protein revealed numerous protein-interacting regions. The amino acid residues involved in the interactions are noted. Mutations in exon IV are within the COOH-terminal oligomerization subdomain and PCNA binding regions (residues 141-159).

Fig. 2 Working model. Genotoxic stresses such as UV irradiation and/or IR will activate numerous signal transduction cascades, including the MAPK pathway. MTK1 activation will lead to the activation of the downstream antiproliferative JNK and p38 MAPKs, which are involved on one hand in phosphorylating and activating p53 and on the other hand in phosphorylating and repressing Cdc25B phosphatase (which promotes G₂-M transition). Activated p53 will in turn transcribe genes, such as *Gadd45a*, whose products are involved in cell cycle regulation and apoptosis. Depending on the stimulus, *Gadd45a* activation may be accomplished by both p53-dependent and independent pathways. A defective MAPK signaling pathway may result in cell cycle, apoptosis, and DNA repair defects, which may culminate in genomic instability and ultimately cancer.



33-61; and (b) COOH-terminal amino acid residues 132-165. Furthermore, *Gadd45a* is able to oligomerize with its two family members, *Gadd45b* and *Gadd45g* (11).

Gadd45a and Cell Cycle Regulation

Initial functional studies with *Gadd45a* revealed that overexpression of this product leads to growth suppression in numerous cell types (*i.e.*, human fibroblasts) primarily through activation of the G₂ checkpoint in the cell cycle (17). Additional studies demonstrated the ability of *Gadd45a* to interact with nuclear factors associated with cell cycle regulation, such as the Cdc2-cyclin B1 kinase complex (8), p21 (7), and PCNA (9). *In vitro* kinase activity experiments performed with immunoprecipitated Cdc2-cyclin B1 established the direct and highly specific inhibitory effect of *Gadd45a* with this complex. Moreover,

whereas *Gadd45a* is able to interact with Cdc2, its suppressive effect is achieved through disruption of the kinase complex (8).

Unlike *Gadd45a*, which specifically targets Cdc2-cyclin B1, p21 is a universal Cdk/cyclin inhibitor known to associate and block the activity of several kinase complexes, including Cdk4/6-cyclin D and Cdk2-cyclin E (involved in G₁ checkpoint and G₁-S progression, respectively) and Cdc2-cyclin B1 (involved in G₂ progression). Whereas p21 is a potent Cdk/cyclin inhibitor, it is unclear how p21-*Gadd45a* association may contribute to suppression of this G₁-M kinase complex. Recent studies establish a strong correlation between p21 and *Gadd45a*, inasmuch as both are costimulated by epidermal growth factor, a known activator of the G₁ checkpoint (18).

In addition to its association with Cdc2-cyclin B1 and p21, *Gadd45a* has also been found to interact with PCNA after IR.

PCNA is part of the DNA replication machinery and is known to be involved not only in DNA synthesis but also in DNA repair and apoptosis (19). Gadd45a is observed to compete with p21 for association with PCNA. Interestingly, whereas p21 will disrupt the homotrimeric PCNA complex, Gadd45a does not do so (20). In accordance with the *in vitro* results, PCNA disruption is also observed in UVR cells, and overexpression of Gadd45a in synchronized H1299 lung carcinoma cells blocked entry into S phase, possibly as a result of Gadd45a-PCNA interactions (9).

Gadd45a and DNA Repair

To effectively demonstrate the involvement of Gadd45a in nucleotide excision repair, an *in vitro* assay was designed to compare the ability of cell extracts, before and after IR, to repair a UVR or chemically damaged DNA target. The degree to which the extracts were able to repair the damaged DNA was measured by the rate of radiolabeled nucleotide incorporation. Whereas extracts from IR-treated cells effectively repaired the target DNA, extracts from untreated control cells did not. Furthermore, overexpression of Gadd45a led to increased repair (as measured by labeled nucleotide incorporation). Conversely, addition of anti-Gadd45a antibodies blocked this effect. Gadd45a not only blocks cells from entering S phase in H1299 cells but also stimulates nucleotide excision repair (9). Therefore, it is possible that the association of Gadd45a with PCNA may do one of two things: it either blocks PCNA from replicating DNA or redirects the replication machinery from DNA synthesis to DNA repair.

The manner in which Gadd45a contributes to DNA repair extends beyond its ability to associate with PCNA. More recently, Gadd45a has been found to associate with core histones around damaged DNA, and in doing so, it alters the chromatin structure and makes it more accessible (21). Like Gadd45a, many proteins that associate and contribute to nucleosome stability and chromatin structure are acidic. Nucleophosmin is one such protein that shares an almost identical match in 9 of 10 amino acid stretch with Gadd45a. In addition to histone-binding proteins, UV radiation also contributes to disruption of DNA-histone interactions by increasing histone acetylation. Interestingly, Gadd45a preferentially associates with chromatin altered by either UV radiation or histone acetylation. In doing so, Gadd45a facilitates the action of repair machinery elements such as topoisomerases, which are involved in relaxing the negative coils in double-stranded DNA (21).

