Prognostic impact of AMP-activated protein kinase expression in ovarian carcinoma: Correlation of protein expression and GC/TOF-MS-based metabolomics

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Abstract. AMP-activated protein kinase (AMPK) plays a central role in regulating energy metabolism in cells. AMPK activation results in down-regulation of anabolic pathways (e.g., fatty acid biosynthesis) and switches on catabolic processes such as glucose uptake, glycolysis or fatty acid oxidation. Recent studies in cell culture models have shown that the growth of tumor cell lines was inhibited by AMPK activation, but the expression of AMPK in human ovarian tumors has not been reported so far. In this study we investigated AMPK expression in a cohort of 70 ovarian carcinomas, 14 borderline tumors and 5 normal ovaries and linked the protein expression data to Gas chromatography/time of flight mass spectrometry (GC/TOF-MS) based metabolomics. We observed a significantly higher expression in ovarian carcinomas compared to borderline tumors and normal ovaries (p=0.038). Decreased AMPK expression correlated significantly with higher tumor grade (p=0.009) and was of adverse prognosis in patients with advanced tumor stages (p=0.016) as well as in patients with serous ovarian carcinomas (p=0.037). GC/TOF-MS based metabolomics revealed a significantly higher concentration of glucose in AMPK-negative carcinomas (p=0.022) as well as over-expression of other metabolites from carbohydrate metabolism. Our results indicate a role for AMPK in progression of ovarian tumors and point towards a prognostic impact of AMPK expression for patient overall survival. Furthermore, our data suggest a deregulation of the AMPK-dependent energy metabolism in human ovarian carcinomas. In future clinical studies, activation of AMPK in ovarian carcinoma patients with advanced tumor stages might be an interesting therapeutic approach.

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Introduction

Ovarian carcinoma is the fifth most common cause of cancer death in women in the Western world. In the United States, an estimated number of 21,550 new cases and an estimated number of 14,600 deaths of ovarian carcinomas were expected for 2009 (1). This poor outcome is mostly due to the advanced tumor stage by the time of diagnosis. Early symptoms of disease are usually missing and no sufficient screening method exists, so far. More than half of the patients present with extensive peritoneal carcinosis. Current treatment options are based on radical cyto-reductive surgery followed by chemotherapy. Nevertheless, 5-year survival rate is only 13% in patients with distant disease [stage IV according to the International Federation of Gynecology and Obstetrics (FIGO)] and 30-50% for FIGO stage III. In contrast, the 5-year survival rate in patients with disease limited to the ovary (FIGO stage I) is 65-90% (2).

Metabolites are the end products of cellular biochemical pathways. The detection and identification of metabolites in tissue samples is called 'metabolomics' in analogy to the terms 'genomics' and 'proteomics'. This analysis provides an impression of the occurring biochemical processes in tissue and can be regarded as a dynamic portrait of the metabolic status of living systems (3). Already in 1924 the scientist Otto Heinrich Warburg observed high levels of lactic acid in cancer cells compared to normal cells. This led him to the hypothesis that in cancer cells the energy generation is switched from oxidative phosphorylation towards anaerobic glycolysis,

which allows malignant cells to grow independently of the oxygen supply (4). Notable metabolic changes in cells might be early detectable signs of malignant transformation and could therefore be especially helpful to identify patients with early stage ovarian carcinoma.

AMP-activated protein kinase (AMPK) has been of growing interest during the last years, since this enzyme plays a central role in regulating energy metabolism in cells. ATP consuming processes such as glucose deprivation or hypoxia cause AMPK activation either via an activating upstream kinase or via changes within the intracellular AMP:ATP-ratio. A large number of downstream targets of AMPK have been identified to date. They are modified through direct phosphorylation as well as through gene expression. Generally, AMPK activation results in down-regulation of anabolic pathways (e.g., fatty acid biosynthesis) and switches on catabolic processes such as glucose uptake, glycolysis or fatty acid oxidation. AMPK activation leads to the release of ATP and restores the energy balance of the cell (5-9). Another effect of activated AMPK is down-regulation of protein synthesis under energy starvation by inhibition of the mTOR pathway (10). This pathway regulates cell growth and proliferation (11) which suggests a role for AMPK in tumorigenesis. The recently identified upstream kinase of AMPK is liver kinase B1 (LKB1) a tumor suppressor mutated in Peutz-Jeghers syndrome (12-14). Patients with this hereditary syndrome develop multiple gastrointestinal polyps with the tendency to malignant transformation (15,16). The relation between LKB1, AMPK and mTOR makes AMPK an interesting target for anticancer research. Furthermore, the central role in regulating energy metabolism might link AMPK to tumorigenesis according to the Warburg hypothesis.

Recent studies in cell culture models have shown that the growth of tumor cell lines was inhibited by AMPK activation (17-20) but no data exist on the expression of AMPK in human ovarian tumors so far. It has also been shown, that pharmacological activation of AMPK can be achieved by metformin, a drug widely used for the treatment of type 2 diabetes mellitus (21).

