Down-regulation of Gadd45 Expression is associated with Tumor Differentiation in Non-small Cell Lung Cancer

HIROSHI HIGASHI[1,2], DANIEL VALLBÖHMER[1], UTE WARNECKE-EBERZ[1], SHUICHI HOKITA[1,2], HUAN XI[1,3], JAN BRABENDER[1], RALF METZGER[1], STEPHAN E. BALDUS[4], SHOJI NATSUGOE MD[4], TAKASHI AIKOU[4], ARNULF H. HÖLSCHER[1] and PAUL M. SCHNEIDER[1]

[1]Department of Visceral and Vascular Surgery and [4]Institute of Pathology, University of Cologne, Cologne, Germany; [3]Department of General Surgery, Beijing Hospital, Beijing, China; [2]First Department of Surgery, Kagoshima University School of Medicine, Kagoshima, Japan

Abstract. *Background: Evidence suggests that the growth arrest and DNA damage-inducible gene 45 (Gadd45) is an effective indicator of poor prognosis or malignant potential in solid tumors. The purpose of this study was to determine the gene expression patterns and clinical relevance of Gadd45 in tumor specimens of non-small cell lung cancer (NSCLC) patients. Patients and Methods: Using a quantitative real-time RT-PCR method, the mRNA expression of Gadd45 was analyzed in tumor and paired normal-appearing tissues of 66 patients with NSCLC. The gene expression data for each patient were matched to the clinicopathological parameters. Results: Gadd45 mRNA expression was detectable in all (100%) specimens analyzed. The overall median mRNA expression level of Gadd45 was approximately 10-fold lower in tumor tissues than in matching normal lung tissues ($p<0.001$). High intratumoral Gadd45 expression was significantly associated with a poorer histological grading ($p=0.041$). Conclusion: The significant decrease in Gadd45 expression in tumors compared with normal tissue and its association with histological grading suggest a role for Gadd45 in the differentiation pathway of NSCLC.*

Lung cancer is the most common cause of death from neoplasia in the Western world. In the USA, 172,570 new cases are expected for the year 2005, with 163,510 expected deaths from this disease (1). Despite improvements in its detection, surgical resection and systemic therapy, only modest progress has been made in the outcome for patients diagnosed with lung cancer. In fact, the relative 5-year survival rate for all stages of lung cancer combined was 13% from 1974-1976 and 15% from 1995-2000 (1). This places a high priority on elucidating the molecular mechanisms underlying this disease with the aim of ultimately developing novel and effective therapeutic strategies to target the malignancy.

The growth arrest and DNA damage-inducible gene 45 (Gadd45) encodes a protein that is induced by genotoxic and certain cellular stress situations, including ionizing and ultraviolet radiation, as well as various chemotherapeutic agents (2-4). Gadd45 is multifunctional, with interference in cell cycle arrest, genomic stability, nucleotide excision repair, chromatin accessibility and apoptosis (2-4). It is one of the downstream mediators of p53 and deactivates p53 that contributes to cell cycle regulation through binding with cyclin-dependent kinase inhibitors and proliferating cell nuclear antigen (5, 6). Interestingly, Gadd45A-null mice exhibit a phenotype similar to that of p53-null mice (3).

Previous studies suggested that Gadd45 is an indicator of poor prognosis or malignant potential in solid tumors. Sengupta *et al.* reported that, in epithelial ovarian cancers, Gadd45 expression was a significant prognostic factor (7). In cervical carcinomas, Santucci *et al.* described that lack of Gadd45α induction after radiotherapy was correlated with good clinical response (8). Recently, Yamasawa *et al.* demonstrated, in patients with invasive ductal carcinomas of the pancreas, that intratumoral overexpression of Gadd45α protein, along with possible p53 loss of function, significantly contributed to poor prognosis (9).

To determine the gene expression patterns and clinical relevance of Gadd45 in NSCLC, quantitative real-time PCR was performed on surgically removed tumor specimens and adjacent benign tissue from 66 patients with NSCLC.

