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Immunohistochemical Characterization of *N*-Methyl-*N*-Nitrosourea-induced Mammary Tumours of Sprague-Dawley Rats

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Abstract. *Background: Single dose of N-methyl-N-nitrosourea (MNU) was shown to induce malignant tumours in susceptible rat strains. However, such tumours are not well-characterized. Material and Methods: We characterized MNU-induced tumours in Sprague-Dawley rats using ultrasonographic, radiographic and immunohistochemical (IHC) methods. Results: In 27 rats, 41 tumours developed, appearing ultrasonographically as hypodense, non-homogenic areas with signal enhancement at their periphery. Out of these, 39 were of malignant epithelial origin, with an IHC phenotype closely-resembling that of human invasive ductal breast carcinoma. One case was diagnosed as carcinosarcoma. IHC analysis revealed that Ki-67 antigen expression correlated positively with tumour volume (r=0.40, p=0.0079). Moreover, tumours with α-smooth muscle actin in the tumour stroma were characterized by a higher proliferative rate as compared to those without its expression (p<0.05). Conclusion: This rat model of chemical carcinogenesis may be suitable for examining breast cancer development and progression.*

Although breast cancer-related mortality decreased in the last decade in the European female population, it is estimated that approximately almost 89,000 women will die of the disease in 2013. This accounts for 15% of cancer-related deaths in women (1). Therefore, animal models of breast cancer are of great interest for studying etiology, prevention and treatment of this malignancy.

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One such model is based on the induction of mammary carcinomas in rats. This goal may be achieved using various methods, however utilization of chemical compounds seems to be the one most commonly implemented (2). In the 1960s and 1970s two potent carcinogens 7,12-dimethylbenz(a)anthracene (DMBA) and *N*-methyl-*N*-nitrosourea (MNU), were shown to rapidly induce mammary tumours after a single dose in susceptible rat strains such as Sprague-Dawley and Wistar-Furth (3-5). In contrast to DMBA, MNU does not require metabolic activation to exert mutagenic effects, the induced tumours seem to be more aggressive and the ratio of malignant: benign tumours is higher (2, 5). However, malignant tumours induced by both carcinogens rarely metastasize (2, 5). Chemically-induced rat mammary tumours resemble some of the features of human breast cancer, including the growth dependence on ovarian hormones and histological progression (6-9). DMBA and MNU were shown to be capable of inducing benign and *in situ* carcinomas, which represent the early stages of carcinogenesis, as well as invasive carcinomas corresponding to advanced stages (5, 10-12). Chemically- induced rat mammary carcinomas often present lymphocytic infiltrates and altered reactive stroma, features characteristic of human breast cancer (10, 11). Moreover, MNU-induced rat mammary cancer was shown frequently to possess a G to A transition in codon 12 of the Ha-*Ras* gene (13-15). In contrast, such mutations are rarely found in human breast cancer. In addition, discrepancies in regard to dysregulation of p53 activity also exist, as these are commonly found in human breast cancer, but not in the MNU-induced counterparts (15, 16).

Recent research indicated that stroma may significantly contribute to initiation and progression of various malignant diseases. Similar role for cancer stroma may be derived from studies which showed that stromal cells may regulate the embryonic development of rat mammary gland, as well as induce tumour progression (17, 18). In addition, some studies revealed that mutation in the Ha-*Ras* gene did not

correlate with initiation of neoplasia, indirectly supporting the role of stromal cells in the carcinogenesis process (19).

Lines of evidence suggest, that single-dose administration of MNU may be capable of inducing mammary tumour with 100% incidence and that such tumours may be potentially utilized for clarifying breast cancer biology in humans. However, the existence of some molecular differences between rat and human mammary tumours may pose problems in implementing the results obtained in animal models into the clinical practice (15, 16). To our knowledge, no detailed immunohistochemical (IHC) analysis of rat mammary tumours exist. Therefore in this study, we determined the expression of the most utilized IHC markers in human breast cancer diagnostics on paraffin sections of MNU-induced rat mammary tumours.

Materials and Methods

Animals. Twenty-seven virgin female Sprague-Dawley rats (Experimental Medicine Center, Medical University of Silesia, Katowice, Poland) of two months of age were utilized for the experiment. Animals were maintained in the Animal Research Facility of the Department of Pathomorphology, Medical University of Wroclaw, controlled light (12 h light and 12 h dark) and temperature conditions. All animals were allowed access to standard rodent pellet food and tap water *ad libitum*. All the experimental procedures were performed according to European Union standards and approved by the Local Bioethics Committee by the Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences (accession number 37/2010).

