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Quantitative Analysis of Ku70 and Ku80 mRNA Gene Expression in Melanoma Brain Metastases. Correlation with Immunohistochemistry and *In Situ* Hybridization

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Abstract. *Background: Altered expression and prognostic significance of DNA double-strand repair genes Ku70 and Ku80 has been shown by our research group for malignant melanomas. High genomic instability known for melanoma brain metastases stimulated us to analyze Ku70 and Ku80 expression in melanoma brain metastases. Materials and Methods: Quantitative evaluation of mRNA Ku70 and Ku80 expression was performed in 13 melanoma brain metastases. Immunohistochemistry and in situ hybridization for Ku70 and Ku80 were applied to 34 metastatic tumours. Results: Quantitative analysis of Ku70 mRNA expression demonstrated values between 0.01 and 0.33. Ku80 mRNA expression ranged between 0.001 and 0.54. Immunohistochemistry demonstrated Ku70 and Ku80 expression in 34 and in 25 metastatic tumours, respectively. In situ hybridization detected Ku70 expression in 19/34 and Ku80 expression in 13/34 metastatic tumours. Correlation between Ku70 and Ku80 expression in melanoma brain metastases was lost. Conclusion: Ku70 and especially Ku80 expression is altered in melanoma brain metastases and corresponds with the high genomic instability of these lesions.*

The incidence of brain metastases increases with age. The most common primary source of brain metastases are carcinoma of the respiratory tract and breast as well as malignant melanomas. The histological and immunohistochemical profile of metastatic melanomas is similar to those of primary tumours. Longer survival of patients with melanoma brain metastases is associated with younger age and high Karnofsky score. The progression of primary tumour, the number and locations of brain metastases as well as sensitivity of tumour to therapy can also have influence on longer survival. Metastatic malignant melanomas form multiple lesions, resulting in a very severe clinical picture (1-3). Because of this very severe clinical picture of melanoma brain metastases, it is important to evaluate the expression of DNA repair genes, as for example Ku70 and Ku80 genes, which could influence the results of therapy.

It is clear, that Ku70 and Ku80 genes are involved in non-homologous end-joining and are active during G1- and early S-phase (4-6). Ku is a heterodimer composed of 69- and 83-kD polypeptides. The heterodimeric Ku protein is the DNA-targeting component of DNA-dependent protein kinase, which plays a critical role in mammalian DNA double-strand breaks repair. In DNA repair, Ku is able to stabilize the broken DNA ends and bring them together for ligation. Ku has been also reported to take part in DNA replication, apoptotic signaling and telomere maintenance.

Our research group was able to demonstrate the down-regulation and prognostic significance of DNA double-strand repair genes Ku70 and Ku80 for primary malignant melanomas (7-9). The high malignancy of melanoma brain metastases and our previous results in primary melanocytic tumours stimulated us to analyze Ku70 and Ku80 expression in metastatic melanoma cells in the brain.

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Materials and Methods

The material investigated was taken from 34 patients with melanoma brain metastases: 16 women and 18 men, aged between 38 and 71 years. The patients were treated in the Neurosurgical Departments of the Universities of Göttingen and of the Saarland Germany.

The sites of the metastases were; frontal lobe – 18; temporal lobe – 4; vertebral canal – 7; frontal and temporal lobe – 5. For 13 patients frozen material was available and high quality mRNA for quantitative mRNA expression analysis could be isolated.

For 12 patients primary melanoma tumours were available and they were located in the head and neck area- 4 tumours, trunk- 3 and lower, 1 and upper extremities, 4. Five of the primary tumours represented pT3 stadium and 7 of them were classified as pT4.

