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# Expression of AGR2 in non small cell lung cancer

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**Summary.** We aimed to evaluate immunohistochemically the expression of the human Anterior Gradient-2 (AGR2), a gene which has recently been proposed as an oncogene for lung carcinoma development, in non small cell lung cancer and to correlate the findings to clinico-pathological data including patient survival. 95 cases of NSCLC were immunostained using a polyclonal AGR2 antibody and statistical analyses were applied to test for prognostic and diagnostic associations. AGR2 was expressed in 66.3% of cases, preferentially adenocarcinomas. There were no relevant associations with clinico-pathological paramaters. A prognostic value of AGR2 could not be demonstrated neither in multivariate nor in univariate analyses. Interestingly, this is the first study to demonstrate AGR2 expression in squamous cell carcinomas. Although a prognostic value of AGR2 seems unlikely further studies are warranted to investigate the biological role of AGR2 in NSCLC and its differential expression according to histology.

**Key words:** Lung cancer, NSCLC, AGR2, Prognosis, Immunohistochemistry

## Introduction

Lung cancer is with 174,470 estimated new cases the second most common cancer type in both sexes and with 162,460 estimated deaths in 2006 the most common cause of cancer mortality in the United States (Jemal et al., 2006). The rather heterogeneous group of non small cell lung cancers constitute the leading histological type (about 80%) of lung cancer. Conventional tumour parameters of the TNM-classification are the most valuable prognostic markers, so far (Mountain, 1997). Complete surgical resection of the tumour still remains the most important curative therapeutic option (Flehinger et al., 1992) but with recurrence rates exceeding 50% in some studies, additional therapy is necessary (Singhal et al., 2005). As even with systemic chemotherapy the mortality rates are high (Betticher, 2005; Lynch and Kim, 2005), clearly, there is a need for novel therapeutic options and therapy targets for this extremely lethal disease (Rosell et al., 2006). Also, since patients with identical tumour staging parameters according to TNM have different survival times, novel prognostic markers are needed (Vielh et al., 2005). Although a variety of novel molecular prognostic markers have been proposed so far for NSCLC, none of them have been validated in larger prospective trials (Kawamukai et al., 2004). Singhal et al. evaluated several studies of prognostic markers for NSCLC and identified p16, p21, p27, cyclin B1, cyclin E, survivin, VEGF and collagen XVIII to be of outstanding independent prognostic value (Singhal et al., 2005).

Very recently, Zhu et al. suggested AGR2 as a potential oncogene in NSCLC (Zhu et al., 2006). AGR2 (synonyms: hAG-2 (Fletcher et al., 2003), Gob-4 (Komiya et al., 1999)) is the human orthologue of the secreted *Xenopus laevis* Anterior Gradient protein XAG-2. In the frog XAG-2 has a putative role in ectodermal patterning of embryo and its expression induces cement gland differentiation and expression of neural marker genes in a fibroblast growth factor dependent way (Aberger et al., 1998). We and others recently described up-regulation of the AGR2 gene in prostate cancer (Kristiansen et al., 2005; Zhang et al., 2005), endometrial carcinomas and renal carcinomas (Fritzsche et al., 2006). Furthermore, AGR2 was proposed for the detection of circulating tumour cells in the peripheral blood in patients with advanced cancers (Smirnov et al., 2005). AGR2 expression has been described in human breast cancer tissue and cell lines by several research groups and its expression was associated with a positive estrogen receptor status of the tumour cells. In Wilms tumours, a common deletion is located on chromosome 7p15-21, a region that also encompasses the AGR2 locus (Sossey-Alaoui et al., 2003).

So far, AGR2 has not been investigated in lung

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cancer yet. Therefore we aimed to investigate the expression of AGR2 immunohistochemically in 95 cases of NSCLC using a well characterised tissue micro array (Kristiansen et al., 2003a) and to evaluate correlations with clinico-pathologic parameters as well as prognostic properties of AGR2. Our data indicate that AGR2 is highly expressed in adenocarcinomas of the lung.

