Altered Expression of DNA Double-strand Repair Genes Ku70 and Ku80 in Carcinomas of the Oral Cavity

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Abstract. *The relevance of double-strand DNA repair genes has been demonstrated in several tumours. The main aim of this study was to analyse the expression of the heterodimers Ku70 and Ku80, building regulatory subunits of the DNA-dependent protein kinase in 40 oral carcinomas. Ku70 expression was found in 87.5% of grade 1 and grade 3 tumours and in 82.9% of grade 2 carcinomas. Ku80 presence was noted in 87.5% of grade 1 tumours, 82.9% of grade 2 tumours and in all grade 3 tumours. Ku70-positive cells were present in 90.5% of tumours without and in 80% of tumours with lymphatic metastases. A similar relationship was found for Ku80 expression. Additionally, the expression of Ku70 was highly significantly related to smoking habits. Our results demonstrated that defects of DNA double-strand repair genes play an important role in the tumour progression of oral carcinomas.*

The heterodimer consisting of Ku70 and Ku80 proteins, functions as a regulatory subunit of the DNA-dependent protein kinase, an enzyme crucial for the repair of double-strand breaks of DNA (1-3). The Ku70 and Ku80 genes are not only active in non homologous end joining but also in cell cycle regulation (4-6). Ku70 is postulated to be a candidate tumour suppressor gene. According to Li *et al.* (7), inactivation of the Ku70 gene can lead to a propensity for malignant transformation both *in vivo* and *in vitro*. The mechanism by which Ku70 exerts its action in human cells is still a subject of discussion. In yeasts, the Ku70 protein regulates adaptation to G2M arrest after DNA damage (8).The role of the Ku80 protein in tumour biology is unclear to date.

The expressions of the Ku70 and Ku80 proteins have not yet been investigated in detail in oral carcinomas. The main goal of this study was to analyse the expression of Ku70 and Ku80 proteins in oral tumours and to relate their expressions to tumour grade, tumour stage, presence of lymphatic metastases and smoking habits.

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Materials and Methods

The material investigated consisted of 40 oral squamous cell carcinomas treated and diagnosed in the University Clinic and Academic Hospitals of the University of Göttingen, Göttingen, Germany. Among the patients were twelve females and 28 males, whose ages ranged between 33 and 84 years, averaging 66 years. The cases investigated were classified as follows: T1N0M0, 14 tumours; T2N0M0, 14 tumours; T2N1M1, 4 tumours; T3N2M1, 8 tumours. Seven cases were classified as grade 1 tumour, 28 cases as grade 2 and five cases were graded as grade 3.

Twenty cases were localised in the floor of the oral cavity and 20 in the tongue. Twenty-eight patients were smokers.

The ethical aspects of this study were approved at the Ethical Center of the Adam Mickiewicz University in Poznan, Poland.

Immunohistochemistry. Immunohistochemistry was performed by using 5-μm paraffin sections, which were deparaffinised in xylene (three times for 5 min) and rehydrated in decreasing concentrations of ethanol (100%, 96%; twice, each time for 10 min), followed by washing in deionised H_2O for 1 min. To unmask the Ku antigens, the slides were covered with 0.01M sodium citrate buffer (pH 6.0) and placed on a hot plate (95˚C) for 10 min. After cooling down, the specimens were rinsed briefly in deionised H_2O (three times). The specific primary (goat polyclonal) antibodies (Ku-70-M19; Ku-80-M20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were applied at 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 Ku70 and Ku80 proteins.

Reagents I-V, used on the second day, were supplied in the ImmunoCruz Staining System (Santa Cruz Biotechnology). Each of these reagents was pre-diluted and ready to use at room temperature. After extensive washing with 0.02 M 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. The 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 deionised 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). The samples were washed again in deionised H_2O, then counterstained with haematoxylin, dehydrated (increasing concentrations of ethanol: 96% and 100%, followed by xylene, each twice for 10 sec) and mounted in DePeX (Merck, Whitehouse Station, USA). For negative control staining, the primary antibodies were omitted. For quantification of the positively stained tumour cells, the CAS200 image analyzer (Becton-Dickinson, Hamburg, Germany) was used and results were expressed as the percentage of positive cells (indices).

Statistics. The minimum, 25th percentile, median, 75th percentile and maximum of Ku70 and Ku80 indices were calculated for each diagnostic group investigated. Comparison of the groups was performed by applying the Mann-Whitney *U*-test. The relationship between the markers was analysed using the correlation coefficient according to Spearman.

