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Lung Cancer Protein Expression Profiles of Smokers and Non-Smokers

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Abstract. *Epidemiological studies have established a causal relationship between cigarette smoking and respiratory tract cancer. The knowledge about the interaction of tobacco constituents with cellular systems is, however, still incomplete. Therefore, we analyzed 36 factors with known or assumed relevance and found that 8 proteins in lung cancer were associated with the smoking habits of 94 patients. These 8 factors belong to different functional classes including products of drug resistance-related proteins (P-glycoprotein, glutathione S-transferase-π, lung resistance protein, catalase), proto-oncogenes and transcription factors (FOS, JUN, HIF-1β), and proliferative factors (cyclin D). By means of hierarchical cluster analysis, we were able to show that the 94 patients analyzed could be separated into three different clusters, of which one contained significantly more patients who smoked than the others (p=0.0026). This cluster also contained significantly more drug-resistant tumors than the others (p=0.0069), pointing to a close interrelationship between the smoking habits of patients and drug resistance of tumors.*

Although epidemiological studies have established a causal relationship between cigarette smoking and respiratory tract cancer (1), the knowledge about the interaction of tobacco constituents with cellular systems is, however, still incomplete. Therefore, we analyzed 36 proteins in non-small cell lung carcinoma including drug resistance-related proteins, oncoproteins, apoptosis-regulating proteins, heat shock proteins, angiogenesis factors, DNA repair proteins, and proliferative factors and investigated their interrelationships with the smoking habits of the patients.

Proteins from the above-mentioned categories have been associated with carcinogenesis in general as well as to cancer development induced by smoking. Oncogenes have been addressed as possible links between cigarette smoking and carcinogenesis. It is known that exposure to chemical carcinogens leads to the activation of proto-oncogenes in animal model systems (2). In human beings, relationships between the up-regulation of proto-oncogenes and smoking have also been found (3,4). After initiation of carcinogenesis, *i.e.* by DNA damage and activation of proto-oncogenes, proliferative stimuli are necessary to promote cancer growth. Cyclin and cyclin-dependent kinases are universal regulators of cellular proliferation (5). An interrelationship has been reported between smoking and drug resistance. Some data indicates that chemical carcinogens not only cause cancer, but are also resistant to xenobiotics including anti-neoplastic drugs and decrease chances for successful chemotherapy (6, 7). The view that oncogenesis and therapy resistance represent parts of common cellular alterations gained considerable attractiveness to explain the inherent unresponsiveness of many tumors. The fate of growing tumors depends on the blood vessel system to obtain a supply of nutrients and oxygen. Otherwise, tumor cells become hypoxic and die. Interestingly, cigarette smoke affects angiogenesis (8) and apoptosis (9). Furthermore, smoking habits correlate with DNA repair capacity, telomerase activity and heat shock protein function (10-12), and these mechanisms participate in the surveillance of the cellular integrity and defense against tumor cells.

In order to understand the complex network of proteins in carcinogenesis, it is not sufficient to investigate single parameters, as was usually done in the past. Instead, analyses of multiple proteins may be more appropriate to gain insight in this process. Therefore, we analyzed whether hierarchical cluster analysis is capable of differentiating between smokers and non-smokers in lung cancer patients.

Materials and Methods

Patients and tumors. Ninety-four patients (83 men, 11 women) with previously untreated NSCLC were admitted to this investigation. The morphological classification of the carcinomas was conducted according to the WHO specifications (13). Of the carcinomas, 48 were squamous carcinomas, 34 were adenocarcinomas and 12 were

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large cell carcinomas. All patients were staged at the time of their surgery according to the guidelines of the American Joint Committee on Cancer (14,15). Sixteen patients had stage I, 12 patients stage II and 66 patients had stage III tumors. The mean age of the patients was 59 years. In our patients collective, 22 patients were non-smokers, and 72 patients smokers. Nine of the smokers smoked 1-10 cigarettes, 25 patients 11-20, 12 patients 21-30, 13 patients 31-40 and 5 patients more than 40 cigarettes daily. Cigarettes consumption could not be determined exactly for 8 smokers.