## Materials and methods

### *Patients*

Our study included 95 patients with non small cell lung cancer, diagnosed at the Institute of Pathology, Charité – Universitätsmedizin Berlin, between 1995 and 1997. Patient age at the time of diagnosis ranged from 34 to 80 with a median of 62 years. Clinical follow-up data was available for 77 patients who where further treated at the Charité – Universitätsmedizin Berlin. The median postoperative observation time was 23 months and ranged from 0 to 92 months. 33 patients (42.9%) died from cancer during follow-up, and eight patients (10.4%) died from non cancer related causes. The selection of cases for the creation of the micro array was based on availability of tissue and these were not stratified for any known preoperative or pathological prognostic factors. Five patients had systemic disease (pM1) at the time of diagnosis. Histological typing of tumours was carried out according to the criteria of the World Health Organization (WHO). Histologically, 46 cases (48.4%) were adenocarcinomas and 49 cases (51.6%) were squamous cell carcinomas. Clinico-pathologic were gathered from the archival pathology reports and are described in Table 1.

### *Immunohistochemistry*

Immunohistochemical analysis was carried out on a tissue micro array from formalin-fixed, paraffin-embedded archival tissue blocks as classified before (Kristiansen et al., 2002, 2003a,b). The array was freshly cut, mounted on a slide, deparaffinised with xylene and gradually rehydrated. Antigen retrieval was achieved by pressure cooking in 0.01M citrate buffer for 5 minutes. For AGR2 we generated polyclonal rabbit antisera against peptides derived from the AGR2 protein sequence (dilution 1:250). The antibody specificity was ascertained by Western blot (Fritzsche et al., 2006). The primary antibody was incubated at room temperature for 1 hour. As a negative control, one array slide was processed without primary antibody. As a positive control we used two whole tissue slides of AGR2 positive breast cancers. Detection took place by the conventional labelled-streptavidin-biotin method (DAKO, Hamburg, Germany) with alkaline phosphatase as the reporting enzyme according to the manufacturer´s instructions. Fast-Red (Sigma-Aldrich, Munich, Germany) served as chromogen. Afterwards the slides were briefly counterstained with haematoxylin and aquaeously mounted.

### *Evaluation of the immunohistochemical stainings*

The immunostainings were examined by two pathologists who were blinded to patient outcome. We used a simple scoring system classifying the staining intensity into three categories: negative (0), weakly to moderately positive (1) and strongly positive (2). Tumours were only considered positive when at least 10% of tumour cells on the spot showed expression of AGR2.

### *Statistical analysis*

Statistical analysis was performed using SPSS, version 13.0. Correlations were calculated according to Spearman. Fisher´s exact and chi-square tests were applied to assess the statistical significance of the associations between expression of AGR2 and various clinico-pathological parameters. Univariate survival analysis was carried out according to Kaplan-Meier, differences in survival curves were assessed with the Log rank test. Multivariate analyses were calculated according to the Cox regression model. P values $< 0.05$ were considered significant.

## Results

### *AGR2 immunostaining in non small cell lung cancer*

32 of the 95 NSCLC cases (33.7%) were negative for AGR2 (Fig. 1A,B). 29 cases (30.5%) showed a weak to moderate (Fig. 1C/D) and 34 cases (35.8%) a strong expression of AGR2 (Fig. 1E/F). For cross-tables and survival analyses tumours were divided in AGR2 negative (score 0) and AGR2 positive (score 1&2) ones. AGR2 expression was significantly higher in cases without residual tumour. AGR2 positivity was also significantly associated and correlated with an adenoepithelial histology, as 28 of the 46 adenocarcinomas (60.9%) stained strongly positive and only 7 adenocarcinomas (15.2%) were completely negative. In contrast, 25 of 49 (51.0%) squamous cell carcinomas were negative and only 6 cases (12.2%) were strongly positive. These differences between AGR2 expression and the histology type was highly significant (Fisher's exact test, p=0.001) Other associations of AGR2 with clinico-pathological parameters were not noted (Tables 1,2).