Gadd45a and Apoptosis

Although there is a clear correlation between Gadd45a expression and apoptosis, it is unclear whether Gadd45a expression is a cause or effect of this complex genetic program (22). On one hand, overexpression of Gadd45a in human fibroblasts will only induce cell cycle arrest in a p53-dependent manner, whereas overexpression of Gadd45 family proteins (either Gadd45a, Gadd45b, or Gadd45g) will sensitize myeloblastic leukemia and lung carcinoma cells to undergo apoptosis if cells are either UVR or γ -irradiated (23). Although controversial, it has recently been proposed that Gadd45a mediates stress-induced apoptosis by activating the MAPK pathway, a signal transduction pathway activated by a variety of genotoxic and

nongenotoxic stresses (6). In a general sense, intracellular responses (cytoplasmic and/or nuclear) to extracellular signals are mediated by a number of signaling cascades, including the MAPK pathways, whereby MAPK kinase kinases phosphorylate specific serine and threonine sites on MAPK kinases and activate them, which in turn do the same to MAPK (24). Ultimately, numerous transcription factors (such as p53, c-Myc, c-Jun, and so forth) and other cellular factors (*i.e.*, Cdc25B phosphatase) are targeted and activated (24, 25). The pathway components that are potentially under Gadd45a influence are MTK1 (a MAPK kinase kinase) and p38 and JNK MAPKs (two antiproliferative and proapoptotic MAPKs). When cell lysates from green monkey kidney cells coexpressing epitope-tagged MTK1 and Gadd45 proteins were used for immunoprecipitation assays, all three Gadd45 proteins (Gadd45a, Gadd45b, and Gadd45g) were coprecipitated along with MTK1. Additionally, Gadd45 proteins were shown to activate MTK1 and its downstream targets p38 and JNK MAPK. When overexpressed in HeLa human cervical epithelium cells, Gadd45 proteins induced rampant apoptosis via MAPK activation. This activation was blocked when Gadd45 and MTK1 were coexpressed with a dominant negative MTK1 mutant, which competed for interaction with Gadd45 proteins (6). In contrast to this set of observations, independent laboratories report conflicting results because *Gadd45a* gene expression and protein synthesis do not precede MTK1/p38/JNK MAPK activation (26, 27). Clearly much remains to be done to elucidate the mechanistic role of Gadd45 in cell signaling and apoptosis.

Gadd45a-null Mouse Model

To further elucidate the biological relevance of Gadd45a, *Gadd45a*-null mice were generated. Although *Gadd45a*-null mice developed normally, they demonstrated an increased frequency of neural tube defects. Similar to *Tp53*-null mice, approximately 8% of newborn pups derived by Cesarean section were exencephalic. Unlike the *Tp53*-null counterparts, this phenotype was observed at equal frequency irrespective of gender. Furthermore, mice lacking Gadd45a exhibited thymic hyperplasia, albeit with normal CD4⁺CD8⁺, CD4⁺CD8⁻, and CD4⁻CD8⁺ distribution (10). Although the mice did not develop spontaneous tumors, they share significant similarities to *Tp53*-null mice. *Gadd45a*-null mice are more prone to IR and dimethylbenzanthracene-induced lymphomas (10, 28). The rate at which Gadd45a-deficient mice developed tumors was 3 times greater than normal, with a median onset of approximately 3 months earlier than wild-type mice (20 weeks *versus* 32 weeks). Additionally, *Gadd45a*-null embryonic fibroblasts lost normal senescence and had a growth advantage compared with wild-type cells. As with *Tp53*-null cells, single oncogene transformation by Ras was also achieved with *Gadd45a*-null embryonic fibroblasts. Interestingly, whereas *Gadd45a*-null mice did not develop spontaneous tumors, embryonic fibroblasts had a demonstrable increase in genomic instability, with more than twice the normal frequency of aneuploidy and tetraploidy, accompanied by numerous chromosomal/chromatid aberrations such as double minutes, centromere fusions, triradials, quadraradials, and chromatid deletions. Moreover, centrosome amplification

commonly found in tumors was also observed at a higher frequency in *Gadd45a*-deficient cells (10, 28).