Detection of drug resistance in vitro. Most of the patients were treated by surgical procedures alone. Only a small group of patients was treated by combined surgical and radiation treatment or chemotherapy, but their additional radiation treatment and chemotherapy had no significant effect on patient survival time ($p>0.1$). For determining the drug resistance of tumors, we used a short-term *in vitro* test that has been described previously (16, 17). Its basic feature is the measurement of changes in the incorporation of radioactive nucleic acid precursors into tumor cells after addition of doxorubicin. Cell suspensions were incubated with doxorubicin at different concentrations for 3 h at 37˚C. ^{3}H-uridine was added during the last hour of incubation. Aliquots of the cell suspensions were pipetted onto filter paper discs, the acid-soluble radioactivity was extracted and the incorporated activity measured by scintillation counting. We found that anthracyclines (*e.g.*, doxorubicin) can be used as reference compounds for multiple resistance. Tumors were defined as being sensitive or resistant depending on whether nucleotide uptake was inhibited by more or less than 65% at a concentration of 10 μg/ml doxorubicin. This threshold was based on prior clinical correlation (16).

Immunohistochemistry. The previously described method was applied for formalin-fixed and paraffin-embedded specimens (18, 19). Briefly, formalin-fixed and paraffin-embedded tissue were deparaffinised. After pre-incubation with hydrogen peroxide and protein blocking solution, the primary antibodies were applied for 16 h at 4˚C. After incubation with secondary antibodies, the streptavidin-biotinylated peroxidase complex was added and the peroxidase activity visualised with 3-amino-9-ethylcarbazole. Counter-staining was performed with haematoxylin. Both negative and positive controls were conducted. Negative controls were prepared by omitting the primary antibodies and by substituting irrelevant antibodies for the primary antibodies. The specificity of the reactions were proved by Western blots. Three observers independently evaluated the results from the immunohistochemical staining without having any prior knowledge of an individual patient's clinical data. The evaluations agreed in 90 % to 95 % of the samples. The other specimens (5% to 10%) were re-evaluated and then classified according to the classification most frequently given by the observers. To evaluate the protein expression, the staining intensity or the staining intensity and the percentages of positive cells were determined. The immunohistochemical parameters were evaluated on either a binary scale ("no reaction" or "reaction", coded as 0 or 1) or an ordinal scale "no reaction", "weak", "moderate" or "strong reaction" (coded as 1, 2, or 3).