Tumour induction and detection. Following two-week quarantine the animals were intraperitoneally injected with 180 mg of MNU/kg body weight. MNU (Sigma-Aldrich, Munich, Germany) was dissolved in warm NaCl and acidified to pH 5.0 with acetic acid. Four weeks after MNU administration, the animals were observed daily and palpated every three days to detect the induction of the tumours until the 13th week, when ultrasound and radiographic (x-ray) imaging was performed. Moreover, during the experiment, the animals were weighed weekly.

All procedures were performed under anesthesia management using a mixture of ketamine 60 mg/kg and medetomidine 0.5 mg/kg *i.m.* Ultrasonography was performed using Aloka α7, 7-10 MHz transducer (Aloka, Tokyo, Japan) to confirm the localization of the induced tumours and detect potential metastases in abdominal organs. Maximal tumour size was documented using 2-D mode and blood flow was visualized using colour Doppler. Moreover, lateral radiographic examination was carried out to identify potential lung metastases.

Tissue collection and histopathological evaluation. On the day after the ultrasonographical and radiographic examination, the animals were euthanized by intraperitoneal injection of 200 mg/kg pentobarbital prior to initial anesthesia by *i.m.* injection of ketamine at 60 mg/kg and medetomidine at 0.5 mg/kg. All tumours detected by palpation and ultrasonography were excised and measured. During autopsy, lungs, liver, spleen, kidney and suspicious, enlarged lymph nodes were collected. All tissues were fixed in 4% buffered formalin, dehydrated and embedded in paraffin.

Histopathological examination was performed on 6-μm-thick paraffin sections stained with haematoxylin and eosin (HE). Tissue sections were reviewed by two pathologists utilizing a double-headed BX41 microscope (Olympus, Tokyo, Japan). Tumour samples were categorized as benign or malignant lesions of particular histology based on the recently published classification of rat mammary gland tumours (11). Briefly, the histopathological criteria of malignancy were the loss of tubular-alveolar pattern, cellular pleomorphism, increased nuclear:cytoplasmic ratio, enlarged nuclei with coarse chromatin, distinct nucleoli, presence of necrosis and haemorrhage, invasion of the surrounding tissues and metastasis. Five main malignant carcinomas of rat mammary gland may be distinguished: papillary, characterized by cancer cells situated on a fibrovascular core; cribriform, solid nests of cancer cells are interrupted by various secondary lumina; solid, solid nests of cancer cells without secondary lumina formations; comedo, multilayered epithelial structures with central necrosis; and tubular, cancer cells form well-differentiated tubular and alveolar structures (11).

Tissue microarray (TMA) construction. Following the histopathological examination of the collected tumours, a TMA was made from selected areas of interest of paraffin donor blocks. Three 2.0-mm core punches with potentially the highest tumour cell content were taken for each tumour using a Manual Tissue Arrayer I (Beecher Instruments Inc, Sun Prairie, WI, USA) and transferred into the recipient paraffin block.

IHC and terminal deoxynucleotidyl transferase (Tdt)-mediated dUTP–biotin nick end labeling (TUNEL). Immunohistochemical reactions were performed on 4-μm-thick TMA sections in an automated staining platform Autostainer Link48 (Dako, Glostrup, Denmark) to ensure constant reaction conditions. In order to de-paraffinize, rehydrate and retrieve the antigens, the sections were boiled in Target Retrieval Solution buffer (Dako) using Pre-Treament Link Platform (Dako). The sections were then washed in TBS/0.05% Tween buffer followed by a 5-min incubation with EnVision FLEX Peroxidase-Blocking Reagent to block the endogenous peroxidase activity. The sections were subsequently rinsed in TBS/0.05% Tween buffer and incubated with primary antibodies directed against the studied antigens listed in Table I. Sections were then washed in TBS/0.05% Tween followed by incubation (20 min at room temperature; RT) with EnVision FLEX/horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako). In the next step the substrate for peroxidase, diaminobenzidine (Dako), was applied and the sections were incubated for 10 min at RT. Finally, the sections were counterstained with Mayer's haematoxylin, dehydrated in alcohol (70%, 96%, 99.8%) and xylene, and then mounted using SUB-X Mounting Medium (Dako).

