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GADD153 is an independent prognostic factor in melanoma: immunohistochemical and molecular genetic analysis

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Summary. The main role of growth arrest and DNA damage-inducible (GADD) genes is to block proliferation at G1 and G2 checkpoints in response to DNA damage. The goal of this study was to examine the expression of GADD genes in primary melanomas with respect to prognosis.

GADD34 was found in 73% of the primary melanomas investigated. GADD45 and GADD153 were positive in 60% and 80% of primary melanomas, respectively. Cox regression demonstrated that only GADD153 had any independent prognostic impact. We therefore decided to establish a PCR assay for detection of GADD153 in paraffin-embedded tissue. GADD153 deletion was found in 3/26 melanomas. None of the 3 cases with GADD153 deletion showed any expression of GADD153. Sequencing analysis detected polymorphism T-C at amino acid position 10 in 6/23 melanomas. In 6 cases with GADD153 polymorphism, GADD153 expression was found in 2 melanomas with a maximum GADD153 index of 10%.

We postulate that the GADD gene family plays an important role in melanoma progression.

Key words: Melanoma, GADD34, GADD45, GADD153

Introduction

The genes GADD7, GADD33, GADD34, GADD45 and GADD153 belong to the GADD (growth arrest and DNA damage-inducible) gene family (Jackman et al., 1994). These genes were isolated from Chinese hamster ovary cells following UV radiation (Fornace et al., 1989). Apart from the UV radiation, a GADD gene expression can be induced by various other factors such as asphyxia, dithiothreitol and various chemotherapeutic agents (Chen et al., 1992, Fornace et al., 1989, Luethy and Holbrook, 1992, Price and Calderwood, 1992).

GADD genes are assumed to have two essential functions within the cell cycle. They suppress cell growth and induce DNA repair (Smith et al., 1994, Zhan et al., 1994). An interruption in the progression of the cell cycle at the G1 and G2 control points in response to genotoxic stress or DNA damage appears to be involved, which presumably happens to allow time for the damaged DNA to be repaired before the S and M phase is entered into. GADD genes are regulated on a transcriptional level in conjunction with a strong posttranscriptional response to DNA damage (Jackman et al., 1994).

Our preliminary reports demonstrated down-regulation of GADD genes in melanoma progression (Korabiowska et al., 1997, 1999). The main aim of this study was to examine the expression of GADD genes in primary melanomas with respect to prognosis.

Multivariate Cox analyses have shown that GADD153 has independent prognostic merit. GADD153 has not been investigated in detail in malignant melanoma. We therefore decided to examine this gene on a molecular genetic level.

Materials and methods

Patients

A well-characterized cohort of 106 primary melanoma patients treated at the teaching hospital of the University of Krakow between 1993 and 1995 was the focus of this study. These cases were selected based on the availability of follow up and the histological slides have been re-examined. Patients' age ranged between 12 and 95 years, averaging 56 years. Twenty-four of the tumors were superficially spreading melanomas and 82 represented nodular melanomas. The anatomical localization of these tumors was distributed as follows:

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head and neck (n=17), trunk (n=46), upper extremities (n=21), lower extremities (n=22). Seventeen tumors were Clark I tumors, 6 Clark II, 20 Clark III, 26 Clark IV and 37 Clark V. Tumor thickness varied between 0.12 mm and 8.4 mm, averaging 2.03 mm. All patients were monitored from the time of diagnosis up to the end of the study (January 1, 2000) (41 patients alive and 65 death). Data on the clinical course of the patients were collected from the attending physicians The median follow up time was 22 months.

Immunohistochemistry

To demonstrate GADD genes, the following antibodies were used:

a) S20, rabbit polyclonal antibody raised against a peptide corresponding to amino acids 719-738, mapping to the carboxyl terminus of GADD34 of human origin (Santa Cruz Biotechnology Inc. Heidelberg, Germany)

b) C20, rabbit polyclonal antibody raised against a peptide corresponding to amino acids 146-165, mapping to the carboxyl terminus of GADD45 of human origin (Santa Cruz Biotechnology Inc. Heidelberg, Germany)

c) R20, rabbit polyclonal antibody raised against a peptide of 20 amino acids in length, mapping at the carboxyl terminus of GADD153 of human origin (Santa Cruz Biotechnology Inc. Heidelberg, Germany)