RNA extraction and RT-PCR for Ku70 and Ku80. RNA was isolated from cryopreserved tissue samples by applying the RNeasy Mini Kit (Qiagen, Hilden, Germany), concentrated by vacuum centrifugation, and subsequently suspended in 10 μl of water. Oligonucleotide primers for RT-PCR were synthesized using GIBCO BRL (Life Technologies Eggenstein, Germany). First-strand cDNA synthesis of β-actin, Ku70 and Ku80 transcripts were performed with RNAse inhibitor, reverse transcriptase and random hexamers (all from Perkin Elmer, Weiterstadt, Germany) according to the manufacturer's instructions, by applying 1 μg mRNA. The standard PCR mix contained 10xPCR buffer (Pharmacia, Freiburg, Germany; 500 mM KCl, 100 mM Tris-HCl, pH 8.3) 0.3 μg primer and 10 μm of each dNTP (Pharmacia, Freiburg, Germany). The final volume was 50 μl. The mixture was overlaid with mineral oil (Sigma, Munich, Germany). Thermal cycling was performed on a DNA thermal cycler 480 (Perkin-Elmer) using thin-walled reaction tubes.

Standard precautions against contamination were taken. The following PCR primers were applied to amplify the β-actin sequence: 5'-CTACAATGAAGCTGCGTGTGGC-3'(sense primer) and 5'-CAGGTCCAGACGCAGGATGGC-3'(antisense primer) yielding a 240 base-pair product. The Ku70 sequence was amplified using primers with the following sequence: (sense T7 primer): 5'-GGATCCTAATACGACTCACTATAGGGAGGCATG GCAACTCCAGAGCAG–3'; (antisense T3 primer): 5'-ATTAA CCCTCACTAAAGGGAGCTCCTTAAACTCATCCACC–3'. For amplification of Ku80 the primers with the following sequence were used: (sense T7 primer): 5'-GGATCCTAATACGACTCACTA TAGGGAGAGAAGAAGGCCAGCTTTGAG–3', (antisense T3 primer):5'ATTAACCCTCACTAAAGGGAAGCTGTGACAGAA CTTCCAG–3.

The first run of β-actin, Ku70 and Ku80 PCR was performed as published previously. Once to 2 μl of the first-run PCR product were used for the second run of nested Ku70 and Ku80 PCR. For amplification of first-run Ku70 and Ku80 DNA, reaction tubes were heated to 95˚C for 5 min followed by 30 cycles at 95˚C for 45 sec, 62˚C for 1 min, and final extension at 72˚C for 7 min. A positive control cDNA generated from the Hela 60 cell-line was performed along with each amplification procedure. Amplified DNA was separated for 2 h at 80 V on a 3% agarose gel (Biozym, Hessisch Oldendorf, Germany) containing 0.5 μg ethidium bromide per ml.

PCR product cloning. The Ku70 and Ku80 riboprobes for *in situ* hybridization were generated by cloning second-round nested PCR products into the pCR4-TOPO vector using the TOPO TA cloning kit for sequencing (Invitrogen, Groningen, Netherlands) according to the manufacturer's instructions. Correct cloning of Ku70- and Ku80-specific PCR fragments was confirmed by cycle-sequencing with the Rhodamine-dye-Terminator Kit using an ABI 310-Sequencer according to the manufacturer's instructions (Perkin-Elmer). Riboprobes were generated by *in vitro* transcription using T3- or T7-polymerase (Boehringer, Mannheim, Germany). The amount of cRNA transcripts was monitored by 1% agarose gel electrophoresis and determined by spectrophotometry.

In situ hybridization. For *in situ* hybridization, paraffin sections were mounted on polylysine-coated slides under RNAse-free conditions. Slides were dried overnight at 37˚C.

Digoxigenin-labeled cRNA probes were prepared using a kit according to the manufacturer's instructions (Boehringer, Mannheim, Germany). In brief, plasmids were linearized with NotI or HindIII and digoxigenin-11-dUTP-labeled antisense; sense probes were generated by *in vitro* transcription using T3- or T7-polymerase (Boehringer). The amount of transcripts was monitored by 1% agarose gel electrophoresis. Labeling efficiency was controlled by dot blot analysis of serial probe dilutions.