Apoptosis detection was performed utilizing the ApopTag® Peroxidase *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA). Four-μm-thick TMA sections were de-waxed in xylene, rehydrated in alcohol and rinsed in distilled water and 1×PBS, pH 7.4. The sections were then incubated in Proteinase K (Dako) for 5 min at RT and rinsed in 1×PBS. Endogenous peroxidase was blocked by 5-min incubation in 3% H_2O_2/1×PBS. Subsequently, the sections were incubated with Equilibration Buffer for 10 min at RT, with subsequent incubation with TdT Enzyme and Reaction Buffer at 37˚C for 1 h. The reaction was stopped by 10 min incubation in Stop Buffer and sections were then rinsed in 1×PBS. Anti-

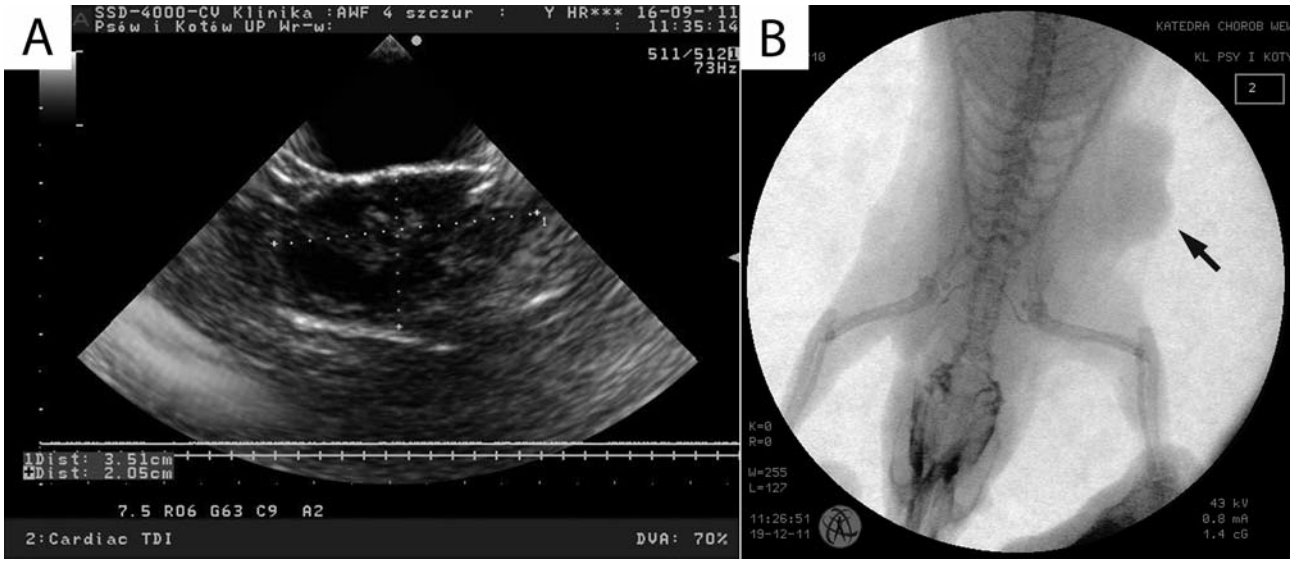

Figure 1. *Tumours upon examination using ultrasonography appeared as hypodense, non-homogenic areas with signal enhancement noted in the tumour periphery (A). Radiographic examination of the thoracic area presenting a tumour mass (arrow) localized laterally in the projection area of the mammary ridge (B).*

Table I. *List of primary antibodies utilized in the study.*

Antibody	Host	Clone	Antigen retrieval	Dilution	Supplier
Cytokeratin-5/6	Mouse	D5/16 B4	pH 9	RTU	Dako+
High molecular weight cytokeratins (Cyt-HMW)	Mouse	34βE12	pH 9	RTU	Dako
Cytokeratins AE1/AE3	Mouse	AE1/AE3	pH 9	RTU	Dako
E-Cadherin	Mouse	NCH-38	pH 9	RTU	Dako
N-Cadherin	Mouse	6G11	pH 9	1:100	Dako
Estrogen receptor α (ERα)	Rabbit	E115	pH 9	1:100	Novus*
Estrogen receptor β (ERβ)	Rabbit	Polyclonal	pH 9	1:100	Novus
Progesterone receptor (PR)	Rabbit	Polyclonal	pH 9	1:100	Novus
Vimentin	Mouse	V9	pH 9	RTU	Dako
α-Smooth muscle actin	Mouse	1A4	pH 9	RTU	Dako
Desmin	Mouse	D33	pH 9	RTU	Dako
Ki-67 antigen	Mouse	MIB-5	pH 6	1:25	Dako

*Novus Biologicals, Cambridge, United Kingdom; +Dako, Glostrup, Denmark; RTU: ready-to-use.

dioxygenin peroxidase-conjugated antibodies were applied for 30 min at RT. Following that, the sections were incubated for 10 min with diaminobenzidine (Dako) to visualize the TUNEL-positive cell nuclei. Finally, the sections were counterstained with Mayer's haematoxylin and, after dehydration in alcohols, mounted in SUB-X Mounting Medium (Dako).