Results

Relationship between Ku70 and Ku80 expressions and tumour grade (G). In G1 carcinomas, Ku70- and Ku80-positive cells were found in 87.5% of tumours (Figures 1, 2). The maximum values of Ku70 and Ku80 in G1 carcinomas reached 10% and 40%, respectively (Figure 3A). In the G2 tumours, Ku70 and Ku80 positivity was observed in 82.9% of cases. Ku70 expression in these tumours reached a maximum of 60% and that of Ku80 of 50% (Figure 3A). In the G3 carcinomas, Ku70 expression was found in 87.5% of the tumours. Ku80 expression was found in all G3 carcinomas. Ku70 expression in these tumours reached a maximum of 40% and that of Ku80 a maximum of 30% (Figure 3A). Comparison of the Ku70 and Ku80 expressions in tumours of different grades did not demonstrate any statistically significant differences ($p>0.05$). Significant correlations between the Ku70 and Ku80 expressions were found only in G2 carcinomas ($p<0.05$).

Relationship between Ku70 and Ku80 expressions and tumour size (T). In T1 carcinomas, Ku70- and Ku80-positive cells were found in 78.6% of tumours. The maximum values of Ku70 and Ku80 expression in T1 carcinomas reached 60% and 40%, respectively (Figure 3B). In T2 tumours, Ku70 and Ku80 positivity was observed in 93.1% of the cases investigated. The Ku70 and Ku80 expression values reached maxima in 60% and 50% of cases, respectively, in the T2 oral carcinomas (Figure 3B). In the T3 tumours, Ku70 expression was found in 62.5% of cases and Ku80 expression was detected in 75% of tumours. The maximal values of the Ku70 and Ku80 indices reached 40% and 30%, respectively (Figure 3B). A comparison of Ku70 and Ku80 expressions in T1, T2 and T3 tumours did not demonstrate any statistically significant differences. No significant correlations between Ku70 and Ku80 expressions were found in T1, or in T2 or T3 carcinomas ($p>0.05$).

Relationship between Ku70 and Ku80 expressions and the presence of lymphatic metastases (N). In N0 carcinomas, Ku70- and Ku80-positive cells were found in 90.5% of tumours. The maximum values of Ku70 and Ku80 in N0 carcinomas reached 60% and 40%, respectively (Figure 3C). In the N>0 tumours, Ku70 positivity was observed in 80% of cases and Ku80 positivity in 83.3% of tumours. Ku70 and Ku80 expression values reached maxima of 50% (Figure 3C). A comparison of Ku70 and Ku80 expressions in N0 and N>0 tumours did not demonstrate any significant difference ($p>0.05$). No significant correlations between Ku70 and Ku80 expressions were found either in N0, or in N>0 carcinomas ($p>0.05$).

Relationship between Ku70 and Ku80 expressions and smoking habits. In carcinomas from smokers, Ku70-positive cells were found in 82.9% of tumours and Ku80 positivity was observed in 85.7% of carcinomas. The maximum values of Ku70 and Ku80 expressions in carcinomas from smokers reached 60% and 50%, respectively (Figure 3D). In tumours from non-smokers, Ku70 positivity was observed in 87.5% of cases and Ku80 positivity in 93.8% of tumours. Ku70 and Ku80 expression values reached maxima of 40% (Figure 3D). A comparison of Ku70 and Ku80 expressions in tumours from smokers and non-smokers demonstrated a highly significant result for Ku70 ($p=0.008$). Significant correlations between Ku70 and Ku80 expression were found in carcinomas from non-smokers ($p<0.05$). In tumours from smokers, these significant relationships were not preserved ($p>0.05$).

Discussion

The importance of the correctly functioning DNA repair genes Ku70 and Ku80 has been demonstrated in several tumours, including malignant melanomas, colorectal carcinomas and cervical carcinomas. In this study, we investigated the role of Ku70 and Ku80 genes in the progression of oral carcinomas. Tumour size and nodal status are the most significant prognostic factors for oral carcinomas (9, 10). Histological grade is reported to