In situ hybridization was performed according to the method described by Breitschopf *et al.* (10). In brief, tissue sections were deparaffinized, rehydrated in serial dilutions of ethanol, and postfixed in 4% TBS-buffered paraformaldehyde. Samples were permeabilized using proteinase K (10 μg/ml) at 37˚C for 30 min. Digestion was stopped by washing the samples in phosphate-buffered saline (pH 7.4). The samples were then dehydrated in serial dilutions of ethanol. Digoxigenine-labeled riboprobes were diluted in hybridization buffer (Amersham, Brunswick, Germany). After sense or antisense probes had been applied, the samples were covered with sterile coverslips and placed on a hotplate at 85˚C for 5 min in order to denaturize the probe. Hybridization was performed overnight at 45˚C in a sealed, humidified chamber containing 50% formamide. Non-specific binding or unbound probes were removed in subsequent post-hybridization washes: 1xSSC/0.1%SDS at room temperature (2 x 5 min) and 0.2xSSC/0.1%SDS at 60˚C (2 x 10 min) followed by RNAse digestion (20 μg/ml, Gibco, Karlsruhe, Germany) at 37˚C for 30 min. Finally, sections were washed in TBS containing 0.1% Tween-20 (Boehringer).

Hybridization signals were detected using a sheep polyclonal antibody F(ab)2 fragment against digoxigenin conjugated with horseradish peroxidase (1:300, Boehringer). After washing in TBST, the slides were incubated in streptavidin-HRP complex (diluted 1:100 in TNB) for an additional 30 min. Three washes with TBS were followed by a 15-min application of biotin-tyramide diluted 1:50 in amplification diluent (NEN). Reactive sites were detected with streptavidin-HRP (1:100 in TBS, 30 min, RT). Peroxidase activity was visualized by using diaminobenzidine (DAB) as chromogen. Sense riboprobes served as controls for each tissue section. Normal skin structures were used as positive controls. The CAS200 image analyzer (Becton-Dickinson, Hamburg, Germany) was used to quantify the tumor cells with positive staining; the results were expressed as percentages of positive cells (indices).

Immunohistochemical analysis. Immunohistochemistry was performed by using 5-μm sections which were deparaffinized in xylene (3 times for 5 min) and rehydrated in decreasing concentrations of ethanol (100%, 96%; 2 times, both for 10 min)

followed by washing in deionized H_2O for one minute. To unmask the Ku antigens, the slides were covered with 0.01M sodium citrate buffer (pH 6.0) and placed on a hotplate (95˚C) for 10 min. After cooling, the specimens were rinsed briefly in deionized H_2O (3 times). Specific primary (goat polyclonal) antibodies (Ku-70-M19; Ku-80-M20; Santa Cruz Biotechnology, Santa Cruz, USA) were applied in a dilution of 1:50 overnight at 4˚C. Both primary antibodies applied were directed against an epitope mapping at the carboxy terminus of the Ku 70 and Ku80 proteins. Reagents I-V used on the second day were supplied in the ImmunoCruz Staining System (Santa Cruz Biotechnology, Santa Cruz, USA). Each of these reagents was prediluted and ready to use at room temperature.

After extensive washing with 0.02M Tris/phosphate buffer (TPBS, pH 7.2), the immunoreactivity was detected with a biotinylated secondary antibody [I] by incubating the specimens for 30 min at room temperature. Slides were rinsed with TPBS for 5 min before a horseradish peroxidase (HRP)-streptavidin complex [II] was added for 30 min. Washing in TPBS for 5 min followed. Subsequently, colour was developed by using an HRP substrate (mixture of 1.6 ml deionized H_2O, 250 µl 10x substrate buffer [III], 50 µl 50x DAB chromogen [IV] and 50 µl 50x peroxidase substrate [V]) which was applied to the sections until light brown staining was visible (approx. 10 min). Samples were washed again in deionized H_2O, then counterstained with hematoxylin, dehydrated (increasing concentrations of ethanol: 96% and 100%, followed by xylene, each 2 times for 10 sec) and mounted in DePeX (Merck, Whitehouse Station, USA). The primary antibodies were omitted for negative control staining. The results were presented as percentages of positive cells (indices) and were quantified by CAS200 (Becton-Dickinson, Hamburg, Germany) image analyzer. Immunohistochemical specimens were evaluated by 2 independent observers (MK, FK). Minor quantitative differences in the interobserver results (mostly below 5%) allowed us to use means of the results for statistical analysis.