Evaluation of IHC reactions. The IHC sections were evaluated under a BX-41 light microscope equipped with Cell^D software for computer-assisted image analysis (Olympus). For the evaluation of Ki-67 antigen and TUNEL-positive cells in TMA sections, three fields with the highest number of tumor cells yielding positive reaction were selected from separate cores ('hot spots'). The percentage of positive cells in each hot spot was evaluated by scoring brown-labeled nuclei of cancer cells under ×400 magnification. The average score of the three hot spots was recorded for each tumor.

The expression of other IHC markers was evaluated independently in tumour cells, as well in corresponding tumour stroma. The expression of particular markers was regarded as positive when found in more than 10% of tumour or stromal cells.

Statistical analysis. Statistical analysis was performed using Prism 5.0 (GraphPad, La Jolla, CA, USA). Data distribution was determined utilizing the Shaphiro-Wilk test. The Mann-Whitney *U*-test was used to compare the groups of data that failed to satisfy the assumptions of the parametric test. Correlations between the scores were tested using Spearman's correlation test. Results were considered statistically significant when $p<0.05$ in all the analyses.

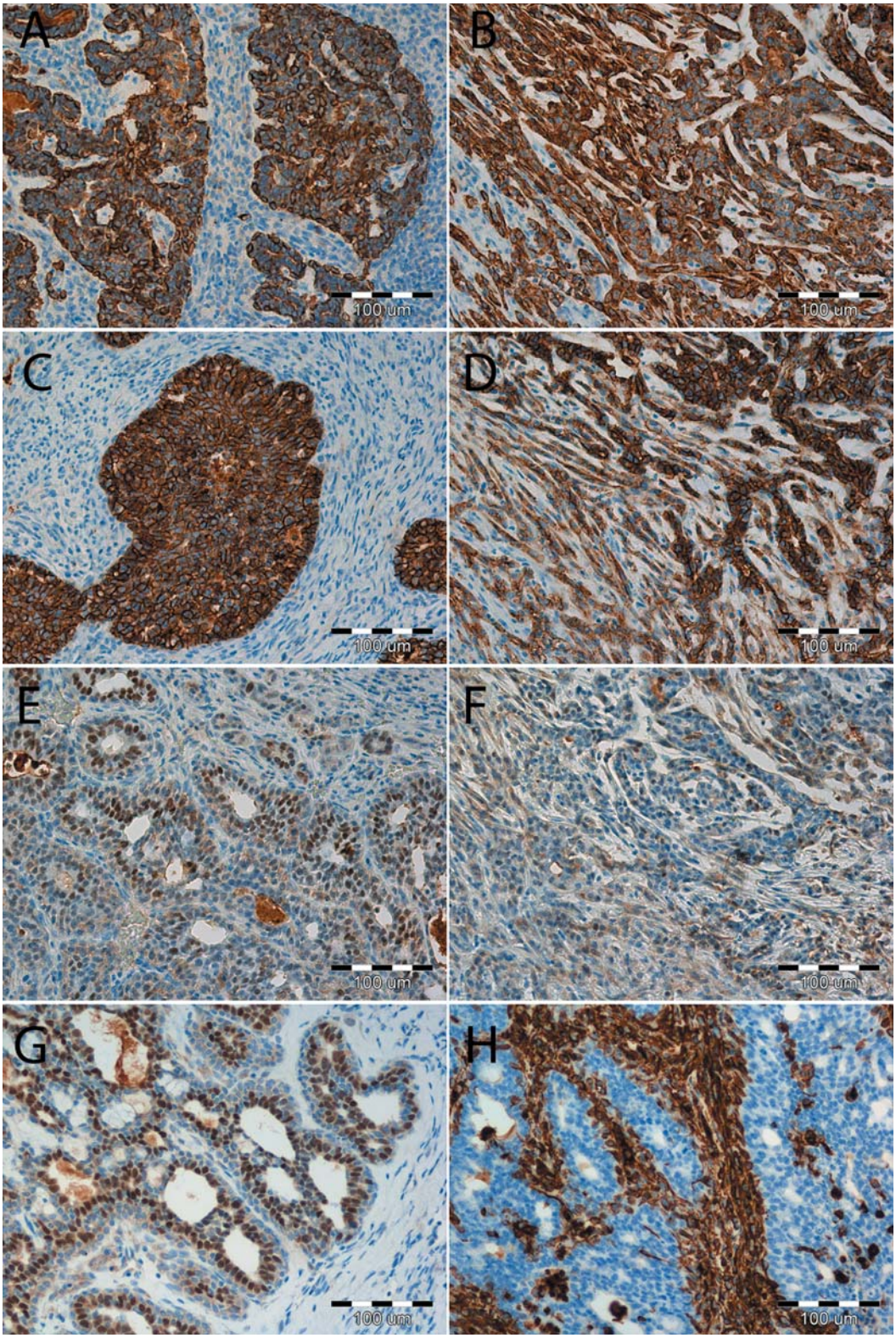

Figure 2. *Immunohistochemical expression of cytokeratin-AE1/AE3 (A, B), E-cadherin (C, D), progesterone receptors (E, F). In epithelial lesions expression of the above mentioned markers is noted only in tumour cells (A, C, E), whereas in carcinomasarcoma their expression was observed in epithelial, as well as mesenchymal-like cells (B, D, F). Expression of estrogen receptor-α in tumour cells (G). Vimentin expression visible in cells of the tumour stroma (H).*

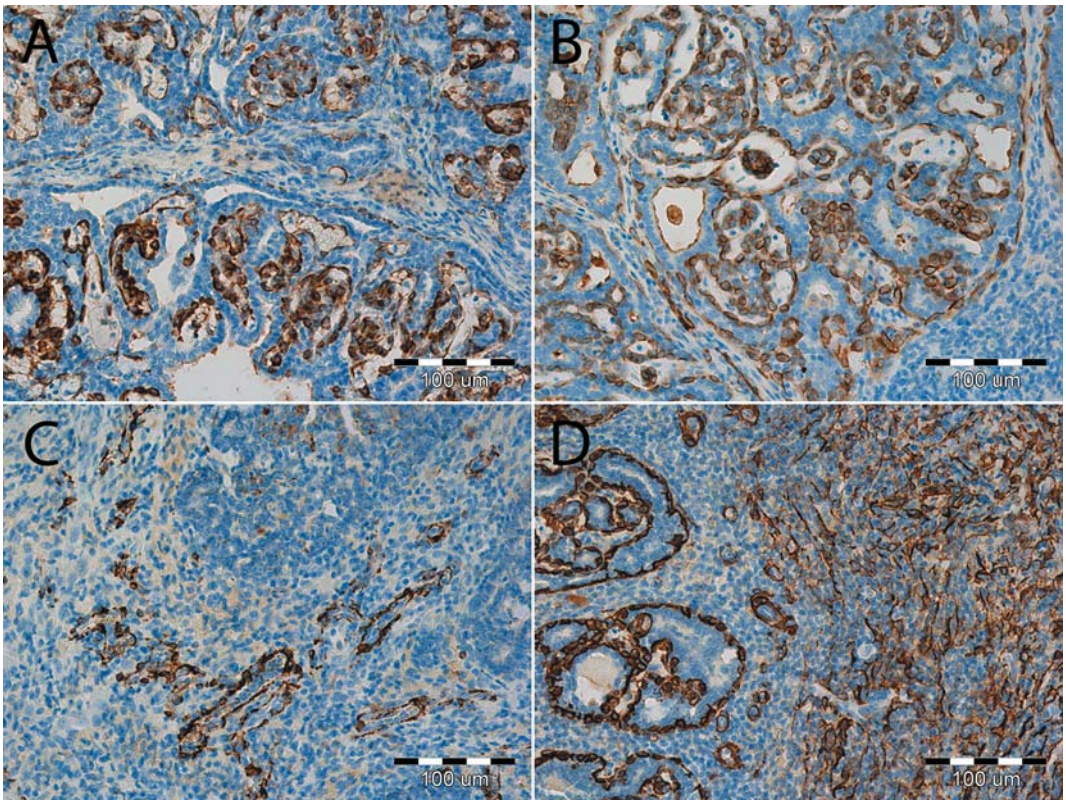

Figure 3. *Immunohistochemical expression of high-molecular weight cytokeratins (A) and cytokeratin-5/6 (B) in cells resembling phenotypically myoepithelial cells. Desmin expression in the tumour stroma in cells forming lumens filled with erythrocytes (C). The expression of α-smooth muscle actin (D) was noted in cells resembling myoepithelial cells (left) and fibroblast-like cells (right).*

