Coexpression of MUC1 Glycoprotein with Multiple Angiogenic Factors in Non-Small Cell Lung Cancer Suggests Coactivation of Angiogenic and Migration Pathways

Alexandra Giatromanolaki, Michael I. Koukourakis, Efthimios Sivridis, Keneth O’Byrne, Giles Cox, Philip E. Thorpe, Kevin C. Gatter, and Adrian L. Harris

Department of Pathology, Democritus University of Thrace, Alexandroupolis 68100, Greece [A. G., E. S.]; Department of Radiotherapy/Oncology, University of Thessalia, Medical School, Larisa, Greece [M. I. K.]; Department of Oncology, Leicester Royal Infirmary, Leicester LE1 5WW, United Kingdom [K. O., G. C.]; Department of Pharmacology, University of Texas Southern Medical Center, Dallas, Texas 75235-9041 [P. E. T.]; and Departments of Cellular Science and Imperial Cancer Research Fund-Medical Oncology Unit, Oxford Radcliffe Hospital, Headington, Oxford OX3 7LJ, United Kingdom [K. C. G., A. L. H.]

ABSTRACT

We investigated the expression of MUC1 protein and its relationship to the microvessel density and the expression of thymidine phosphorylase, vascular endothelial growth factor (VEGF), VEGF-receptor KDR, basic fibroblast growth factor (bFGF), and bFGF-receptor (FGFR-2) in non-small cell lung cancer. Although MUC1 expression was found equally in poorly and highly vascularized tumors, a significant coexpression with multiple angiogenic factors and their receptors was noted ($P = 0.0002, 0.03, 0.19, 0.10, and 0.01$ for thymidine phosphorylase, VEGF, KDR, bFGF, and FGFR-2, respectively). In multiple regression analysis, both angiogenesis and MUC1 expression were independent prognostic variables. The present study suggests the existence of an early genetic event leading to the activation of both migration and angiogenesis pathways in non-small cell lung cancer.

INTRODUCTION

MUC1, also known as episialin and as PEM, EMA, or CA-15-3 antigen, is a transmembrane glycoprotein expressed at the apical side of the normal glandular epithelial cells. In cancer cells, a depolarized expression throughout the entire cell surface has been reported (1, 2). In vitro studies suggested that MUC1 reduces the E-cadherin-mediated cell-cell adhesion by steric hindrance, which results in an increased metastatic ability (3). High MUC1 levels also reduce the integrin-mediated cell adhesion to the extracellular matrix (4). An important interaction of MUC1 glycoprotein with the immune system has also been shown. A MHC-non-restricted T-cell cytotoxicity elicited by MUC1 has been observed (5). However, MUC1-derived peptides are processed in the context of HLA3 class I antigen presentation and are targets of HLA class I-restricted T-cell cytotoxicity (6, 7).

Despite the large number of publications on the subject, the exact role and the clinical importance of MUC1 glycoprotein are not well understood. In a recent investigation, we showed that MUC1 is up-regulated in NSCLC and, although not associated with lymph node metastasis, its expression confers a poor prognosis in early operable cases with nodal involvement (1). Lymph node metastasis is strongly related to intratumoral angiogenesis in NSCLC (8). However, links between MUC1 glycoprotein and angiogenesis have not been reported. In the present study, we examined the overexpression of MUC1 glycoprotein in conjunction with the microvessel density and the expression of the angiogenic factors TP, VEGF, and bFGF and of the receptors KDR and FGFR-2 in an extended series of NSCLCs.

MATERIALS AND METHODS

We examined 199 tumor surgical samples from patients with operable NSCLCs treated with surgery alone without radiotherapy or chemotherapy. Patients and disease characteristics are shown in Table 1. The follow-up was available in 181 patients. Patients who died within 2 months after surgery were excluded from survival analysis to avoid bias from perioperative death. For patients alive, the median follow-up was 1400 days (range, 710-3110 days).

MUC1 Immunohistochemistry. Although in a recent study in gastric cancer, MUC1 expression was associated with poor outcome irrespective of the glycosylation status, the patterns of glycosylated and nonglycosylated MUC1 expression were not identical (9). The glycosylated form of episialin MUC1 expression was assessed on paraffin-embedded material using the Ma695 (IgG1) MAb (YLEM, Italy), recognizing a carbohydrate epitope of the MUC1 glycoprotein. Results from staining with the 214D4 MAb (IgG1) recognizing the protein backbone

Received 10/25/99; revised 1/24/00; accepted 1/31/00.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the Tumor and Angiogenesis Research Group (TARG) and the Imperial Cancer Research Fund (ICRF).