Patients and Methods

Sixty-six patients with histopathologically confirmed NSLC were included in this study, the data for whom were available from a previous prospective trial of 103 consecutive patients with completely

Correspondence to: P.M. Schneider, MD, Professor of Surgery, Department of Visceral and Vascular Surgery, University of Cologne, 50931 Cologne, Germany. Tel: 49-221-4784829, Fax: 49-221-4786258, e-mail: paul.schneider@medizin.uni-koeln.de

Key Words: Non-small cell lung, Gadd45.

resected (R0 resection) NSCLC (10). There were 54 men and 12 women, with a median age of 65 years (range 34-80). Thirty-five (53%) patients had squamous cell carcinomas, 21 (32%) had adenocarcinomas and 10 (15%) had large cell carcinomas. The primary tumors were graded histopathologically as moderately-differentiated (G2, 14 patients) and poorly-differentiated (G3, 52 patients). Tumor staging was performed according to the International Union Against Cancer (UICC) TNM classification (10). Thirty-three patients (50%) had stage 1 tumors, 13 (20%) had stage 2 tumors and 20 (30%) had stage 3a tumors. All 66 patients underwent thoracic surgery and the tumors were radically removed (R0 resection). Patients with histopathological stage 3a tumors received postoperative radiotherapy. Informed consent was obtained from each patient.

The median follow-up was 86.8 months (min. 63.3; max. 105.2 months). Patients were seen at 3-month intervals during the first postoperative year, every 6 months in the second and third year and once a year thereafter. Evaluation consisted of physical examination, biochemical profile, chest radiograph, CT scan of the brain, chest and abdomen, abdominal ultrasound and technetium bone scan. Data on recurrence and cause of death were obtained for all the patients.

The data and tissue collection were in accordance with the regulations of the local ethic committee.

Tissue acquisition. For the evaluation of gene expression levels, tumor samples were obtained immediately after lung resection before starting radical mediastinal lymphadenectomy and were frozen in liquid nitrogen. Tissue was analyzed from the following two locations: tumor and histologically normal appearing lung tissue taken from the greatest distance to the tumor. Six-mm frozen sections were taken from blocks of tumor tissue and, starting with the first section, every fifth one was routinely stained with H&E and evaluated by a pathologist (S.E.B.). Only areas of estimated 75% malignant cells were pooled for the analysis.

RNA extraction and cDNA synthesis. Total cellular RNA was isolated using Trizol reagent (Life Technologies/GIBCO, Grand Island, NY, USA) and quantitated at A260/280 nm (Smart Spec; Biorad, Hercules, CA, USA). After RNA isolation, cDNA was prepared from each sample as described previously (11).

Real-time polymerase chain reaction quantification. Primers and probes for Gadd45 were designed by U. W-E using the Primer Express® Software v2.0 (Applied Biosystems, Darmstadt, Germany) and were purchased from Eurogentec Deutschland GmbH (Cologne, Germany). The DNA sequences are listed in Table I.

The quantification of Gadd45 and an internal reference gene (β-actin) was done using a fluorescence-based real-time detection method (ABI PRISM 7900 Sequence Detection System [Taqman], Applied Biosystems) as previously reported (12). Briefly, the PCR reaction mixture contained 300 nM of each primer, 200 nM probe in a final volume of 20 ml. The PCR conditions were 50˚C for 2 min, 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and 60˚C for 1 min. Gene expression levels were calculated using standard curves generated by serial dilutions of placental cDNA (BD Biosciences Clontech Lab., Palo Alto, USA). All reactions were done in triplicate.