Results

Tumour induction, ultrasonography, x-ray and histopathological examination. In the 27 rats, first palpable tumours appeared in the 6th week in one of the animals following MNU administration. In 7th week, additional tumours developed in a second animal. In the 9th week of the experiment tumours were detected in nine animals (33.3%), whereas in the 10th, 11th, 12th and 13th week of the experiments, respectively 15 (55.6%), 22 (81.5%), 25 (92.6%) and 27 (100%) of the animals had detectable tumours. At the end of the 13th week, 41 tumours were detected by palpation and using both ultrasonography and x-ray (Figure 1).

The tumours were located in different regions of the mammary gland. The tumours in ultrasonographical examination were characterized by differentiated echogenicity and the extent of the vascularization in all of the analyzed tumours was weak. Moreover, the larger tumours were characterized by changes due to tumour necrosis.

Histopathological examination of the excised tumours revealed a 100% mammary tumour incidence, with numbers of tumours per animal ranging 1 to 3. Out of the 41 examined tumours, 40 (97.6%) were diagnosed as malignant, whereas one (2.4%) tumour was diagnosed as benign tubular adenoma. Most of the malignant lesions were mixed in their histological structure, with the papillary-cribriform pattern being the most frequent (31 tumours; 77.5%). However, when the dominant pattern was acknowledged as a cut-off point for the histopathological diagnosis, the following malignant tumours were diagnosed: 19 (47.5%) cribriform carcinomas, 12 (30.0%) papillary carcinomas, six (15.0%) tubular carcinomas, two (5.0%) solid carcinomas and one (2.5%) carcinosarcoma. The mean tumour volume was 1431±2331 mm^3 and no significant differences in volume were noted according to histopathological lesion type (Mann-Whitney test).

Ultrasonographical, radiographical, as well as histopathological examination revealed no metastatic changes in lungs, liver, kidney and spleen. No differences in number of detected tumours were noted between the ultrasonography, radiography and palpation. Moreover, no signs of acute MNU toxicity in sections from the collected organs were noted.

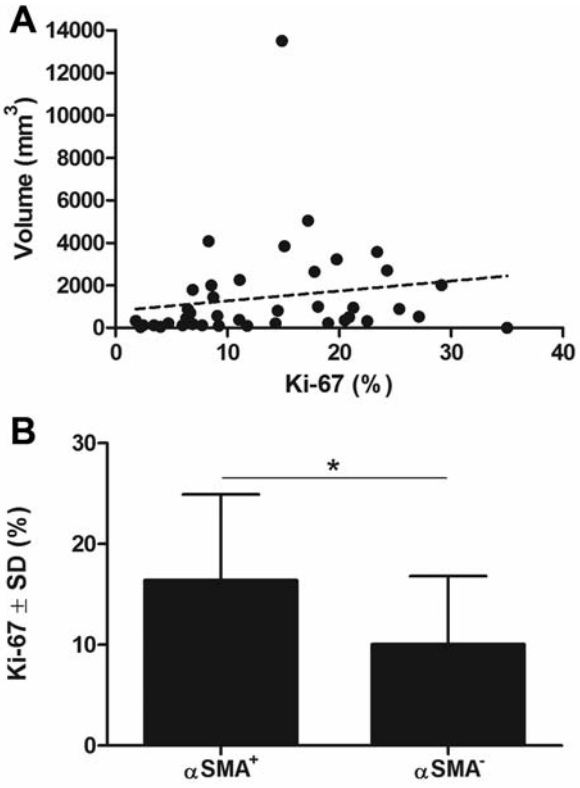

Figure 4. *Positive correlation between the expression intensity of the Ki-67 antigen and tumour volume (r=0.40, p=0.0079, Spearman correlation test) (A). Ki-67 antigen expression in regard to stromal α-smooth muscle actin (αSMA) expression (B). *p<0.05, Mann-Whitney test.*

Immunohistochemical characterization of MNU-induced tumours. Although histopathological examination revealed one benign and other differentiated malignant tumour, all tumour cells were characterized by the expression of cytokeratin (CK) AE1/AE3, E-cadherin and progesterone receptor (Figure 2). In, almost all of the analyzed tumours, expression of estrogen receptor-α (ERα) was detected except for the carcinosarcoma and one of the solid-type tumours. None of the analyzed tumour cells showed expression of N-cadherin and ERβ. Vimentin expression was however, noted only in spindle-like cells resembling fibroblasts within the stroma (Figure 2).