2 To whom requests for reprints should be addressed, at Tumour and Angiogenesis Research Group, 18 Dimokratias Avenue, Iraklion 71306, Crete, Greece. Phone: 0030-932-480808; Fax: 0030-81-284661; E-mail: targ@her.forthnet.gr.

3 The abbreviations used are: HLA, human lymphocyte antigen; NSCLC, non-small cell lung cancer; VEGF, vascular endothelial growth factor; TP, thymidine phosphorylase; FGF, fibroblast growth factor; bFGF, basic FGF; FGFR, FGF receptor; MAb, monoclonal antibody; APAAP, alkaline phosphatase/antialkaline phosphatase.
of episialin were also available in a subset of 110 patients, analyzed in previous studies of ours (1, 2). The avidin biotin complex immunoperoxidase technique was used as described previously. Overexpression of the episialin results in a circumferential cytoplasmatic and membrane immunoreactivity, which is never seen in normal cells (1, 2). The percentage of cancer cells with episialin overexpression was recorded.

**Angiogenesis Assessment.** The JC70 MAb (DAKO) recognizing CD31 (platelet/endothelial cell adhesion molecule; PECAM-1), was used for microvessel staining on 5-μm paraffin-embedded sections using the APAAP procedure as described previously (8).

Microvessel counting was used for angiogenesis assessment as reported previously (8). The areas of the highest vascularization were chosen at low power (×100), and microvessel counting followed on three chosen ×200 fields of the highest density. The microvessel score (MS) was the sum of the vessel counts obtained in these three fields. Vessels with a clearly defined lumen or well-defined linear vessel shape but not single endothelial cells were taken into account for microvessel counting. Microvessel score >74 defined high vascular grade, 45–75 defined medium vascular grade, and <45 defined low vascular grade. These cutoff points were based on a previous study of ours (8).

**Angiogenic Factor Immunohistochemistry.** TP expression was assessed with the P-GF.44C MAb using the streptavidin-biotin-peroxidase technique as described previously (10). Two staining groups were considered according to our previous study (10): low/medium reactivity (0–50% of cells stained or weak diffuse staining intensity) and high reactivity (strong intensity in >50% of cells).

VEGF expression was assessed with the VG1 MAb (recognizing the 121, 165, and 189 isoforms) with the APAAP technique and microwaving for antigen retrieval (11). The mean percentage of positive cancer cells (56%) was used as a cutoff point to distinguish between cancers with low and high VEGF reactivity. Microvessel counting of the VEGF/KDR positive vessels was also performed, in ×200 optical fields, in the tumor-invading front. The mean microvessel score (mean, 15; range, 0–45) was used as a cutoff point to distinguish between tumors with or without VEGF/KDR up-regulated angiogenic pathway.

The cytoplasmic bFGF and its “bek” receptor (FGFR-2) expression was assessed in cancer cells, using the APAAP technique. We used the FGF-2 (147)-G and the Bek(C-17)-G MAb respectively (Santa Cruz Biotechnology). The immunostaining was performed in a subset of 105 randomly selected cases that were also stained for the Ma695 MAb. The mean percentage of positive cells was used as a cutoff point to define two groups of low and high reactivity (66% and 17% for bFGF and FGFR-2, respectively).

**Statistical Analysis.** Statistical analysis and graphs were performed using the Pism 2.01 and the Instat 3.0 packages (GraphPad, San Diego, CA).4 Fisher’s exact t test was used for testing relationships between categorical tumor variables as appropriate. Nonparametric analysis was used to assess correlation between continuous variables. The Cox proportional hazard model was used to assess the effects of patient and tumor variables on response, local relapse, and survival. A P < 0.05 was considered significant.

**RESULTS**

**MUC1 Overexpression.** The mean percentage of cells with MUC1 overexpression was 27 ± 32 (range, 0–100) for the glycosylated form-detecting antibody and 24 ± 30 (range, 0–100) for the core-detecting antibody. MUC1 overexpression in more than the mean was noted in 73 (36%) of 199 and in 43 (39%) of 110 cases for the two MAb, respectively. These cases were considered as positive. There was a significant concordance between the two MAb (P = 0.0001; r, 0.60). There was no association of MUC1 expression with histological type, T stage, N stage, histological grade, age, and sex.