Statistical analysis. The Gadd45 mRNA expression value was expressed as a ratio between two absolute measurements: Gadd45 and the internal reference gene, β-actin. Associations between gene expression levels of Gadd45 expression in tumor and histologically normal appearing tissue were tested by using the Wilcoxon signed rank test. The associations of gene expression levels of Gadd45 in tumor and histologically normal appearing tissue and their ratio (tumor:normal) with clinicopathological variables were tested using the Kruskal-Wallis test. To assess the associations of the expression levels of Gadd45 in tumor tissue, histologically normal appearing tissue and their ratio (tumor:normal) with overall survival, the expression level was categorized into a low and a high value at optimal cut-off points for all patients and for males and females separately. The maximal χ^2 method of Miller, Siegmund and Halpern was adapted to determine which gene expression (optimal cut-off) best segregated patients into poor and good prognostic subgroups (in terms of likelihood of survival) (13, 14). To determine a *p*-value that could be interpreted as a measure of the strength of the association based on the maximal χ^2 analysis, 2000 bootstrap-like simulations were used to estimate the distribution of the maximal χ^2 statistics under the null hypothesis of no association. The corrected *p* value was calculated as the proportion of the 2000 simulated maximal χ^2 statistics that was greater than the original maximal χ^2.

The overall survival time was calculated as the period from the day of operation until death from any cause or until the date of the last follow-up which was at least 5 years for all surviving patients. The associations between demographic and baseline clinical factors and overall survival were analyzed individually using the log-rank tests.

All reported *p* values were two-sided. All analyses were performed using the SPSS statistical package version 12.0.

Table I. *PCR primers and probes for the analysis of mRNA levels.*

Gadd45 primers FP: 5'-AGT CAG CGC ACG ATC ACT GT- 3'
Gadd45 primers RP: 5'-GGA TCA GGG TGA AGT GGA TCT G-3'
Gadd45 probe 6FAM (carboxyfluorescein)5'-AAG CTG CTC AAC GTC GAC CCC GAT AAC -3'TAMRA

Results

Gadd45 mRNA expression in tumor and histologically normal appearing lung tissues and its correlation to clinicopathological factors in patients with NSCLC. Gadd45 mRNA expression was detectable by quantitative real-time PCR in 66 out of 66 tumor tissues and in 66 out of 66 histologically normal appearing tissues. The median gene expression level of Gadd45 in tumor tissue was 2.36 (range, 0.38-779.64), while in histologically normal appearing tissue it was 26.34 (range, 0.96-948.65). The median Gadd45 ratio between tumor and histologically normal appearing tissue was 0.09 (range, 0.001-209.53). The intratumoral expression levels of Gadd45 were significantly lower than in matching histologically normal appearing lung tissue ($p<0.001$, Wilcoxon test).

The associations between patient clinicopathological data are shown in Table II. High intratumoral Gadd45 expression

Table II. *Association between gene expression levels of Gadd45 and clinicopathological variables.*

		Gadd45 tumoral		Gadd45 normal		Gadd45 ratio (tumor:normal)	
Factors	No.	median (range)	*p*-value	median (range)	*p*-value	median (range)	*p*-value
All patients	66	2.36 (0.38–779.64)		26.34 (0.96–948.65)		0.09 (0.001–209.53)	
Gender							
Male	54	2.323 (0.383-779.64)	0.464	26.34 (0.963-926.03)	0.642	0.091 (0.001-209.53)	0.19
Female	12	5.203 (0.632-35.26)		17.88 (3.7-948.65)		0.226 (0.001-4.373)	
Smoking							
Non-smoker	8	3.63 (0.7-14.03)	0.582	29.36 (7.193-167.68)	0.455	0.064 (0.031-1.951)	0.96
Smoker	58	2.26 (0.383-779.64)		23.068 (0.963-948.65)		0.126 (0.001-209.53)	
Stage							
1	33	2.751 (0.383-111.55)	0.46	25.63 (0.963-926.03)	0.702	0.113 (0.001-115.73)	0.89
2	13	3.085 (0.632-35.4)		46.88 (0.986-821.59)		0.17 (0.003-4.373)	
3A	20	1.695 (0.4-779.64)		21.45 (0.986-948.65)		0.064 (0.001-209.53)	
Histology							
Squamous	35	2.215 (0.4-779.64)	0.835	18.81 (0.986-821.59)	0.49	0.113 (0.001-209.53)	0.76
Adeno	21	2.853 (0.383-111.55)		46.88 (0.963-948.65)		0.081 (0.001-115.73)	
Large Cell	10	2.722 (0.879-16.19)		20.22 (1.42-167.68)		0.135 (0.013-4.373)	
Grade							
Mod. diff.	14	1.3 (0.383-111.55)	0.041	6.312 (0.963-948.65)	0.11	0.165 (0.001-115.73)	0.92
Poorly diff.	52	2.87 (0.4-779.64)		29.77 (0.986-926.03)		0.094 (0.001-209.53)	

was significantly associated with a poorer histological grading ($p<0.041$; Kruskal-Wallis test).