The analyzed tumours were characterized by the differential expression of HMW CKs, CK 5/6, desmin and α-smooth muscle actin (αSMA) (Table II; Figure 3). HMW CKs were expressed in tumour cells in 29 (70.7%) of the analyzed tumours, whereas CK 5/6 expression was noted on the tumour edge of 24 (58.5%) of cases. CK 5/6 expression was noted in cells surrounding tumour cells and was co-expressed with αSMA in these cases. Except for the single diagnosed carcinosarcoma case, none of the other tumours presented with αSMA expression in tumour cells, although its expression was noted in spindle-like cells in the tumour stroma (not the myoepithelial cells) in 22 (53.6%) cases. Similarly, no desmin expression was noted in the tumour cells. However, its expression was noted in cells forming lumens filled in with erythrocytes in 18 (43.9%) of the analyzed tumours (Figure 3).

The MNU-induced tumours were also characterized by differential expression intensity of the Ki-67 proliferation antigen, with the lowest being noted in tubular adenoma (1.82%) and the highest in one of the tubular carcinomas (35.1%). The tumours varied also in regard to TUNEL-positive cells, with the least and highest number noted in two of the cribriform carcinomas (0.39% and 9.36%, respectively). Statistical analysis revealed no significant differences regarding expression intensity of the Ki-67 antigen and the proportion of TUNEL-positive cells between particular histological types (Table III), although the tumour volume correlated positively with the expression intensity of the Ki-67 antigen (r=0.40, p=0.0079, Spearman correlation test; Figure 4A). Moreover, tumours with αSMA expression in the tumour stroma were characterized by a significantly higher expression of the Ki-67 antigen compared to cases lacking its expression (p=0.0108, Mann-Whitney test; Figure 4B).

Discussion

In this article using histopathological assessment, as well as IHC, we examined a series of rat mammy tumours induced by the single administration of 180 mg/kg MNU. However, earlier studies successfully used smaller doses of MNU (<180 mg/kg) to induce mammary tumours in rats (10, 12). Moreover, to our knowledge, for the first time we utilized ultrasonographic and radiographic methods to detect and fully-characterize the MNU-induced tumours. This procedure was routinely employed in our research schedule. In regard to the tumour detection methods, based on the obtained results, the tumour palpation method was as accurate as the tumour detection performed using ultrasonographic and radiographic methods. These ancillary methods did not find any lesions in addition to those detected by palpating the animals. However, we did not actually carry out a whole-mount histological analysis of the mammary ridge, which would allow us to fully-define the sensitivity and specifity of these two methods in detecting small lesions such as hyperplastic ducts or ductal carcinomas *in situ* (11, 12). Besides the resected lesion no gross abnormalities upon macroscopic examination could be detected. Moreover, as the rats did not present lung metastasis, the utility of radiographic examination in dissemination of cancer cells within the thoracic regions remains to be further determined.

In our research, we noted similar incidences in histological types of malignant epithelial lesions as observed in earlier studies with rat strains susceptible to

Table II. *Expression of iunohistochemical markers in the malignant epithelial lesions of the analysed tumours.*

Carcinoma type	HMW CKs		CK 5/6		Desmin		αSMA	
	+	–	+	–	+	–	+	–
Cribriform	17	2	12	7	8	11	15	5
Papillary	10	2	8	4	5	7	3	9
Tubular	4	2	2	4	2	4	3	3
Solid	2	0	2	0	2	0	2	0
Carcinosarcoma	1	0	0	1	0	1	0	1
Adenoma	0	1	0	1	1	0	0	1

HMW CKs: High molecular weight cytokeratins; CK: cytokeratin; αSMA, α-smooth muscle actin.