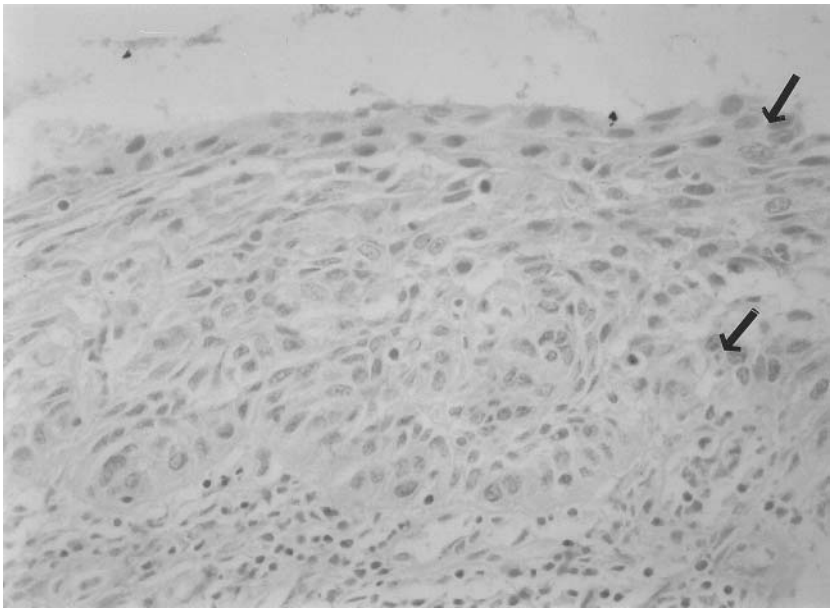

Figure 1. *Immunoreactivity for Ku70 in squamous cell carcinoma of the floor of the oral cavity (x400). Arrows demonstrate cells with K70 immunoreactivity.*

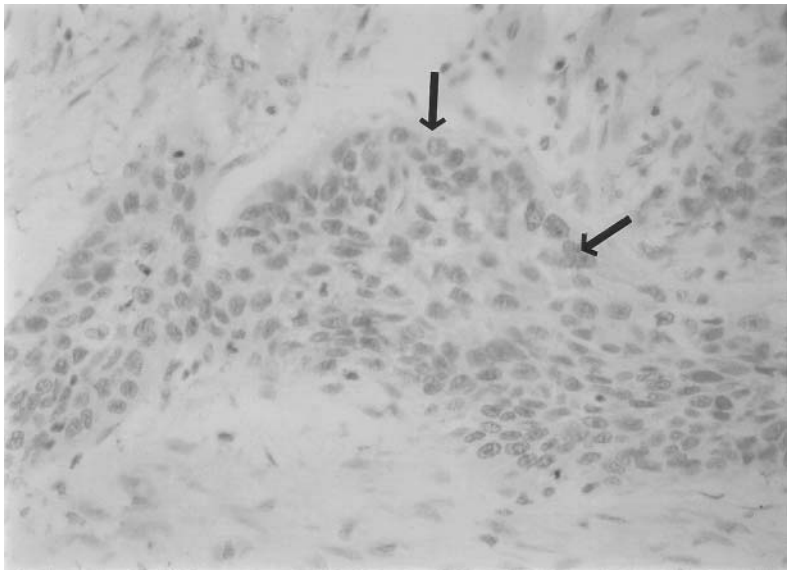

Figure 2. *Immunoreactivity for Ku80 in squamous cell carcinoma of the floor of the oral cavity (x400). Arrows demonstrate cells with K80 immunoreactivity.*

correlate poorly with patient outcome (9, 10). Therefore, we decided to correlate the expressions of Ku70 and Ku80 genes with these variables. No direct significant relationships between Ku70 and Ku80 expressions and tumour size, nodal status or tumour grade were found. Ku70 expression, however, correlated very significantly with smoking habits. This finding is extremely important, tobacco smoking being one of the dominant risk factors for oral and oropharyngeal malignancies (11-13).

The Ku70 and Ku80 proteins are very closely related functionally. Significant correlations between the two markers were found only in tumours from non-smokers. This fact could indicate that dysregulation of the Ku70 and Ku80 axis may be influenced by smoking.

There are only a few reports in the scientific literature dealing with the role of Ku70 and Ku80 in oropharyngeal carcinomas. Interestingly, both genes seem to have therapeutic application in head and neck carcinomas. The