### *AGR2 expression and survival times*

We analyzed the impact of AGR2, histologic tumour type, pT-status, pN-status, disease stage, histologic tumour grade, age and gender on disease specific survival time. The conventional histo-pathologic parameters nodal status, disease stage and histological grade reached significance while AGR2 like all other

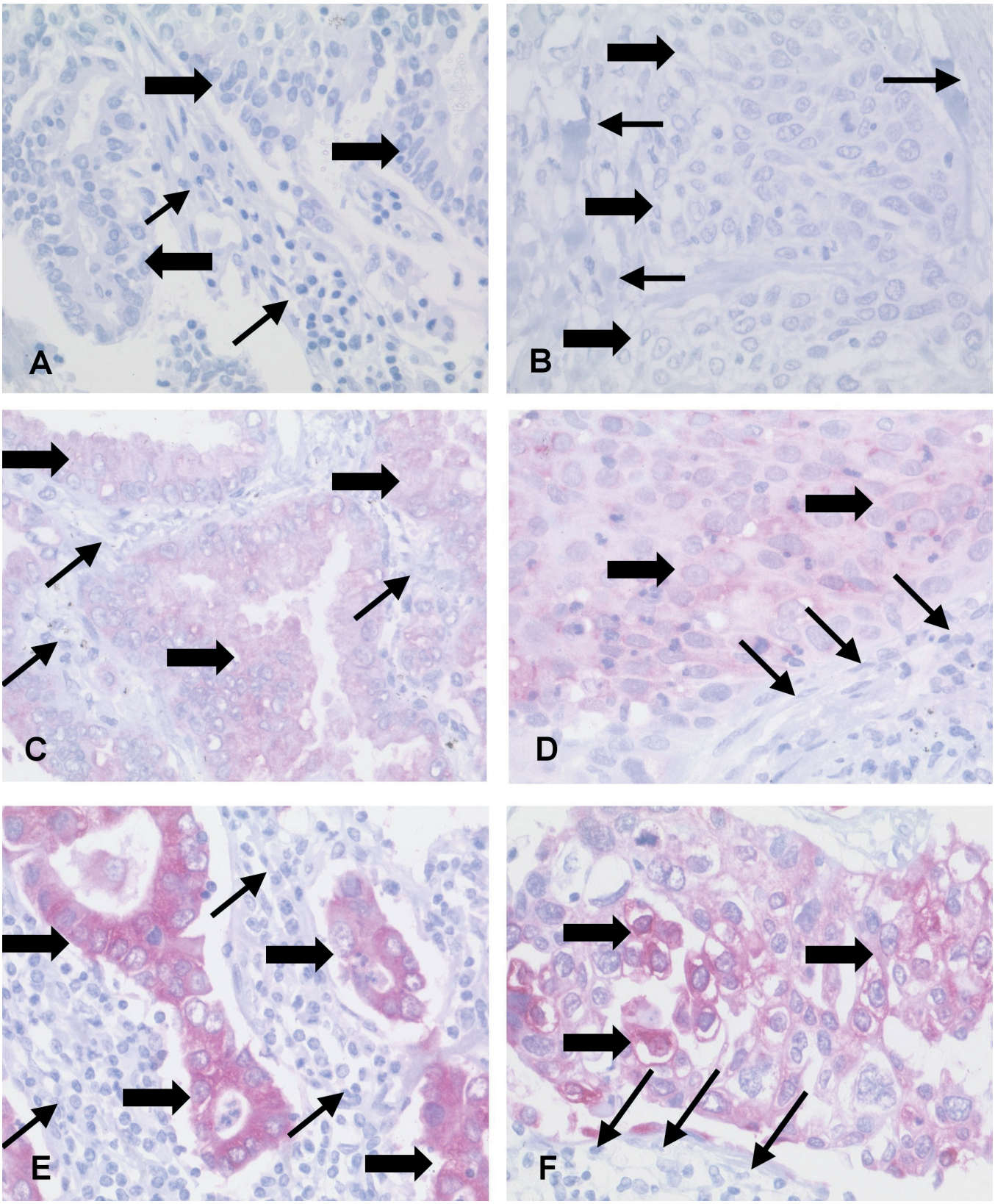


**Fig. 1.** AGR2 Expression in NCSLC. **A/B.** AGR2 negative adenocarcinoma (ADC, **A**) and squamous cell carcinoma (SCC, **B**) of the lung, bold arrows indicate tumour epithelia, surrounding stromal tissue was not immunoreacticve (thin arrows). **C/D.** Weak to moderate AGR2 expression in epithelial cells of ADC (bold arrows, **C**) and SCC (bold arrows, **D**) of the lung. The surrounding stromal cells (thin arrows) are negative for AGR2. **E.** Adenocarcinoma of the lung (bold arrows) with strong AGR2 expression and negative surrounding stroma with prominent lymphocytic infiltrate (thin arrows). **F.** Squamous cell carcinoma of the lung (bold arrows) with moderate to strong AGR2 expression and AGR2-negative stroma (thin arrows). x 400