Association of clinicopathological factors and Gadd45 mRNA expression levels in tumor and histologically normal appearing lung tissue with survival of patients with NSCLC. With a median follow-up of 85.9 months for the 66 patients analyzed in this study, the median survival was 53.8 (range, 3.8-101.7) months. To determine whether there was any prognostic significance associated with quantitative differences in Gadd45 mRNA expression levels, the maximal χ^2 method was adapted to determine which Gadd45 expression level best segregated patients into poor and good prognostic subgroups (13, 14). Using this method, it was not possible to identify an adequate prognostic cut-off value for Gadd45 mRNA expression. In addition, when using the median Gadd45 expression values of each tissue group or their ratio, no statistically significant differences in terms of overall survival were detected between the groups using the log-rank test (data not shown). Finally, univariate and multivariate analyses displayed the UICC tumor stage and the pT- and pN-categories as independent prognostic factors, as already reported (10).

Discussion

This was a comprehensive study of the mRNA expression of Gadd45 in 66 patients with curatively resected NSCLC. It was demonstrated that down-regulation of the mRNA expression of Gadd45 in tumor compared with matching histologically normal appearing lung tissue was a frequent event. Furthermore, an association between the intratumoral Gadd45 mRNA expression and histological grading, suggesting an important role for tumor differentiation, was identified.

There are three members in the family of growth arrest and DNA damage-inducible genes: Gadd45α, Gadd45β and Gadd45γ (15). All Gadd45 members are thought to play a role in the regulation of DNA repair, cell proliferation and apoptosis. Gadd45α is a p53-regulated gene that has been shown to be involved in DNA repair (16). In addition, Zhan *et al.* demonstrated that Gadd45α associates with Cdc2 kinase and disrupts the interaction between Cdc2 and cyclin B1, suggesting the suppression of cell growth by induction of G2/M arrest (17). Furthermore, evidence suggests that Gadd45γ may also induce cell growth arrest by blocking the G1/S transition (18). Finally, Takekawa and Saito demonstrated that the expression of all three Gadd members induced DNA fragmentation *in vitro*, indicating an important role in the regulation of apoptosis (15).

Our observation of impaired expression of Gadd45 in lung cancer tissues suggests a strong dysregulation of these growth arrest and DNA damage-inducible genes in this type of cancer. Similar results were reported by Korabiowska *et al.* analyzing the expression of growth arrest DNA damage genes in oral melanomas (19). In 21 out of 29 oral melanomas a loss of

Gadd45α expression was described, indicating that the loss of Gadd45 is not restricted to lung tumors. Moreover, Zhang *et al.* assessed the mRNA expression of Gadd45γ in human pituitary tumors (20). Although mRNA expression was found in all normal human pituitary tissue samples, only in one out of 18 clinically non-functioning pituitary tumors was it detectable by RT-PCR. In addition, in colony formation assays the study group showed that transfection of human Gadd45γ cDNA into a human pituitary tumor-derived cell line resulted in a dramatic decrease in cell growth. These data and our observations suggest that Gadd45 maintains genomic stability.

The mechanisms leading to an inappropriate expression of Gadd45 in lung cancer are still unknown. Intratumoral down-regulation of Gadd45 can occur through genetic events like mutations or epigenetic inactivation by aberrant methylation of CpG islands. Yamasawa *et al.* recently identified point mutations in over 13% of tumors from 59 patients with invasive ductal carcinomas of the pancreas, suggesting an association between impaired expression of Gadd45 and this genetic event (9). Ying *et al.* demonstrated in multiple tumor cell lines, including lung cancer cells, that Gadd45 expression was frequently disrupted by promoter hypermethylation (21). Therefore, further studies are warranted to determine the underlying mechanisms leading to altered Gadd45 expression in lung tumorigenesis.