The reaction product was demonstrated using the Novostatin Super ABC kit NCL/ABCp from Novocastra (Newcastle upon Tyne, Great Britain) and AEC as chromogen. Microwave pre-treatment was only necessary in the GADD45 reaction (2x5 min, pH=6.0, citrate buffer, 750W). In all reactions, the sections were incubated with primary antibodies overnight at 4 °C. The control reactions were conducted without primary antibodies. Epidermis and sweat gland were used as positive controls for the GADD gene family. The results were presented as percentages of positive cells (indices) and were quantified by CAS200 (Becton Dickinson, Hamburg, Germany) image analysis. Immunohistochemical evaluation was performed by at least 2 independent investigators (M.K. and H.B.). Each observer determined the percentage of tumor cells displaying nuclear immunolabeling reactivity in 10 high-power fields (areas measured at x400 magnification) per histological specimen. Minor quantitative differences in the interobserver results (mostly below 5%) allowed us to use means of the results for statistical analysis.

DNA isolation

The histological material was cut and placed into sterile Eppendorf microfuge tubes. After washing once with xylene and twice with ethanol (96%) to remove the xylene residue, cells and cellular debris were obtained by centrifugation at 500 rpm at 4 °C. DNA was isolated from the samples with QIAamp DNA Mini Kit (Qiagen, Hilden, Germany).

Analysis of GADD153 Gene Deletion by Multiplex DNA PCR

MDM2 DNA and GADD153 DNA were co-amplified using primer pairs 5'-GTGTGATTTGTCAA GGTCGA-3' (MDM2, sense), 5'-ATTGGTTGTCTAC ATACT-3' (MDM2, anti-sense), 5'-AGATGTGCTTTTC CAGACTG-3' (GADD153, sense) and 5´-TCCAGGAG GTGAAACATAG-3' (GADD153, anti-sense). The MDM2 primers yield a 133 bp product. GADD153 primers yield a 160 base-pair product. In each run, test tubes were heated to 95 °C for 7 min, followed by 40 cycles at 95 °C for 45 sec, 58 °C for 45 sec, and 72 °C for 45 sec with a final extension at 72 °C for 7 min. PCR mix consisted of 10x PCR buffer (Pharmacia, Freiburg, Germany), 0.3 g primer, 10 uM of each dNTP (Pharmacia, Freiburg, Germany) and 1 unit of Taq DNA polymerase (Pharmacia, Freiburg, Germany). The final volume was 50 μl. All PCR mixtures were coated with mineral oil (Sigma, Munich, Germany). Thermal cycling was performed on a DNA thermal cycler 480 (Perkin-Elmer, Weiterstadt, Germany) using thin-walled reaction tubes. Resulting PCR products were separated for 2 hours at 80 V on a 3% (wt/v) agarose gel (Biozym, Hessisch Oldendorf, Germany) containing 0.5 μg ethidium bromide per ml. Negative controls were performed with each PCR run.

Sequencing of PCR fragments

Amplification products were purified with the QIA Quick PCR Purification Kit (Qiagen, Hilden, Germany). DNA was labeled with the PRISM - Ready Reaction Dye Deoxy - TM Terminator Cycle Sequencing Kit (Applied Biosystems, Weiterstadt, Germany) and analyzed in an Applied Biosystems DNA sequencer model 310.

Statistics

Data processing

The data were analyzed using the Statistical Analysis System (SAS, Version 7.5) on an IBM-compatible PC under Windows NT 4.0 and had been previously scanned into the spread sheet (Microsoft Excel 97), where they were made available to the statistics program via an ODBC (open database connectivity).

Analytical strategy

In general, all indices-related data were rank scaled, i.e. no means or standard deviations were calculated since indices are not metrical data per se. The median and the other percentiles were calculated for all expression indices. Mann-Whitney-U tests were used for the group comparisons. The relation between markers was investigated by applying the correlation coefficient according to Spearman.