parameters tested remained insignificant (Table 3, Fig. 2). These results were confirmed in a Cox regression analysis (Table 4) with histological grading loosing significance. To evaluate the prognostic value of AGR2 expression in selective patient groups, we repeated the univariate survival analyses in subgroups stratified according to pT-status, pN-status, tumour grade, and

**Table 1.** Clinico-pathological parameters and association with AGR2 expression of the tumour set.

| Variable | Patients | No. of patients (%) AGR2 neg. | AGR2 pos. | p value |
|---|---|---|---|---|
| Patient age | | | | |
| < 60 years | 31 | 7 (22.6) | 24 (77.4) | 0.164 |
| >= 60 years | 64 | 25 (39.1) | 39 (60.9) | |
| Gender | | | | |
| male | 77 | 28 (36.4) | 49 (63.6) | 0.406 |
| female | 18 | 4 (22.2) | 14 (77.8) | |
| Histology | | | | |
| adeno | 46 | 7 (15.2) | 39 (84.8) | <0.001 |
| squamous | 49 | 25 (51.0) | 24 (49.0) | |
| pT-status | | | | |
| pT1 | 18 | 7 (38.9) | 11 (61.1) | 0.823* |
| pT2 | 66 | 21 (31.8) | 45 (68.2) | |
| pT3 | 8 | 3 (37.5) | 5 (62.5) | |
| pT4 | 3 | 1 (33.3) | 2 (66.6) | |
| pN-status | | | | |
| pN0 | 56 | 18 (32.1) | 38 (67.9) | 0.346* |
| pN1 | 16 | 4 (25.0) | 12 (75.0) | |
| pN2 | 22 | 9 (40.9) | 13 (59.1) | |
| pN3 | 1 | 1 (100) | 0 (0) | |
| Histological grade | | | | |
| G1 | 2 | 0 (0) | 2 (100) | 0.199* |
| G2 | 50 | 15 (30.0) | 35 (70.0) | |
| G3 | 43 | 17 (39.5) | 26 (60.5) | |
| Disease stage | | | | |
| IA | 12 | 4 (33.3) | 8 (66.7) | 0.311* |
| IB | 39 | 13 (33.3) | 26 (66.7) | |
| IIA | 2 | 1 (50.0) | 1 (50.0) | |
| IIB | 12 | 1 (8.3) | 11 (91.7) | |
| IIIA | 21 | 7 (33.3) | 14 (66.7) | |
| IIIB | 4 | 2 (50.0) | 2 (50.0) | |
| IV | 5 | 4 (80.0) | 1 (20.0) | |
| Residual status | | | | |
| negative R0 | 80 | 23 (28.8) | 57 (71.3) | 0.054 |
| positive R1 | 9 | 6 (66.7) | 3 (33.3) | |

*: Chi square test for trends

**Table 3.** Univariate disease specific survival analyses (median survival time in months).

| Variable | Disease specific survival No. of cases | No. of events | Median survival time (±SE) | p-value |
|---|---|---|---|---|
| AGR2 | | | | 0.918 |
| negative (0). | 25 | 10 | not reached | |
| positive (1/2) | 52 | 23 | 38.0±11.8 | |
| Age | | | | 0.542 |
| <60 years | 25 | 13 | 30.0± 8.2 | |
| ≥60 years | 52 | 20 | 63.0±19.1 | |
| Gender | | | | 0.180 |
| male | 62 | 28 | 35.0 ± 7.3 | |
| female | 15 | 5 | 63.0 ± 12.8 | |
| Histology | | | | 0.287 |
| adeno | 38 | 18 | 38.0±11.5 | |
| squamous | 39 | 15 | not reached | |
| pT-status | | | | 0.292 |
| pT1 | 17 | 6 | 63.0±0.0 | |
| pT2/3/4 | 60 | 27 | 35.0±6.1 | |
| Nodal status | | | | <0.001 |
| pN0 | 46 | 14 | not reached | |
| pN1 | 11 | 6 | 23.0±4.1 | |
| pN2 | 20 | 13 | 15.0±6.3 | |
| Disease stage | | | | <0.001 |
| IA-IIA | 43 | 11 | not reached | |
| IIB-IV | 34 | 22 | 23.0±13.3 | |
| Histological grade | | | | 0.004 |
| G1 | 2 | 0 | not reached | |
| G2 | 37 | 13 | 63.0±0.0 | |
| G3 | 38 | 20 | 20.0±3.7 | |
| Residual status | | | | 0.337 |
| negative (R0) | 65 | 26 | 63.0±23.2 | |
| positive (R1) | 7 | 5 | 35.0±11.1 | |