Quantification of β-actin, Ku70 and Ku80 mRNA expression using real-time RT-PCR. Reverse transcription was performed in a total volume of 20 µl reaction mixture containing 1 µg RNA, 200 units Superscript II RNase H-reverse transcriptase (Invitrogen Cop., Karlsruhe, Germany), 4 µl 5 x first-strand buffer [250 mMTris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$] 2 µl DTT solution (0.1 M), dNTPs at a concentration of 500 µM each (Roche Molecular Biochemicals, Mannheim, Germany), 3 µg random primers (Invitrogen) and 40 units RNase OUT recombinant Ribonuclease inhibitor. The reaction mixture was incubated at 42˚C for 50 min and subsequently inactivated by heating at 70˚C for 15 min. Gene-specific PCR fragments were amplified from human cDNA in a thermocycler (Biometra, Göttingen, Germany) and were purified from agarose gel using the QIAquick gel extraction Kit (Qiagen) according to the manufacturer's recommendations. Serial dilutions of these fragments (0.01, 0.1, 1.0, 10 and 100 attomol product) were used to construct standard curves. Primers were: β-actin sense 5'-CAT CAC CAT TGG CAA TGA GC-3' and β-actin anti-sense 5'-TCG TCA TAC TCC TGC TTG C-3' (product size 351 bp); Ku70 sense 5'-CAT GGC AAC TCC AGA GCA-3' and Ku70 anti-sense 5'-GCT CCT TAA ACT CAT CCA CC-3' (product size 226 bp); Ku80 sense 5'-AGA AGA AGG CCA GCT TTG AG-3' and Ku80 anti-sense 5'-AGC TGT GAC AGA ACT TCC AG-3' (product size 283 bp). The PCR reactions were performed in a total volume of 50 µl containing 2 µl cDNA, 2.5 units Taq DNA polymerase (Amersham Pharmacia Biotech, Freiburg, Germany), 5 µl 5 x reaction buffer [100 mM Tris-HCl (ph 9.0), 500 mM KCl, 15 mM $MgCl_2$], dNTPs (Roche Molecular Biochemicals) at a concentration of 200 µM each, and 5 pmol sense and 5 pmol antisense primer. Quantitative RT-PCR reactions were performed in a total volume of 20 µl using an i-CyclerTM (Bio-RAD Laboratories, München, Germany). Each reaction mixture contained 100 ng cDNA, 1 unit HotStarTaq DNA polymerase (Qiagen), 2 µl 10 x reaction buffer [Tris-HCl (pH 8.7), KCl, NH_42S0_4, 15 mM $MgCl_2$], dNTP's (Roche Molecular Biochemicals) at a concentration of 200 µM each, 5 pmol sense - and 5 pmol antisense primer, SYBR green I (Molecular Probes Incorporation, Eugene, USA) in a final dilution 1 : 40,000, and fluorescein calibration dye (Bio-RAD Laboratories) diluted 1 : 10,000. The thermal cycling conditions for the i-Cycler consisted of an initial 15 min step at 95˚C to denaturate the cDNA and to activate the HotStarTaq-DNA polymerase. The amplification conditions for the

Table I. *Expression of Ku70 and Ku80 in melanoma brain metastases, results of immunohistochemistry (ICH) and in situ hybridization (ISH)*