It remains unclear whether impaired Gadd45 expression is a cause or a consequence of lung tumorigenesis or merely a surrogate marker. Studies in Gadd45α-deficient mice by Hollander *et al.* exhibited several of the phenotypes characteristic of p53-deficient mice, including genomic instability and increased radiation carcinogenesis (3). In addition, the same group investigated the dimethyl-benzanthracene (DMBA)-induced carcinogenesis in Gadd45α -null mice (22). They found a dramatic increase in the frequency of malignant tumors, a substantially reduced nucleotide excision repair system and a higher mutation rate after DMBA treatment. These data indicate the importance of maintaining genomic stability, suggesting the loss of Gadd45 expression could be an important factor in tumorigenesis.

We also showed a significant association between the intratumoral Gadd45 mRNA expression and histological grading. These findings are consistent with recent studies describing a significant association between Gadd45 expression and clinicopathological parameters. Sengupta *et al.* reported that, in epithelial ovarian cancer, Gadd45 expression was a significant prognostic factor (7). In cervical carcinomas, Santucci *et al.* described that lack of Gadd45α induction after radiotherapy was correlated with good clinical response (8). Recently, Yamasawa *et al.* demonstrated, in patients with invasive ductal carcinomas of the pancreas, that intratumoral overexpression of Gadd45α protein, along with possible p53 loss of function, significantly contributed to poor prognosis (9).

In conclusion, it was shown that decreased expression of Gadd45 is a frequent event in NSCLC. Furthermore, an association was demonstrated between the intratumoral Gadd45 mRNA expression and histological grading, suggesting an important role for Gadd45 in lung tumor differentiation.

References

1 Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ and Thun MJ: Cancer statistics. CA Cancer J Clin *55*: 10-30, 2005.

2 Wang XW, Zhan Q, Coursen JD, Khan MA, Kontny HU, Yu L, Hollander MC, O'Connor PM, Fornace AJ Jr and Harris CC: Gadd45 induction of a G2/M cell cycle checkpoint. Proc Natl Acad Sci USA *96*: 3706-3711, 1999.

3 Hollander MC, Sheikh MS, Bulavin DV, Lundgren K, Augeri-Henmueller L, Shehee R, Molinaro TA, Kim KE, Tolosa E, Ashwell JD, Rosenberg MP, Zhan Q, Fernandez-Salguero PM, Morgan WF, Deng CX and Fornace AJ Jr: Genomic instability in Gadd45α-deficient mice. Nature Genet *23*: 176-184, 1999.

4 Harkin DP, Bean JM, Miklos D, Song YH, Truong VB, Englert C, Christians FC, Ellisen LW, Maheswaran S, Oliner JD and Haber DA: Induction of Gadd45 and JNK/SAPK-dependent apoptosis following inducible expression of BRCA1. Cell *97*: 575-586, 1999.

5 Smith ML, Chen IT and Zhan Q: Interaction of the p53-regulated protein Gadd45 with proliferating cell nuclear antigen. Science *266*: 1376-1380, 1994.

6 Marx J: New link found between p53 and DNA repair. Science *266*: 1321-1322, 1994.

7 Sengupta PS, McGown AT, Bajaj V, Blackhall F, Swindell R, Bromley M, Shanks JH, Ward T, Buckley CH, Reynolds K, Slade RJ and Jayson GC: p53 and related proteins in epithelial ovarian cancer. Eur J Cancer *36*: 2317-2328, 2000.

8 Santucci MA, Barbieri E, Frezza G, Perrone A, Iacurti E, Galuppi A, Salvi F, Bunkeila F, Neri S, Putti C and Babini L: Radiation-induced Gadd45 expression correlates with clinical response to radiotherapy of cervical carcinoma. Int J Radiat Oncol Biol Phys *46*: 411-416, 2000.