For the detection of proto-oncogene products and transcription factors, we used the following antibodies: FOS (clone Ab-2), JUN (clone c-Jun/AP-1), ERBB1 (clone Ab-4), ERBB2 (clone AB-3), MYC (clone Ab-3) and pan-RAS (clone Ab-1). All antibodies for these proteins were purchased from Dianova (Hamburg, Germany) and were applied at a concentration of 10 μg/ml. The tumor suppressor protein p53 was analyzed using antibody clone DO-1 (Oncogene Science, Cambridge, USA) at a concentration of 1:100. Staining for p16INK4A protein was carried out using clone C20 sc-468 from Santa Cruz Biotechnology (Heidelberg, Germany) at a dilution of 1:50. Anti-HSP70 was from DAKO Diagnostika (Hamburg, Germany; dilution 1:500). Mouse monoclonal anti-HIF-1α (clone HIF-1α 67; dilution 1:1,000) and rabbit polyclonal and anti-HIF-1β (dilution 1:1,700) were from Novus Biologicals (Littleton, CO, USA). For detection of the proliferative activity, anti-cyclin A (clone H-432, dilution 1:50) from Santa Cruz Biotechnology, anti-cyclin D1 (clone Ab-3, dilution 1:10) from Calbiochem/ Novabiochem (Baden-Soden, Germany), anti-CDK2 (clone M2; dilution 1:200) from Santa Cruz Biotechnology, and anti-CDK4 (clone C22; dilution 1:100) from Santa Cruz Biotechnology were used. Anti-E2F1 clone KH95 was purchased from Santa Cruz Biotechnology and used at a dilution of 1:50. The polyclonal anti-DNA topoisomerase IIα (Topo IIα) antibody was obtained from Dr. L. Liu (John Hopkins Oncology Center, Baltimore, MD, USA; working dilution 1:500) and the polyclonal anti-thymidylate synthetase (TS) antibody from Dr. B. Yates (Burroughs Welcome, Research Triangle Park, Cornwallis, USA; working dilution 1:500). Anti-glutathione S-transferase-π (dilution 1:2,000) was kindly donated by Dr. K. Satoh (University School of Medicine, Hirosaki, Japan). The catalase antibody was purchased from Calbiochem (La Jolla, CA, USA;) and used in a working dilution of 1:100. Anti-P-glycoprotein (JSB-1, 10μg/ml) was obtained from Sanbio (Uden, Netherlands) and anti-lung resistance protein (LRP-56, dilution 1:20) from Dunn Labortechnik (Asbach, Germany). The metalliothionein antibody (dilution (1:100) was from Dr. P.C. Huang (John Hopkins University, Baltimore, MD, USA). The DNA repair enzyme O^6-MGMT was detected with clone 5H7 obtained from Dr. B. Li (Singapore) in a dilution of 1:100. As apoptotic factors, we analyzed CD95 (clone UB-2; Immunotech, Hamburg, Germany; dilution 1:100), Fas ligand (clone Q20; Santa Cruz Biotechnology; dilution 1:500) and caspase-3 (clone CPP32p20[E-8], Santa Cruz Biotechnology; dilution 1:500). The antibodies for staining angiogenic factors were anti-VEGF (clone AB-2, dilution 1:10) obtained from Dianova, anti-TF (clone TBF, dilution 1:50) from Biodesign (Kennebunk, MA, USA), anti-bFGF (clone 147, dilution 1:200) from Santa Cruz Biotechnology, anti-PD-ECGF (clone 654-1, dilution 1:50) which was a generous gift of Dr. Tanaka (Nippon Roche Research Center, Kamakura, Japan), anti-angiostatin from Oncogene Research Products (clone Ab-1; Cambridge, MA, USA); dilution 1:150), TSP (clone 11.4; Roche Diagnostics, Mannheim, Germany; dilution 1:10), FGFR (flg, Santa Cruz Biotechnology; dilution 1:100), and VEGFR (flk/KDR, Santa Cruz Biotechnology; dilution 1:100) were further proteins that were analyzed in this study. For the determination of telomerase (TRT), we used clones C-20 and H-231 from Santa Cruz Biotechnology.

Statistical analysis. The immunohistochemical parameters were evaluated on an ordinal scale. All objects were assembled into a cluster tree (dendrogram). Hierarchical cluster analysis is an explorative statistical method and aims to group at first sight heterogeneous objects into clusters of homogeneous objects. Objects are classified by calculation of distances according to the closeness of inter-individual distances. The merging of objects with

Table I. *Expression of immunohistochemical markers (scores) in NSCLC.*

Proteins	Non-smokers	Smokers	Ratio
P-gp	0.73	1.58	**2.16**
CAT	0.27	0.50	**1.85**
JUN	0.58	1.02	**1.76**
LRP	0.68	1.09	**1.60**
FOS	0.77	1.10	**1.43**
GST-π	1.41	2.00	**1.42**
HIF-1α	0.32	0.43	**1.34**
Cyclin D	1.39	1.73	**1.24**
O^6-MGMT	1.59	1.89	1.19
p16INK4A	1.00	1.17	1.17
E2F1	0.43	0.50	1.16
CDK4	1.11	1.27	1.14
Angiostatin	1.42	1.62	1.14
MT	0.59	0.66	1.12
ERBB1	1.58	1.74	1.10
FGF	1.28	1.41	1.10
TS	0.68	0.72	1.05
HIF-1β	2.18	2.31	1.05
PD-ECGF	0.98	1.01	1.03
HSP70	1.71	1.77	1.03
TF	0.89	0.91	1.02
Telomerase	2.61	2.67	1.02
RAS	0.87	0.87	1.00
MYC	0.77	0.76	0.98
VEGF	1.35	1.32	0.98
TSP	1.90	1.85	0.97
FAS Ligand	1.84	1.79	0.97
CDK2	2.02	1.93	0.96
CD95	0.58	0.55	0.95
Caspase 3	0.75	0.70	0.93
P53	1.04	0.97	0.93
TOPO II	0.59	0.54	0.92
Cyclin A	1.73	1.59	0.92
FGFR	1.87	1.71	0.91
VEGFR	1.94	1.70	0.88
ERBB2	1.42	1.16	0.82