chemical carcinogenesis (4, 11, 12, 17, 19, 20). In our study, in addition to the malignant epithelial tumours, a single tubular adenoma and a malignant carcinosarcoma were noted. However, although various factors influencing mammary gland carcinogenesis were examined in earlier studies, many of the studies give only limited information concerning the induced tumour histology (21-24). Therefore, in our study we sought to analyse the expression of IHC markers in rat mammary tumours, frequently used in diagnostic algorithms and to characterize the biology of human breast cancer. We found that most of the analyzed tumours were characterized by the expression of pan-cytokeratin marker (CK AE1/AE3) and E-cadherin with simultaneous lack of vimentin expression in tumour cells, which confirms the ductal character of the induced tumours (25). We have also corroborated the results of other studies which analyzed the steroid receptor expression status and hormone dependency of MNU-induced tumours (26-28). We have shown that ERα and PR are expressed in tumour cells and may be the major contributors to the observed effects of steroid hormones on MNU-induced tumour growth (26-28). Interestingly, no ERβ expression was noted in the analyzed tumours in contrast to its expression in the human counterpart (29).

The analyzed tumours differed with regard to the expression of HMW CKs and CK 5/6. High molecular weight cytokeratins (CK 5/6, CK 14, 34βE12) are expressed in myoepthelial cells in non-malignant breast tissue and in a minority of human breast cancer cases (30, 31). In our study, similarly as in human ductal carcinoma *in situ*, we observed the expression of these antigens in myopepithelial cells surrounding the tumour cells in some of the cases. Although these markers are utilized in human breast pathology to determine the invasiveness of the lesion, their utility in rat mammary cancers, remains to be clarified. No differences regarding tumour size, Ki-67 antigen expression or TUNEL-positive cells between tumours classified as positive or negative in relation to CK 5/6 and HMW CKs was noted. In our study we also, analyzed the expression of desmin, which

Table III. *Intensity of Ki-67 antigen- and TUNEL-positive expression in regard to histopathological diagnosis. Data are presented as means with standard deviation.*

Carcinoma type	Ki-67±SD (%)	TUNEL(+)±SD (%)
Cribriform (n=19)	12.89±7.53	2.50±2.14
Papillary (n=12)	12.39±8.51	1.64±0.77
Tubular (n=6)	17.87±10.89	3.10±0.87
Solid (n=2)	15.18±8.57	2.04±0.60
Carcinosarcoma (n=1)	17.20	3.90
Adenoma (n=1)	1.82	3.03

SD, Standard deviation.

was observed in the stroma of the tumours, in particular in cells forming lumens filled with erythrocytes. These cells are presumably pericytes, identified by their morphological appearance and characteristic localization in the IHC sections. Moreover, desmin expression was recently shown to be expressed in a subset of pericytes during rat pituitary gland development and in those of the microvessels of human colorectal carcinoma (32, 33).

In this study, we also found that αSMA, besides its well-known expression in myoepithelial cells, was also expressed in spindle-like cells in the tumour stroma, which phenotypically resembled fibroblasts (30, 31). Recently, these spindle-like cells expressing αSMA were identified as cancer-associated fibroblasts (CAFs), which were shown by our group and others to affect tumour progression *via* induction of invasiveness and proliferation of cancer cells in several human malignancies (34, 35). Interestingly, tumours characterized by the αSMA expression in stromal cells were also characterized by an increased proliferative rate as measured by the Ki-67 antigen expression intensity. This observation is consistent with those for human tumours (34, 35). In addition, our results strongly support the hypothesis of stromal regulation of neoplastic development during MNU-induced carcinogenesis and underlie the importance of

stromal cells in tumour progression (12, 17, 19, 36). It was shown that stromal cells, but not epithelial cells, exposed to MNU *in vivo* are crucial for neoplastic transformation (19). We have also shown that the proportion of Ki-67 antigen-positive, but not of TUNEL-positive cells, correlate positively with tumour size. These results are comparable to those obtained on human invasive ductal breast carcinomas, which showed that increased proliferation of cancer cells results in larger primary tumour size (37).

In this study we also observed an induction of a single case of carcinosarcoma. This tumour presented with IHC features similar to these observed in other epithelial tumours, however it lacked ERα expression. Moreover, the expression of all the other analyzed markers was noted in the epithelial, as well the mesenchymal cells, which confirmed the histopathological diagnosis derived from the HE sections. In other malignant lesions which were characterized by the αSMA expression, stromal cells did not express epithelial markers (CK AE1/AE3, E-cadherin, HMW CKs, CK 5/6).

In summary, our results support and underlie the role of stroma, in particular cells expressing αSMA, on tumour progression in this carcinogenesis model, pointing to its potential usefulness in studying their role in breast cancer.

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