The Kaplan-Meier method was employed to calculate the survival rates (Kaplan and Meier, 1958). The significance of the difference in the survival curves was calculated using the log-rank tests. Cox regression was the multivariate method used for predicting the survival rate based on several parameters (Cox, 1972). This method estimates the regression coefficients that make it possible to form a prediction equation. The first step of this method tests all coefficients globally (chi^{2}-distributed statistics) to see if at least one of the estimated coefficients deviates from zero. Then, each regression coefficient is tested individually for significance by means of the statistical method of Wald.

Additionally, the probability of survival was started at the 95% confidence interval. This parameter is often called the relative risk. When modeling the data using Cox regression a differentiation was made as to whether the parameters were to be considered as constant or as categorical (e.g. Clark levels differentiating between 5 levels of invasion). Categorical parameters were coded by assessing the significance of deviation contrasts in relation to the data of the highest category. The interpretation of probability of survival must take into account the categories of each parameter. Different parameters require different types of categorization. Thus, whenever the level of an individual parameter changes, a change in the probability of survival has to be considered in relation to the respective parameter.

The data set was modeled using the methods offered by the SPSS forward selection and backward selection. The first method adds parameters to a survival prediction model until the exclusion criterion is reached. In contrast, backward selection eliminates parameters from the model until the inclusion criterion is reached. The terminate criteria (for inclusion or exclusion) was the likelihood ratio based on partial likelihood estimators.

Results

GADD expression in primary melanomas

GADD34 was found in 73% of primary melanomas investigated. The GADD34 index ranged between 0 and 94%. GADD45 was positive in 60% of primary melanomas where the GADD45 index ranged between 0 and 97%. GADD153 was expressed in 80% of melanomas and the GADD153 index did not exceed 96% (Fig. 1) (Table 1).

Clark I

GADD34 was positive in 94% of Clark I melanomas where the GADD34 index did not exceed 94%. GADD45 was found in 76% of Clark I melanomas and the index values ranged between 0 and 97%. GADD153 was present in all Clark I melanomas and the GADD153 index was not higher than 96% (Table 2).

Clark II

All Clark II tumors showed expression of GADD34 and GADD45. The GADD34 index fluctuated between 11 and 70%. The GADD45 index ranged between 7 and 74%. GADD153 was found in 83% of tumors and the GADD153 index did not exceed 81% (Table 2).

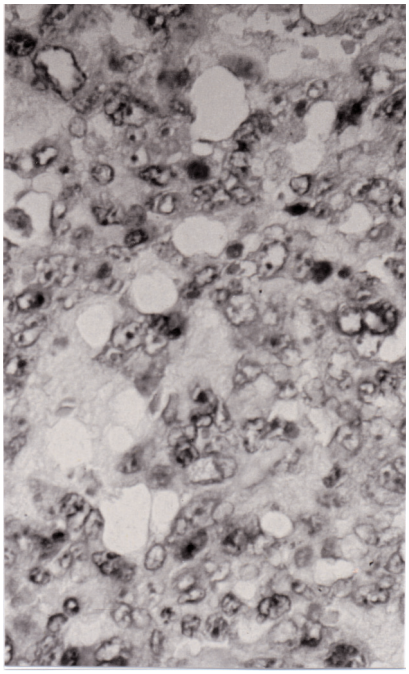


Fig. 1. GADD153-positive nuclei of Clark V melanoma. A similar nuclear reaction was found for GADD45 and GADD34. x 100

Table 1. Expression of GADD genes in prmary melanomas.

MARKER	PERCENTAGE OF POSITIVE CASES	PERCENTAGE OF POSITIVE CELLS				
		Min.	25th Perc	Median	75th Perc	Max.
GADD34	73	0	0	20	62	94
GADD45	60	0	0	12	51	97
GADD153	80	0	0	30	70	96

Table 2. Percentage of marker positive cases in different clark levels.

CLARK LEVEL	MARKER		
	GADD34	GADD45	GADD153
I	94%	76%	100%
II	100%	100%	83%
III	35%	55%	90%
IV	88%	73%	85%
V	68%	41%	70%

Clark III

GADD34 was found in 35% of Clark III melanomas where the GADD34 index did not exceed 85%. GADD45-positive cells were present in 55% of Clark III melanomas where the GADD45 index ranged between 0 and 74%. GADD153 was positive in 90% of Clark III melanomas where the GADD153 index reached a maximum of 83% (Table 2).