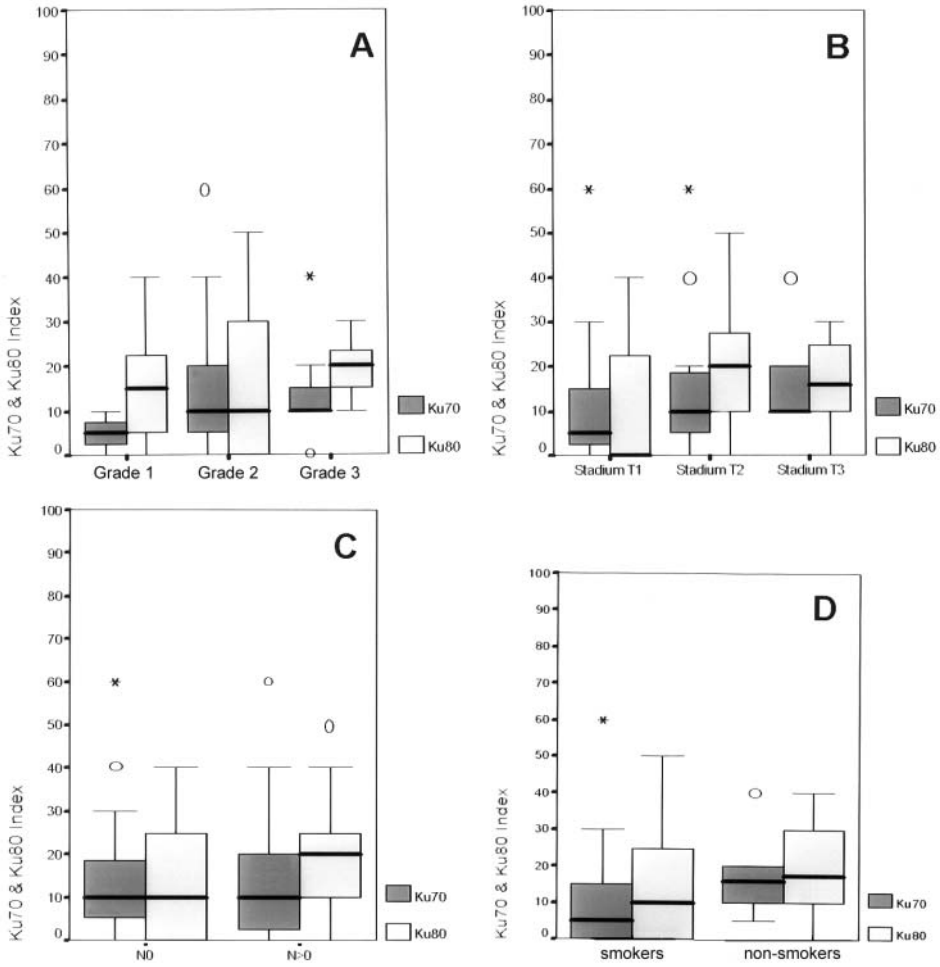

Figure 3. *A) Box-plot demonstrating the distribution of Ku70 and Ku80 expressions in tumours of different grades. B) Box-plot demonstrating the distribution of Ku70 and Ku80 expressions in tumours of different stages. C) Box-plot demonstrating the distribution of Ku70 and Ku80 expressions in tumours with and without involvement of lymph nodes in metastatic process. D) Box-plot demonstrating the distribution of Ku70 and Ku80 expressions in tumours from smokers and non-smokers. *, single values out of box-plot range.*

expression of Ku genes and of DNA-dependent protein kinase were down-regulated by the celecoxib-cyclooxygenase 2 inhibitor in the HN5 cell line derived from head and neck carcinoma (14). Inhibitors of cyclooxygenase 2 enhanced the response of tumour cells to radiation.

In the study by Shintani *et al.,* the expressions of the DNA-dependent protein kinase complex proteins, and especially that of Ku70, in oral squamous cell carcinoma, increased after radiation treatment and were associated with radiation resistance (15). Therefore, DNA-dependent protein kinase might be a molecular target for a novel radiation sensitisation therapy of oral cancer. In the study by Lee *et al.* performed on nasopharyngeal carcinoma, univariate analysis indicated that the overexpression of Ku70 can be regarded as an independent factor for locoregional control. Seventy-five percent of the patients with locoregional recurrences of the tumour displayed an overexpression of Ku70 (16). A similar tendency was found in our study, along with an increase of Ku70 and Ku80 expressions correlated to higher tumour grade and stage.

Interestingly, Ku70 was demonstrated to interact with the human papillomavirus 16 E7 oncogene, known to be a high risk factor for cancer development. Additionally, the interaction between Ku70 and HPV16 E7 resulted in up-regulation of Ku70 expression (17).

Our study is a small contribution to the elucidation of the complicated mechanisms of Ku70 and Ku80 participation in the tumour progression of oral cancer. Because of potential clinical applications concerning the prediction of recurrences and the influence of response to radiotherapy, as well as the strong relationship between Ku70 expression and smoking habits, molecular genetic investigations should be performed to fully clarify the role of these genes in the progression and therapy of oral carcinomas.

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