**Table 4.** Multivariate disease specific survival analysis (Variables were subdivided analogous to Table 3).

| Variable | Relative risk | 95% confidence interval | p-value |
|---|---|---|---|
| AGR2 | 0.809 | 0.346-1.893 | 0.635 |
| Disease stage | 4.884 | 2.014-11.844 | <0.001 |
| Histological grade | 1.720 | 0.786-3.765 | 0.174 |
| Residual status | 0.488 | 0.149-1.596 | 0.236 |
| Gender | 0.791 | 0.289-2.163 | 0.648 |

**Table 2.** Correlation of AGR2 expression in NSCLC with conventional clinical or tumour parameters.

| AGR2 | pT-status | pN-status | Grading | R-status | Histology | Age |
|---|---|---|---|---|---|---|
| correlation coefficient | 0.025 | -0.017 | -0.147 | -0.235 | -0.508 | -0.109 |
| significance (two-sided) | 0.811 | 0.867 | 0.154 | 0.026 | <0.001 | 0.292 |
| N | 95 | 95 | 95 | 89 | 95 | 95 |

R-status: residual status; Histology: adenocarcinoma, 0; squamous cell carcinoma, 1.

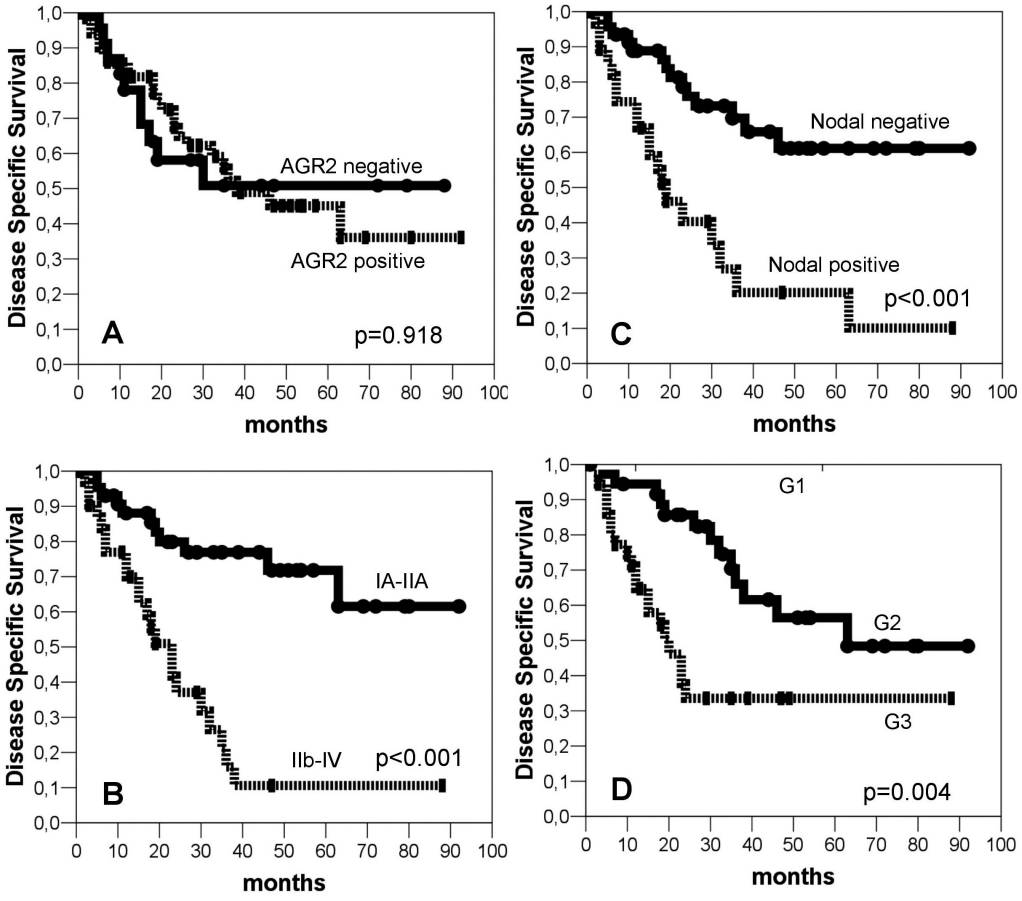


**Fig. 2**. Kaplan-Meier curves for Disease Specific Survival. **A**. Survival curves for AGR2 positive (dotted line) vs. negative (bold line) NSCLC (n=77). **B**. Survival curves for tumours of disease stage IA-IIA (bold line) vs. tumours of disease stage IIB-IV (dotted line). **C.** Survival curves for nodal negative (pN0, bold line) vs. nodal positive (pN1/2, dotted line) tumours. **D.** Survival curves for NSCLC delineated by histological grade (G2 = bold line, G2 = dotted line).

histologic tumour type, respectively. Only in the small sub-group (n=17) of small tumours (pT1) a significant difference (p=0.026) in favour of AGR2 positive carcinomas became apparent if AGR2-negative cases (n=7; events:4) were grouped versus AGR2-positive ones (n=10; events:2).

## Discussion

AGR2, the human orthologue of the *Xenopus* Anterior Gradient-2 (XAG-2) protein, is a small, possibly secreted molecule (Liu et al., 2005) of yet weakly defined functions that is expressed in several human tissues. In the *Xenopus* embryo XAG-2 is thought to be responsible for the patterning of the cement gland which is involved in the attachment of the Xenopus embryo to a solid support prior to swimming and feeding. In a former AGR2 expression study we have shown up-regulation of AGR2 (on mRNA and protein level) in breast tumour tissue and its association with estrogen receptor status, better tumour differentiation and a lower proliferative