Clark IV

GADD34 was present in 88% of Clark IV melanomas. The GADD34 index ranged between 0 and 92%. GADD45 was positive in 73% of Clark IV melanomas where the GADD45 index did not exceed 83%. GADD 153 was expressed in 85% of Clark IV melanomas where the GADD153 index fluctuated between 0 and 84% (Table 2).

Clark V

GADD34 was positive in 68% of Clark V melanomas where the GADD34 index did not exceed 81%. GADD45 was found in 41% of these cases. The GADD45 index ranged between 0 and 62%. GADD153 was found in 70% of Clark V melanomas where the GADD153 index reached a maximum of 95% (Table 2).

Comparison of CLARK levels

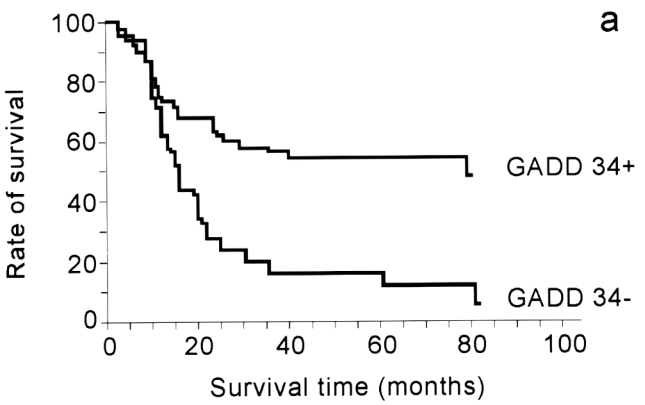
GADD34

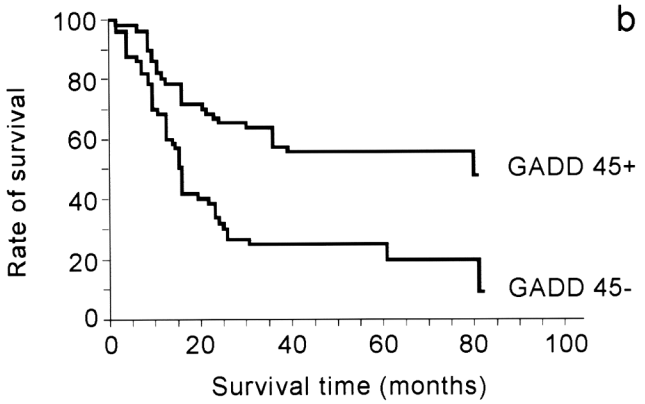
The GADD34 index differed significantly between Clark I and Clark II melanomas (p=0.0421) as well as between Clark III and Clark IV melanomas (p=0.0394).

Table 3. Spearman's correlation between the indices of GADD genes in primary melanomas.

	GADD34	GADD45	GADD153
GADD34	# XX	XX	
GADD45	XX	#	XX
GADD153	XX	XX	#

x: correlation with r>0.6 and p<0.05; XX: correlation with r>0.7 and p<0.01





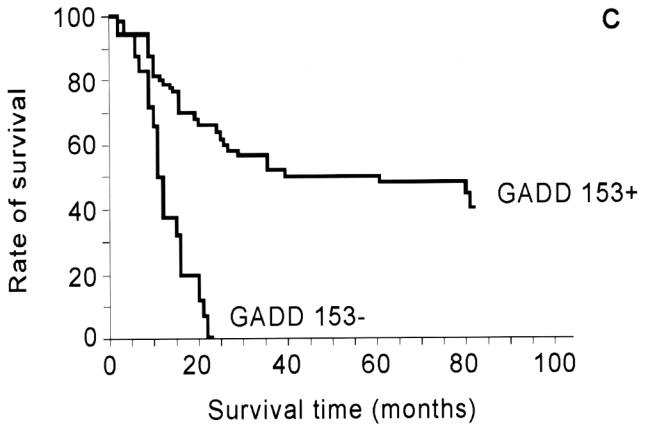


Fig. 2.a. Kaplan-Meier survival curves of GADD34-positive (n=77) and negative (n=29) melanomas. The difference between survival curves was significant (p<0.05). **b.** Kaplan-Meier survival curves of GADD45-positive (n=63) and negative (n=43) melanomas. The difference between survival curves was significant (p<0.05). **c.** Kaplan-Meier survival curves of GADD153-positive (n=84) and negative (n=22) melanomas. The difference between survival curves was significant (p<0.05).