Table 2 shows the association of the glycosylated MUC1 (Ma695) expression with the vascular grade and the expression of other molecular variables. Although MUC1 expression was not related to the vascular grade, a strong association of positive MUC1 expression with high TP expression was noted (P = 0.0003). This was verified for squamous cell carcinomas (P = 0.0001). In adenocarcinomas, although cases with high MUC1 expression frequently showed high TP reactivity (13 of 27 versus 16 of 45), the difference did not reach significance because of the small number of cases analyzed (P = 0.22).

Continuous variable analysis showed that MUC1-positive cases had a significantly higher mean number of TP positive cells ($P = 0.0002$).

MUC1 expression was also linked to high VEGF expression ($P = 0.04$ and 0.03 for VG1 and 11B5 MAbs, respectively). Separate analyses in squamous cell carcinomas and adenocarcinomas showed a similar trend in both groups but the differences were no longer significant (for VG1 MAb: $P = 0.18$ and 0.43, respectively) because of the smaller number of patients in the subgroups. Continuous variable analysis showed that MUC1-positive cases had a significantly higher mean number of VEGF-positive cells ($P = 0.04$).

Although MUC1 overexpression was more frequent in cases with up-regulated VEGF/KDR angiogenic pathway, the difference did not reach significance ($P = 0.19$). Analysis in 105 patients showed a direct association of MUC1 overexpression with the FGFR-2 expression ($P = 0.01$) and a trend with bFGF ($P = 0.10$). The smaller number of cases analyzed for the variables did not allow a subanalysis in the two histology subgroups.

Analysis of MUC1 with the 214D4 core MAb in 110 cases (data not shown) showed a significant association with TP expression ($P = 0.004$), whereas the association with VEGF (VG1) did not reach significance ($P = 0.12$). Similarly, the association of the 214D4 reactivity was marginally associated with the FGFR-2 ($P = 0.06$), but there was no association with the bFGF expression ($P = 0.29$).

Other Correlations. VEGF expression was statistically correlated with VEGF/KDR up-regulated angiogenic pathway ($P < 0.0001$) and bFGF expression ($P < 0.0001$, $r = 0.36$ for the VG1 MAb; $P = 0.006$, $r = 0.26$ for the 11B5 MAb). No association of VEGF with the FGFR-2 was observed. A direct association of bFGF with FGFR-2 expression was noted ($P = 0.001$; $r = 0.28$).

Survival Analysis. In univariate analysis, advanced T and N stage, MUC1 (Ma695) overexpression, and high VG were statistically associated with worse prognosis ($P < 0.001$). Table 3 shows the prognostic significance of the analyzed variables in two multivariate models. All of the 4 variables maintained their independent prognostic meaning (multivariate mode 1). In early operable stages ($T_{1-2}$-$N_0$), only the high VG and MUC1 expression maintained their independent prognostic significance (multivariate model 2).

DISCUSSION

MUC1 is a glycoprotein with multiple functions including cancer cell migration and immune response (3–6). A strong association of MUC1 overexpression with prognosis has been reported in breast (13), colon (14), and gastric cancer (15). The expression of several mucin genes in lung carcinoma cell lines has been recently reported (16). The MUC1 protein is expressed in both adenocarcinoma and squamous cell carcinomas of the lung (2). Ohgami et al. (17) reported that high expression of MUC1 (assessed with reverse transcriptase-PCR) is associated with early recurrence and hematogenic metastasis in stage I lung adenocarcinoma. In a recent study (1), we also showed a strong association of MUC1 overexpression with poor prognosis in NSCLC. Although N stage is considered the most important prognostic factor in early operable disease, surprisingly MUC1 expression defined a group of patients with poor prognosis in the N1 stage. In the present study, we confirmed an important prognostic role of MUC1 expression in NSCLC, which is independent from T and N stage or even from intratumoral angiogenesis.

We compared the MUC1 expression with the angiogenesis and with the expression of several angiogenic factors in a large series of NSCLCs. Although MUC1 overexpression was found with equal frequency in cases with low or high microvessel density, a strong association with the expression of the angiogenic factor TP was noted. A significant coexpression of MUC1 with VEGF and the bFGF receptor was also observed. MUC1 association with bFGF did not reach significance, but this may be a consequence of the large number of modified isoforms for example, cleavage and phosphorylation) impossible to detect with the used MAb. On the other hand, bFGF was significantly correlated with the FGFR-2 as well as the VEGF expression. These observations strongly suggest that malignant transformation includes a simultaneous activation of both angiogenic and migration-related pathways. The interaction between MUC1 and ICAM-1 of endothelial cells has been recently reported to be a critical event for the appearance of blood-borne metastases (18). Furthermore, this points to a coordinated transformation with two complementary aims: metastasis and angiogenic ability that will allow higher chances of cell survival and colony formation in distant organs. Whether the display of these two pathways is necessary for the metastasis to occur requires further investigation. The existence of highly angiogenic tumors with low metastatic ability is well known. In support of the idea of a coor-