fraction (Fritzsche et al., 2006). The former two associations were recently further validated by Innes et al. (2006). In our study on breast cancer (Fritzsche et al., 2006) expression of AGR2 was significantly associated with longer overall survival times whereas Innes et al. (2006) found contrary results in the subgroup of estrogen receptor positive carcinomas. Pohler et al. (2004) found ARG2 over-expressed in Barrett´s epithelium, a condition defined by a metaplasia of persistent squamous epithelium into an intestinal epithelium, from which adenocarcinomas of the esophagus can arise. Very recently, Zhu et al. analysed three lung adenocarcinoma cell lines with array comparative genomic hybridisation and quantitative real time polymerase chain reaction. They demonstrated a strong up-regulation of AGR2 (Zhu et al., 2006) and concluded that AGR2 is a strong candidate oncogene in lung cancer. In our study we carefully analyzed the expression of AGR2 in 95 non small cell lung cancers on protein level and correlated these data to clinico-histopathological parameters. Two third of all NSCLC and specifically 85% of the adenocarcinomas of the lung expressed AGR2, which is likely to support the results of Zhu et al. (2006). It is of additional interest to notice, that our study is the first to demonstrate AGR2 expression in squamous cell carcinomas.

Of the conventional clinico-histopathological parameters histology (adenocarcinomas) and negative residual status were correlated with AGR2 expression (Table 2). The significant survival advantage of patients with AGR2 positive tumours in the sub-group of small tumours (pT1) is of exploratory value only. Although a favourable prognostic value of AGR2 would compare to our findings in breast cancer the very small number of cases in this sub-group does not allow any reasonable assumption in this direction. Therefore this finding might just be taken as a hint towards the necessity for additional studies with larger tumour cohorts.

In conclusion we demonstrated that AGR2 is commonly expressed in NSCLC and particularly in adenocarcinomas of the lung while a prognostic value of AGR2 could not be demonstrated. Functional studies to investigate the biological role of AGR2 in NSCLC as well as its differential expression according to histology are clearly warranted.

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