GADD45

A significant difference in the GADD45 expression was found between Clark IV and Clark V melanomas only (p=0.0102).

GADD153

A significant difference in the GADD153 index was found between Clark I and Clark II melanomas only (p=0.0116).

Correlation of expression among GADD genes

All GADD genes showed a highly significant correlation with each other (Table 3).

Prognostic significance (Kaplan-Meyer Survival Curves)

GADD34

54% of GADD34-positive and 16% of GADD34-negative melanoma patients survived 5 years. This difference was highly significant (p=0.0001) (Fig. 2a).

GADD45

55% of GADD45-positive and 25% of GADD45-negative patients survived more than 5 years. This difference was also highly significant (p=0.0001) (Fig. 2b).

GADD153

50% of patients with GADD153-positive and none with GADD153-negative melanomas survived more than 5 years. Even this difference was of great significance (Fig. 2c).

Cox regression model

Multivariate analyses with Cox regression included: GADD34 index, GADD45 index, GADD153 index and tumour thickness according to Breslow. Results demonstrated that only GADD153 and tumor thickness according to Breslow had any independent prognostic impact (same results as in backward and forward regression) (Table 4).

Analysis of GADD153 deletion

It was possible to isolate DNA from 26 MDM2-positive melanomas. GADD153, analyzed by multiplex PCR, was deleted in 3 out of 26 cases (Fig. 3). Sequencing analysis revealed polymorphism T-C at amino acid position 10 in 6 out of 23 cases (Fig. 4).

None of the 3 melanomas with GADD153 deletion showed any expression of GADD153. Among 6 cases with GADD153 polymorphism, GADD153 expression was found in 2 tumors with a maximum GADD153 index of 10%.

Table 4. Results of multivariate Cox analysis.

FACTOR	P VALUE	COEFFICIENT IN REGRESSION	HAZARD RATIO	CONFIDENCE LIMITS	
				lower	upper
GADD153	0.0024	-0.02	0.982	-0.6%	2.9%
Breslow	0.048	-0.07	0.76	-42%	0%

Discussion

GADD genes represent a unique family of genes that work concurrently to block proliferation and initiate the DNA repair process. We investigated the presence of 3 members of GADD family (GADD34, GADD45 and GADD153) in paraffin-embedded malignant melanomas with known follow up. Considering the very close functional connections between members of the GADD gene family, highly significant correlations between them are hardly surprising.

GADD153 was the only gene of the GADD family with an independent prognostic influence on melanoma patients in Cox regression model. The GADD153 gene is a homologue of CHOP 10 (Chinese hamster ovary cell protein) and codes nuclear protein with a molecular weight of about 28 kDa (Ron and Habener, 1992). The function of GADD153 is to induce G1-S arrest, as was shown in cultivated fibroblasts (Friedman, 1996). The

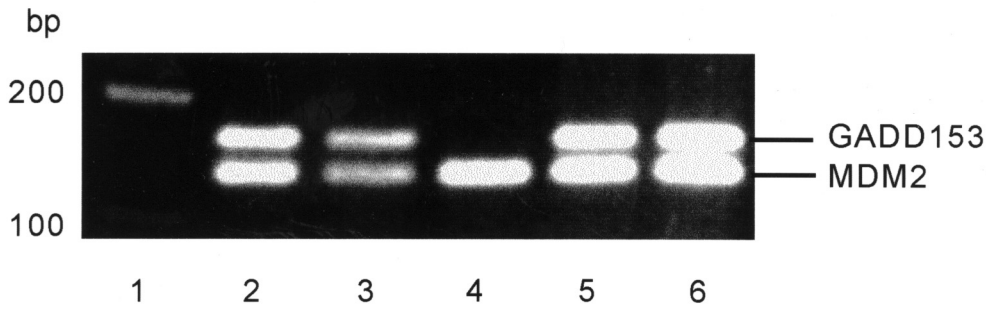


Fig. 3. Detection of PCR-fragments resulting from GADD153 (upper lane) and MDM2 (lower lane) on 3% agarose gel. The size of the markers is shown on the left. GADD153 was found in 4 samples (lanes 2,3,5,6). A good example of GADD153 deletion can be seen in lane 4.