Table 2  Correlation of MUC1 expression (Ma695 MAb) with vascular grade and angiogenic factor and receptor expression

<table>
<thead>
<tr>
<th>Parameter</th>
<th>No. of patients</th>
<th>MUC1 Negative</th>
<th>MUC1 Positive</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vascular Grade</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>199</td>
<td>55</td>
<td>35</td>
<td></td>
</tr>
<tr>
<td>Medium</td>
<td>22</td>
<td>13</td>
<td></td>
<td>0.79</td>
</tr>
<tr>
<td>High</td>
<td>49</td>
<td>25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP</td>
<td>199</td>
<td>95</td>
<td>36</td>
<td>0.0003</td>
</tr>
<tr>
<td>Low</td>
<td>31</td>
<td>37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>53</td>
<td>42</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF (VG1)</td>
<td>199</td>
<td>73</td>
<td>31</td>
<td>0.04</td>
</tr>
<tr>
<td>Low</td>
<td>26</td>
<td>11</td>
<td></td>
<td>0.03</td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF/KDR vessels</td>
<td>90</td>
<td>34</td>
<td>21</td>
<td>0.19</td>
</tr>
<tr>
<td>No</td>
<td>16</td>
<td>19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>29</td>
<td>13</td>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td>bFGF</td>
<td>105</td>
<td>42</td>
<td>20</td>
<td>0.01</td>
</tr>
<tr>
<td>Low</td>
<td>26</td>
<td>23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>24</td>
<td>29</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Not-up-regulated VEGF/KDR angiogenic pathway.

* Up-regulated VEGF/KDR angiogenic pathway.
Table 3  Multivariate analysis of overall survival in 181 cases of NSCLC

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Categories</th>
<th>Model [1]**</th>
<th></th>
<th></th>
<th>Model [2]**</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>P</td>
<td>t ratio</td>
<td>SE</td>
<td>P</td>
<td>t ratio</td>
</tr>
<tr>
<td>T stage</td>
<td>(T3 vs. T2) T2 vs. T1</td>
<td>0.001</td>
<td>3.31</td>
<td>0.06</td>
<td>0.001</td>
<td>1.92</td>
</tr>
<tr>
<td>N stage</td>
<td>(N2 vs. N1) N1 vs. N0</td>
<td>0.04</td>
<td>1.97</td>
<td>0.05</td>
<td>0.04</td>
<td>1.76</td>
</tr>
<tr>
<td>Vascular grade</td>
<td>High vs. medium/low</td>
<td>0.005</td>
<td>2.79</td>
<td>0.07</td>
<td>0.005</td>
<td>2.29</td>
</tr>
<tr>
<td>MUC1 (Ma695)</td>
<td>Positive vs. negative</td>
<td>0.0001</td>
<td>4.45</td>
<td>0.07</td>
<td>0.0001</td>
<td>4.24</td>
</tr>
</tbody>
</table>

**All cases.

* Excluding T3 and N2 stage cases.

The nature of this early pathogenetic pathway leading to the appearance of a double migratory/angiogenic phenotype should be sought with in vitro studies. Immune surveillance, recognizing migration-related proteins, may have a critical role in the selection of clones with predominantly quiescent, migratory, or angiogenic properties.

REFERENCES
10. Koukourakis, M., Giatromanolaki, A., Kakolyris, S., O’Byrne, K., Apostolikas, N., Skarlatos, J., Gatter, K. C., and Harris, A. L. Different patterns of stromal and cancer cell thymidine phosphorylase reactivity in
Coexpression of MUC1 Glycoprotein with Multiple Angiogenic Factors in Non-Small Cell Lung Cancer Suggests Coactivation of Angiogenic and Migration Pathways

Alexandra Giatromanolaki, Michael I. Koukourakis, Efthimios Sivridis, et al.


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/6/5/1917

Cited articles  This article cites 21 articles, 15 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/6/5/1917.full#ref-list-1

Citing articles  This article has been cited by 10 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/6/5/1917.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.