Abbreviations:
CAT, catalase; CDK, cyclin-dependent kinases; FAS (CD95), apoptosis-related receptor; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; GST-π, glutathione S-transferase-π ; HIF-1, hypoxia-inducible factor; MT, metallothionein ; O^6-MGMT, O^6-methylguanine-DNA-methyltransferase; PD-ECGF, platelet-derived endothelial growth factor; P-gp, P-glycoprotein 170; TF, tissue factor; Topo II, topoisomerase II; TS, thymidine synthase; TSP, thrombospondin; VEGF, vascular endothelial growth factor; VEGF-R, vascular endothelial growth factor receptor.

similar features leads to the formation of a cluster, where the length of the branch indicates the degree of relatedness. Thus, objects with tightly related features appear together, while the separation in the cluster tree increases with progressive dissimilarity. Cluster analyses applying the complete linkage method were done by means of the WinSTAT program (Kalmia Company). In order to calculate distances of all variables included in the analysis, the program automatically standardizes the variables by transforming the data with a mean=0 and a variance=1. To construct clustered-image maps (CIM) two dendograms were related to each other. Fisher's exact test was used as an implement of the WinSTAT program.

Results

The objective of this study was to evaluate the expression profiles of proteins of non-small cell lung carcinomas (NSCLC) of smokers and non-smokers. As a first step, we analyzed the expression of 36 proteins (Table I). The scoring of protein expression was associated with the smoking habits of the lung cancer patients. As can be seen in Table I, the expression of 8 out of 36 proteins was higher in smokers than in non-smokers with a ratio of 1.2 or higher (FOS, JUN, Cyclin D, P-gp, GST-π, CAT, LRP, HIF-1α).

As a next step, we aimed to ascertain, whether these 8 factors in lung cancer were indeed capable of distinguishing smokers and non-smokers. For this reason, we subjected the protein expression of these 8 factors in primary NSCLC of 94 patients to hierarchical cluster analysis and clustered image mapping. In the dendrogram shown in Figure 1 (right side) that shows the 94 patients included into the analysis, we obtained three main clusters of tumors. The cluster image map (CIM) derived from this dendrogram and from the clustering of the 8 proteins (Figure 1, top) can be subdivided into three areas. Most of the investigated proteins were more frequently up-regulated in area 2. In Table II, the mean values ± SE of expression levels of the immunohistochemical markers (scores) are given for the carcinomas of these clusters.

Then, we assigned the smokers and non-smokers to the corresponding clusters. As shown in Table III, the distribution of smokers and non-smokers was statistically different between the three clusters ($p=0.034$). If the cases of cluster 1 and 3 were combined (19 non-smokers *vs*. 35 smokers) and compared to the cases of cluster 2 (3 non-smokers *vs*. 37 smokers), the relationship was also statistically significant ($p=0.0026$, Fisher's exact test). Although this relationship was statistically significant, the separation of smoking and non-smoking patients by these 8 factors was incomplete in the cluster analysis indicating that other factors may also play a role for the cluster formation.

In order to prove whether or not interrelationships exist, we correlated different clinical factors with the affiliation to the three clusters. Indeed, we found that the clustering separated sensitive tumors from resistant ones as well indicating a close relationship between smoking habits of patients and drug resistance of tumors (Table IV).

In contrast, we did not find such an interrelationship with other clinical factors (histology, metastasis). For instance, cluster 1 contained 14 adenocarcinomas, 21 squamous cell carcinomas and 3 large cell carcinomas and cluster 2, 11