9 Yamasawa K, Nio Y, Dong M, Yamaguchi K and Itakura M: Clinicopathological significance of abnormalities in Gadd45 expression and its relationship to p53 in human pancreatic cancer. Clin Cancer Res *8*: 2563-2569, 2002.

10 Schneider PM, Praeuer HW, Stoeltzing O, Boehm J, Manning J, Metzger R, Fink U, Wegerer S, Hoelscher AH and Roth JA: Multiple molecular marker testing (p53, C-Ki-ras, c-erbB-2) improves estimation of prognosis in potentially curative resected non-small cell lung cancer. Br J Cancer *83*: 473-479, 2000.

11 Warnecke-Eberz U, Metzger R, Miyazono F, Baldus SE, Neiss S, Brabender J, Schaefer H, Doerfler W, Bollschweiler E, Dienes HP, Mueller RP, Danenberg PV, Hoelscher AH and Schneider PM: High specificity of quantitative excision repair cross-complementing 1 messenger RNA expression for prediction of minor histopathological response to neoadjuvant radiochemotherapy in esophageal cancer. Clin Canc Res *10*: 3794-3799, 2004.

12 Xi H, Baldus SE, Warnecke-Eberz U, Brabender J, Neiss S, Metzger R, Ling FC, Dienes HP, Bollschweiler E, Moenig S, Mueller RP, Hoelscher AH and Schneider PM: High cyclooxygenase-2 expression following neoadjuvant radiochemotherapy is associated with minor histopathologic response and poor prognosis in esophageal cancer. Clin Cancer Res *11*: 8341-8347, 2005.
13 Miller R and Siegmund D: Maximally selected Chi-square statistics. Bio-metrics *38*: 1011-1016, 1982.
14 Halpern J: Maximally selected Chi-square statistics for small samples. Biometrics *38*: 1017-1023, 1982.
15 Takekawa M and Saito H: A family of stress-inducible Gadd45-like proteins mediate activation of the stress-responsive MTK1/MEKK4/MAPKKK. Cell *95*: 521-530, 1998.
16 Smith ML, Kontny HU, Zhan Q, Sreenath A, O'Connor PM and Fornace AJ Jr : Antisense Gadd45 expression results in decrease DNA repair and sensitizes cells to UV-irradiation or cisplatin. Oncogene *13*: 2255-2263, 1996
17 Zhan Q, Antinore MJ, Wang XW, Wang XW, Carrier F, Smith ML, Harris CC and Fornace AJ Jr: Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene *18*: 2892-2900, 1999.
18 Fan W, Richter G, Cereseto A, Beadling C and Smith KA: Cytokine response gene 6 induces p21 and regulates both cell growth and arrest. Oncogene *18*: 6573-6582, 1999.
19 Korabiowska M, Betke H, Brinck U, Grohmann U, Honig JF and Droese M: Loss of growth arrest DNA damage gene expression in oral melanomas. In Vivo *13*: 483-485, 1999.
20 Zhang X, Sun H, Danila DC, Johnson SR, Zhou Y, Swearingen B and Klibanski A: Loss of expression of Gadd45 gamma, a growth inhibitory gene, in human pituitary adenomas: implications for tumorigenesis. J Clin Endocrinol Metab *87*: 1262-1267, 2002.
21 Ying J, Srivastava G, Hsieh WS, Gao Z, Murray P, Liao SK, Ambinder R and Tao Q: The stress-responsive gene Gadd45γ is a functional tumor suppressor, with its response to environmental stresses frequently disrupted epigenetically in multiple tumors. Clin Cancer Res *11*: 6442-6449, 2005.
22 Hollander MC, Kovalsky O, Salvador JM, Kim KE, Patterson AD, Haines DC and Fornace AJ Jr: Dimethylbenzanthracene carcinogenesis in Gadd45α-null mice is associated with decreased DNA repair and increased mutation frequency. Cancer Res *61*: 2487-2491, 2001.

Received March 15, 2006
Accepted April 14, 2006