Case	Percentage of positive cells			
NO	Ku70 ICH	Ku70 ISH	Ku80 ICH	Ku80 ISH
1	23	0	15	19
2	11	9	0	0
3	16	12	2	7
4	5	0	24	0
5	76	0	3	0
6	7	5	0	0
7	24	19	16	21
8	11	0	11	0
9	42	31	22	14
10	36	0	26	0
11	8	25	0	0
12	25	43	2	0
13	32	0	4	0
14	69	0	16	23
15	17	0	1	0
16	12	21	0	0
17	27	32	11	9
18	43	29	23	17
19	58	0	0	0
20	9	17	5	0
21	6	4	14	10
22	13	0	21	27
23	17	10	0	0
24	64	51	23	19
25	31	0	0	0
26	8	0	3	0
27	42	34	16	19
28	16	3	0	0
29	21	0	9	0
30	14	10	12	7
31	6	0	2	0
32	28	23	0	0
33	65	59	6	0
34	24	0	8	13

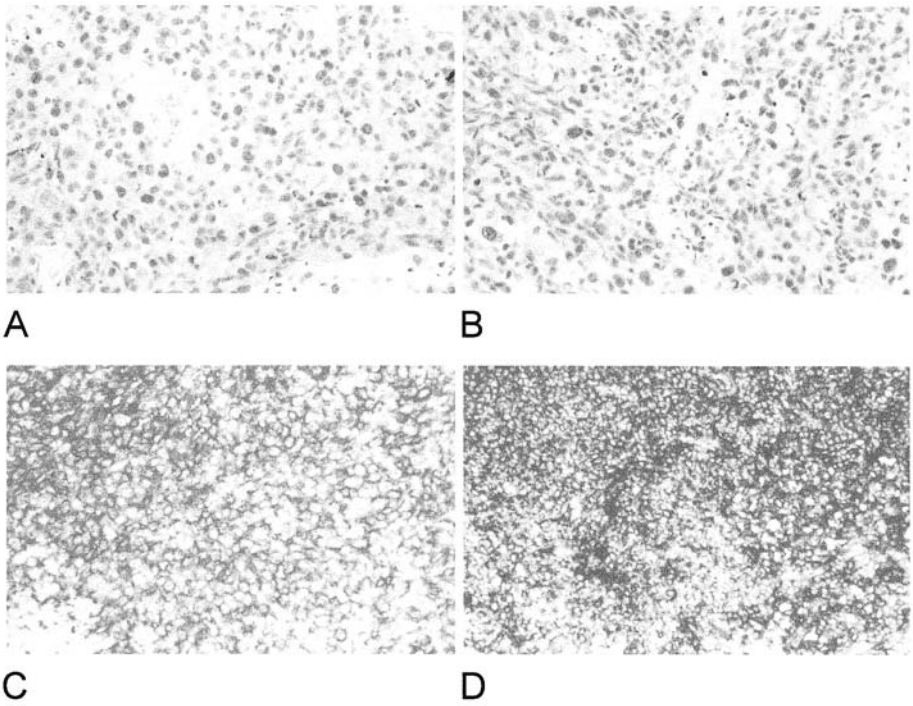

Figure 1. *Demonstration of Ku70 (A) and Ku80 (B) expression by immunohistochemistry in melanoma brain metastases. Note nuclear positive reaction much more expressed for Ku70 than Ku80. In situ hybridization product for Ku70 (C) and for Ku80 (D) in cytoplasm of melanoma brain metastases.*

various genes were as follows: β-actin: 94˚C 45sec - 60˚C 45sec - 72˚C 45sec, Ku70: 94˚C - 45sec, 56˚C - 45sec, 72˚C - 30 sec, Ku80: 94˚C 45sec - 58˚C 45sec - 72˚C 30 sec. The tumor samples and the normal probes were analyzed simultaneously in two separate runs. The mean expression value of the threshold cycles from the two runs was standardized to an external gene-specific standard curve. The calculated values of Ku70, Ku80 mRNA were divided through the expression value of the housekeeping gene β-actin.