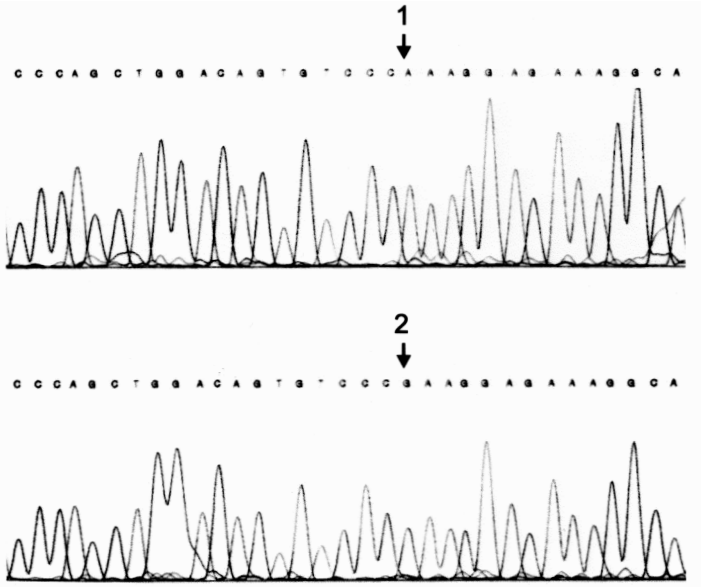


Fig. 4. Sequencing analysis demonstrated polymorphism T-C at amino acid position 10. 1: normal sequence, 2: sequence with polymorphism. (Both 1 and 2 with antisense primers).

question is, why is GADD153 so important in melanoma prognosis?

Despite the important role of the GADD153 gene in malignant tumorigenesis, especially in oral tumors (Gujuluva at al., 1994) and in liposarcomas (Crozat et al., 1993), mutations and polymorphic variants of this gene in malignant melanomas have not been reported previously. The gene alteration we found occurred in about 25% of tumors. Interestingly, we have found loss of GADD153 expression in 3 cases with GADD153 deletion. Furthermore, 4 out of 6 melanomas with reported polymorphism did not show any expression of GADD153. Immunonegativity of melanomas with GADD153 deletion and polymorphism could suggest that genetic abnormalities may be partly responsible for loss of GADD153 expression on the protein level.

GADD153 seems to play an important role not only in melanoma biology but also with regard to other tumors. For example, in gastric cancer GADD153 modulates the sensitivity to certain anticancer agents by activating the AP-1-associated signal transduction pathway leading to apoptosis (Kim et al., 1999). GADD153 and GADD45 were reported to contribute to growth arrest or protection from metabolic damage during poor glutamine conditions (Abcouwer et al., 1999).

It is not yet clear whether GADD45 plays a direct or indirect role in DNA repair. GADD45 has the ability to form a complex with PCNA as well as with the DNA polymerase delta (Shivji et al., 1992; Xiong et al., 1993; Zhang et al., 1993; Smith et al., 1994). GADD45 has a similar structure with cDNA clone MyD118 (Abdollahi et al., 1991). GADD34 was discovered as a homologue of MyD116 gene (Jackmann et al., 1994). The experiments of Lord et al. (1990) demonstrated that MyD genes can induce terminal differentiation of M1-myeloblastic leukemia cells and their proliferation can block and induce apoptosis. Although the exact function of GADD34 and GADD45 is unclear, one can speculate that in malignant melanomas properly functioning GADD34 and GADD45 genes are able to block proliferation and to induce apoptosis.

For sufficient DNA repair to be accomplished, proliferation must be blocked at the G1 and G2 check points. Gujuluva et al. (1994) demonstrated that normal cells react to UV radiation by increasing the gene transcription for GADD45 and GADD153 genes. In contrast, malignant cells were not able to increase the production of GADD transcripts. This might be an indirect result of defects in the genes encoding the GADD family and the inability of malignant cells to protect against UV radiation. This factor is especially important in malignant melanoma since this tumor is induced by UV radiation (Kopf et al., 1984). Therefore, we postulate a very important protective role for the GADD gene family in melanoma progression.

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