In line with the *in vitro* observations mentioned in previous sections, *Gadd45a*-null lymphoblasts had defective G₂ checkpoint induction when treated with genotoxic agents such as UV irradiation or methyl methanesulfonate. Moreover, nucleotide excision repair was significantly reduced in lymphoblasts and embryonic fibroblasts derived from *Gadd45a*-null mice. These same mice also demonstrated an increased number of dimethylbenzanthracene-induced lymphomas, as well as intestinal, ovarian, hepatocellular, and vascular tumors. In contrast, no G₁ checkpoint defects or apoptotic differences have been noted in the absence of *Gadd45a* to date (10, 28).

In addition to increased cancer susceptibility, *Gadd45a*-null mice also manifested deregulated T-cell receptor-mediated T-cell proliferation and a lupus-like phenotype, which is characterized by high titers of anti-DNA and histone antibodies as well as severe leukopenia, lymphopenia, proteinuria, and glomerulonephritis, all of which lead to premature death (12).

Gadd45a in Human Cancer: Integrating the Facts

Unlike *Tp53* mutations, which are commonly found in over 50% of all tumors (and almost 90% of pancreatic cancers), mutations in *Gadd45a* have not been identified in any form of cancer until now (1, 29, 30). As a matter of fact, previous screenings in breast cancer patients and human tumor cell lines were to no avail (29, 30). Interestingly, however, combined high levels of *Gadd45a* and mutant p53 protein have been reported for several cancer cell lines, such as breast (*i.e.*, HS578T), central nervous system (SF-268), lung (NCI-H226), lymphoid (K562 and WI-L2-NS), prostate (PC3), and renal (TK10) cell lines (31, 32). High p53 levels are usually directly associated with mutations that stabilize the p53 protein. Additionally, because most of the mutant forms of p53 render the protein dysfunctional, in all likelihood cells harboring such mutations become resistant to the protective (yet toxic) effects of p53. Therefore, *Tp53*-mutant cells may develop a higher level of "tolerance" for cell signaling deregulation and/or overexpression of factors, such as *Gadd45a*, that are directly or indirectly involved in cell cycle arrest, apoptosis, and so forth. The Yamasawa *et al.* laboratory (4) not only validated this *in vitro* correlation between p53 mutation status and high *Gadd45a* protein levels but also presented the identification of point mutations in approximately 13% of pancreatic cancers screened. Considering the fact that *Gadd45a* is likely to be involved in numerous cytoplasmic (signal transduction) and nuclear (cell cycle regulation, DNA repair, and genomic stability) events, mutations in the *Gadd45a* gene could conceivably contribute significantly to tumorigenesis. Additionally, because *Gadd45a* is a p53 effector gene, mutations in *Tp53* can certainly contribute to altering *Gadd45a* expression levels in a cell and/or tissue. Whereas *Gadd45a* protein normally localizes in the nucleus, overexpression can lead to an increased presence of *Gadd45a* in the cytoplasm. Small alterations in the relative amounts of *Gadd45a* protein in different cellular compartments could have detrimental effects. For instance, if indeed *Gadd45a* contributes to MTK1 activation, a scenario could be construed where high cytoplasmic levels of mutant *Gadd45a* would interfere with

normal activation of the MAPK signaling cascade under stress conditions. This interference would compromise downstream events such as cell cycle regulation, DNA repair, and apoptosis, all of which are important components of the cellular defense mechanism against DNA damage. Moreover, because *Gadd45a* is likely to form homodimers and heterodimers, mutant proteins could potentially disrupt these associations as well. It is important to note that all of the mutations identified are within the PCNA and oligomerization domains. Disrupted PCNA interactions may interfere with DNA repair, whereas disrupted oligomerization may prevent *Gadd45a* activity altogether. Because of the numerous proteins *Gadd45a* associates with, it is imperative to determine biochemically whether the mutations identified do in fact alter *Gadd45a* protein interactions/function. Additionally, because *Gadd45a* is apparently also regulated posttranscriptionally, mutations may potentially alter mRNA and/or protein stability. For instance, not only have *Gadd45a* mRNA stabilizing sequences been identified, but *Gadd45a* protein has been observed to be protected against degradation by protein kinase C δ -dependent deubiquitination (33, 34).

A better understanding of the molecular basis of pancreatic cancer has already led to the design of several new therapeutic agents that are currently being tested, such as farnesyl transferase inhibitors and synthetic antisense RNA against deregulated *ras*, ONXY-015 cytotoxic adenovirus against cells with mutant p53, and fumagillin analogues with antiangiogenic properties. If *Gadd45a* joins the ranks of other known abnormalities in pancreatic cancer, it will certainly be the focus of yet more agents that will hopefully improve the prognosis of pancreatic cancer patients in the future.

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