Statistics. Data processing. The data were analysed 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 XP) where they were made available to the statistics *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. The Mann-Whitney *U*-test was used for the group comparisons. The relationship between markers was investigated by applying the correlation coefficient according to Spearman.

Results

Protein expression of Ku70 and Ku80. Ku70- and Ku80- positive cells were found in 34/34 and in 25/34 melanoma metastases, respectively. The reaction product demonstrated intranuclear localization (Figure 1A-B). The Ku70 index (percentage of Ku70-positive cells) ranged between 4 and 80% (Table I). The Ku80 index reached values between 0 and 26% (Table I). Correlation between the Ku70 and Ku80 indices in melanoma brain metastases was not found ($p>0.05$).

We also analyzed Ku70- and Ku80- positive cells in primary melanomas (n=12) from which brain metastases were derived. Ku70 expression was found in 8/12 primary melanomas. The Ku70 index ranged in these tumours between 0 and 60%. Ku80 expression was observed in 10/12 primary melanomas. The Ku80 index reached values between 0 and 37% in this tumour group. Correlations between the Ku70 and Ku80 expressions in primary melanomas was preserved ($p<0.01$). Comparison of Ku70 and Ku80 expression in primary melanomas and their brain metastases did not demonstrate any significant differences ($p>0.05$).

In situ hybridization for Ku70 and Ku80. In situ hybridization detected Ku70 expresion in 19/34 and Ku80 expression in 13/34 metastatic tumours. The reaction product demonstrated cytoplasmic localization (Figure 1C-D). The Ku70 index reached maximally 64%, while the Ku80 index did not exceed 36% (Table I). Correlation between Ku70 and Ku80 expression detected by *in situ* hybridization was not statistically significant ($p>0.05$).

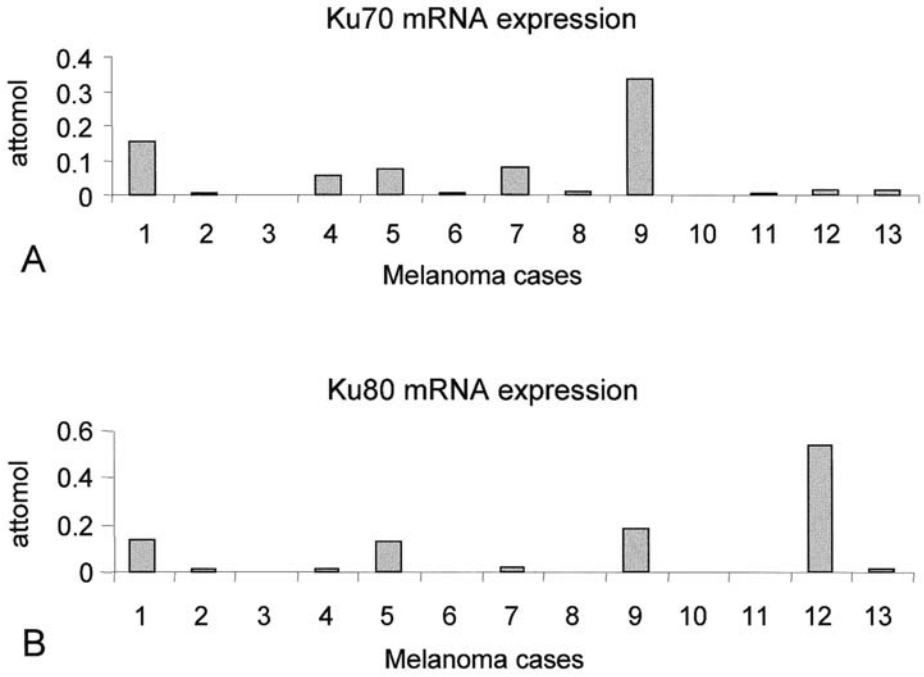

Figure 2. *Diagram showing Ku70 (3A) and Ku80 (3B) mRNA expression in melanoma brain metastases.*