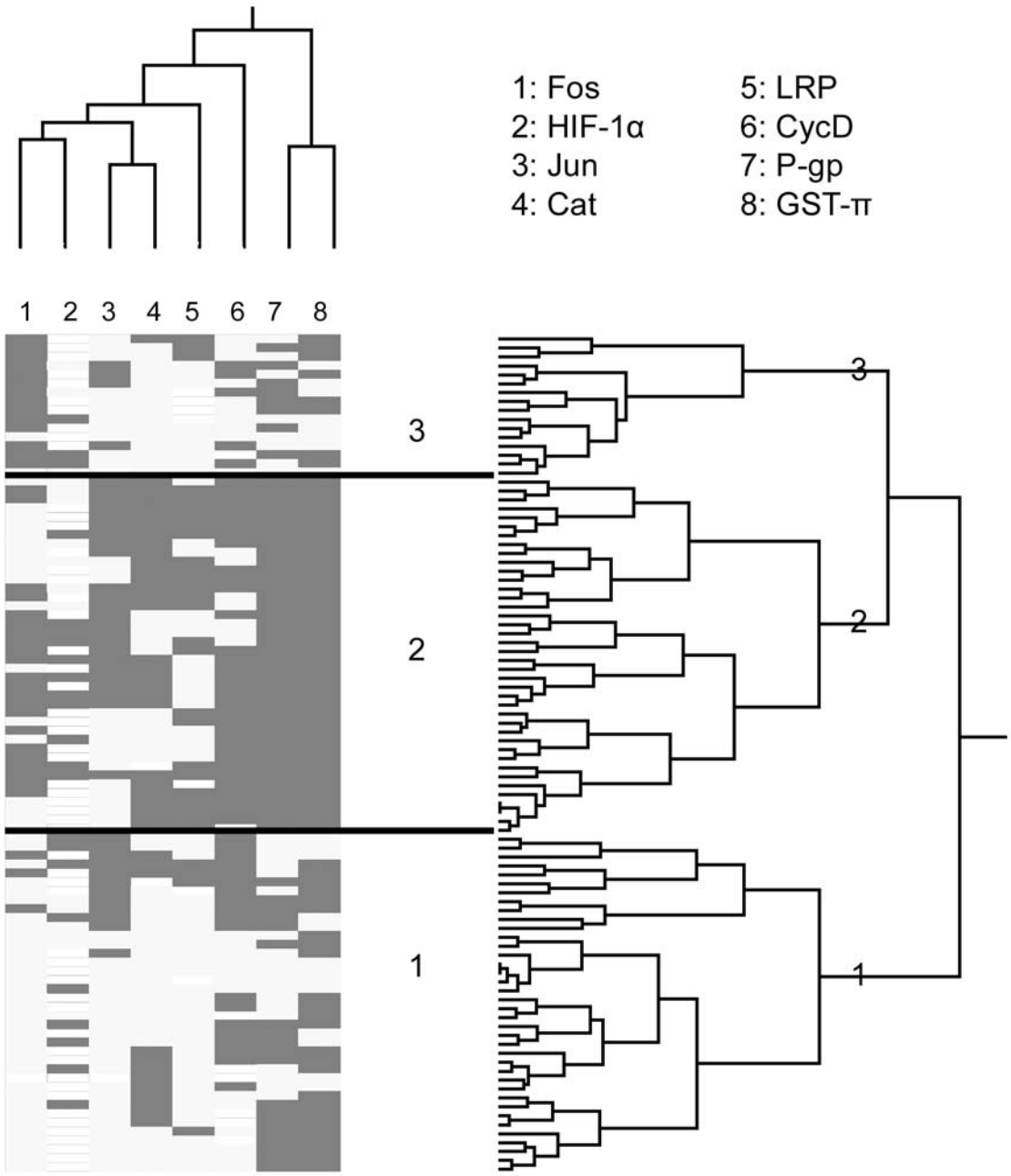

Figure 1. *Dendrograms and clustered image map obtained by hierarchical cluster analysis (complete linkage method). Light fields indicate "not expressed" and dark fields indicate "expressed". Missing values are depicted in white.*

adenocarcinomas, 21 squamous cell carcinomas and 8 large cell carcinomas. The distribution in cluster 3 was: 9 adenocarcinomas, 6 squamous carcinomas and one large cell carcinoma. The distribution of tumors with different histology among the three clusters was statistically not significant.

The same applies for the distribution of metastasizing and non-metastasizing tumors. In cluster 1, 11 carcinomas were metastasizing and 26 were not. Cluster 2 contained 16 metastasizing and 26 non-metastasizing tumors, while cluster 3 consisted of 6 carcinomas without and 9 tumors with metastases. Again, there was no significant relationship.