Quantitative mRNA expression analysis. Quantitative analysis of Ku70 mRNA expression demonstrated values between 0.01 and 0.33. Ku80 mRNA expression ranged between 0.001 and 0.54 (Figure 2A-B). Coincident expression of Ku70 and Ku80 was found in 8/13 tumours. Comparison of Ku70 and Ku80 mRNA expression in melanocytic and amelanocytic melanoma brain metastases did not demonstrate any significant differences ($p>0.05$). Correlation between mRNA Ku70 and Ku80 expression was not present ($p>0.05$).

Discussion

In this study we focused our attention on a DNA double-strand repair system with two active participating genes, Ku70 and Ku80. Heterodimers consisting of Ku70 and Ku80 proteins are able to activate DNA-dependent protein kinase. Mutations in any of the subunits of protein kinase increase their sensitivity to ionizing radiation and to DNA double-strand breaks (4-6).

The relationship between Ku70 and Ku80 expression and the results of radiotherapy was demonstrated for cervical carcinomas (11). Generally a reduced level of Ku70, and especially of Ku80, expression in both primary melanomas and brain metastatic lesions derived from them suggest the potential application of Ku70 and Ku80 genes as predictors of radiotherapy in patients with melanoma brain metastases. A reduced level of Ku80 expression already in primary melanomas could also be regarded as a warning factor for development of melanoma metastases.

In one of our previous studies on Ku70 and Ku80 in melanomas, we demonstrated down-regulation of both Ku antigens along with melanoma progression (7). However, differences between primary melanomas and metastatic entities did not reach the level of statistical significance. The Ku70 and Ku80 proteins are functionally very closely related. Significant correlation between both markers was preserved in melanoma primary lesions but this correlation was not more significant in melanoma brain metastases. This fact indicates that dysregulation of the Ku70 and Ku80 axis may support metastatic tumour spread.

In this study we were able to demonstrate the expression of Ku70 and Ku80 in paraffin-embedded cells using *in situ* hybridization. To our knowledge, no other research group has yet been able to produce *in situ* hybridization probes directed at regulatory subunits of DNA-dependent protein kinase.

In 13 cases fresh material was available and quantitative analysis of Ku70 and Ku80 mRNA expression was performed. To our knowledge, it is the first in scientific literature study based on the quantitative evaluation of Ku70 and Ku80 mRNA expression. The reduced level of Ku70 and Ku80 observed in immunohistochemical and *in situ* hybridization methods was confirmed by quantitative analysis of mRNA expression. The Ku80 expression level

was generally lower than the Ku70 expression level. This fact could potentially explain lost correlation between Ku70 and Ku80 in melanoma brain metastases.

The expression pattern of Ku genes has been reported to correlate with tumour radiosensitivity. In melanoma metastases such investigations have not yet been performed. Ku70 and Ku80 raise the predictive possibility of tumour radiosensitivity and has been reported to be a useful parameter for selecting patients with rectal carcinoma for preoperative radiotherapy (12). On the basis of our results demonstrating decreased and partly lost expression of Ku70 and Ku80 genes in melanoma brain metastases, one can also postulate their eventual value for prediction of therapy outcome.

Previous reports demonstrated that Ku70 and Ku80 genes play an essential role in DNA double-strand repair by nonhomologous DNA endjoining, thus maintaining genomic instability (13-15). These genes also participate in several other basic processes such as cell-cycle control, being active in proliferating cells at the transition of the G1- to the S-phase and during late S- and G2-phase, probably interfering with homologous DNA recombination (16). The Ku70 and Ku80 genes have been reported to take part in telomere maintenance and in regulation of ribosomal gene transcription (17,18).

Reduced expression of both Ku genes, and by this fact loss of their function in melanoma brain metastases, correlates with the high genomic instability of melanoma brain metastases and supports metastatic spread of melanomas.

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