This indicates a clear relationship between the smoking habits of patients and drug resistance of lung cancers. If one compares the distribution of smokers and drug-resistant tumors between the different clusters, a concordance of 88% can be found in cluster 2, while a

Table II. *Expression of immunohistochemical markers (scores) in NSCLC of the three clusters obtained in the dendrogram shown in Figure 1.*

	Cluster 1+3 (score)[a]	Cluster 2 (score)[a]	Ratio Clusters 2/1+3
CAT	0.25±0.01	1.13±0.03	4.5
JUN	0.52±0.02	1.94±0.06	3.7
P-gp	0.67±0.01	2.28±0.02	3.4
FOS	0.90±0.02	2.77±0.04	3.1
GST-π	1.19±0.02	2.70±0.01	2.3
LRP	0.38±0.02	0.73±0.01	1.9
CYCD	1.17±0.03	1.74±0.03	1.5
HIF-1α	1.80±0.06	1.38±0.02	0.8

[a] mean values±SE
Abbreviations: see Table I

Table III. *Clusters of lung tumor patients obtained by hierarchical cluster analysis shown in Figure 1 and comparison to smoking habits of patients.*

	Non-Smokers	Smokers	Exact Test
Cluster 1	14	24	
Cluster 2	3	37	
Cluster 3	5	11	$p=0.034$
Cluster 1+3	19	35	
Cluster 2	3	37	$p=0.0026$

Table IV. *Clusters of lung tumor patients obtained by hierarchical cluster analysis shown in Figure 1 and comparison to drug resistance of lung cancers.*

	Sensitive	Resistant	Exact Test
Cluster 1	15	23	
Cluster 2	4	36	
Cluster 3	4	12	$p=0.0079$
Cluster 1+3	19	35	
Cluster 2	4	36	$p=0.0069$

concordance of 62% and 43 % in clusters 1 and 3, respectively, was found. The total concordance of all three clusters between smoking of patients and drug-resistance of tumors was 70%.

Discussion

The present investigation focuses on cigarette smoking and the expression of proteins in NSCLC. An association between smoking habits and FOS and JUN was found. The correlation of cyclin D with the smoking habits of NSCLC patients points to observations that carcinogens stimulate the growth of malignant cells (20, 21). Activated cyclin D1 and cdk4 complexes lead to cell progress into late G1- and early S- phase. The fact that the expression of typical drug resistance genes are significantly related with smoking habits is surprising at first sight. Glutathione S-transferases and catalase counteract DNA damage and are involved in both carcinogenesis of DNA-damaging xenobiotics and resistance to DNA-damaging anti-tumor agents (22-24). This kind of bifunctionality explains the link of these two genes between carcinogenesis and drug resistance. It is of interest that a tight connection between the smoking status of patients and drug resistance of tumors was found by means of hierarchical cluster analysis. This points to a long-lasting concept of carcinogenesis in general. As many carcinogens act in an anti-proliferative manner, the selection of cells resistant to these carcinogens is thought to be one of the very early steps in tumor development (25). This has been shown in experimental hepatocarcinogenesis models (6, 26) as well in cigarette smoke-induced lung carcinogenesis (27). Along with the adaptive cellular and molecular responses to carcinogenic compounds, the tumor cells become inherently resistant to chemotherapeutics (7). From these studies it became obvious that multidrug resistance proteins (P-glycoprotein and lung-resistance protein) also act in a carcinogenic fashion (28, 29).

The coordinated overexpression of FOS, JUN and drug resistance genes in smoking-related NSCLC found in our investigation points to a possible molecular mechanism by which drug resistance genes may be activated by cigarette smoking. The transcription factors FOS and JUN, both of which constitute the transcription factor "activating protein-1" (AP-1), may up-regulate drug resistance genes. The promoter sequences of P-glycoprotein and glutathione S-transferase genes harbor AP-1 binding motifs and both genes are transcriptionally activated upon AP-1 binding (30, 31). Whether the other investigated resistance proteins are also regulated by AP-1 is currently unknown. Although a role of hypoxia has been proposed in the carcinogenetic process (32), the connection between HIF-1β and the smoking habits of patients was rather poor.

Summing up, by means of hierarchical cluster analysis, we were able to show that the 94 patients analyzed could be separated into three different clusters of which one contained significantly more patients who smoked than the others.

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