In this study we investigated whether AMPK is differentially expressed in normal ovaries and ovarian tumors and whether this expression has influence on prognosis. Furthermore, our hypothesis was that AMPK expression is linked to metabolic abnormalities since AMPK directly and indirectly influences a large amount of downstream targets that regulate glucose and fatty acid metabolism. To address these questions, we investigated AMPK expression in normal ovaries, borderline ovarian tumors and in ovarian carcinomas by immunohistochemistry. Furthermore, we used GC-TOF-MS based metabolomics to analyze the metabolic profile of ovarian carcinomas and compared the results with protein expression data.

Materials and methods

Study population and histopathological examination. Immunohistochemical analysis was performed retrospectively on tissue samples collected from patients who underwent surgery for diagnostic or therapeutic purpose at the Charité University Hospital, Berlin, Germany between 1989 and 2005. This study has been approved by the institutional review board of the Charité Hospital. The tissue specimens included 70 primary ovarian carcinomas, 14 borderline tumors and 5 samples of normal ovaries. The mean (median) age of patients with malignant ovarian tumor was 56.4 (55.7) years with a range from 32 to 85 years. Data on histology, tumor size, nodal status and FIGO stage were extracted from the pathological reports at primary diagnosis. Tumor grading was carried out according to the Silverberg grading system, which includes nuclear polymorphism, mitotic figure count and architectural features (22). The clinicopathological data for the patients with invasive carcinomas are shown in Table I. Data on progression-free survival were available for 64 of 70 (91.4%) patients. Progression-free survival was defined as the time between diagnosis and the first clinical or pathological evidence of local or distant disease recurrence. The mean (median) progression-free survival time was 22.5 (18.5) months. The median follow-up time was 34.0 months. Follow-up data on overall survival were available for all patients. Overall survival was defined as the time between diagnosis and death. The mean (median) overall survival time was 37.8 (37.0) months. Fourteen patients (20.0%) were FIGO stage I with tumor limited to the ovaries. Data on intra-operative residual tumor were available for further 49 patients (70.0%) with FIGO stage II-IV tumors. Of these patients 29 (59.2%) had no residual tumor on intra-operative macroscopic examination. Data on postoperative chemotherapy were available for 64 patients (91.4%). Of these patients, 60 (93.8%) received platinum-based chemotherapy and 4 (6.2%) did not receive any chemotherapy at all. In three out of these four cases chemotherapy was not indicated since disease was limited to the ovary (FIGO I) and the carcinomas were well differentiated (G1). Of the borderline tumors 5 cases (35.7%) were serous, 8 (57.15%) were mucinous and 1 case was sero-mucinous (7.15%).

Immunohistochemical staining. Immunohistochemistry was performed on tissue microarrays (TMAs). These were assembled by punching out representative tumor areas, which were selected by a trained pathologist. Per tumor 4 tissue cores of 1.5 mm diameter were used and transferred into a recipient paraffin block. For detection of AMPK on tissue samples, we used a rabbit polyclonal antibody directed against the phosphorylated ß subunit of AMPK at Ser182 (Cell Signaling Technology, Danvers, MA, USA). Slides were first deparaffinated and rehydrated in a series of descending alcoholic concentration. For antigen retrieval, slides were boiled for 5 min in 0.01 M sodium citrate buffer at pH 6.0 in a pressure cooker and afterwards put in TBS-buffer for the same time. After blocking the endogenous peroxidase, slides were incubated with the primary antibody, diluted 1:50 in antibody diluents solution (Zytomed Systems, Berlin, Germany) for 1-2 h at room temperature. For visualization Dako Real Detection System (Dako, Glostrup, Denmark) was applied according to a standard protocol as provided by the manufacturer using DAB+ Chromogen. Counterstaining was carried out with Haemalaun (Dr Hollborn, Leipzig, Germany). Afterwards the tissue was dehydrated and cover-slipped with Vitroclud (Medizintechnik Langenbrinck, Emmerdingen, Germany).

Table I. Clinical and pathological patient characteristics in our cohort of ovarian carcinomas.

Characteristic	All cases (%)
Histological type	
Serous	46 (65.7)
Undifferentiated	4 (5.7)
Non-serous	20 (28.6)
FIGO stage	
I	14 (20.0)
II	7 (10.0)
III	44 (62.9)
IV	5 (7.1)
pT	
pT1	16 (22.9)
pT2	7 (10.0)
pT3	47 (67.1)
pN (n=57)	
pN0	36 (63.2)
pN1	21 (36.8)
Histological grade (Silverberg)	
G1	8 (11.4)
G2	34 (48.6)
G3	28 (40.0)
Mitoses/10 HPF	
0-9	16 (22.9)
10-24	16 (22.9)
>24	38 (54.2)
Nuclear polymorphism	
Low	11 (15.7)
Medium	37 (52.9)
High	22 (31.4)
Tumor growth pattern	
Glandular	7 (10.0)
Papillary	27 (38.6)
Solid	36 (51.4)
Age at surgery (years)	
≤60	42 (60.0)
>60	28 (40.0)
Intra-operative residual tumor (n=49)	
No residual tumor	29 (59.2)
Residual tumor <2 cm	14 (28.6)
Residual tumor ≥2 cm	6 (12.2)

The immunohistochemical expression of AMPK was evaluated by two pathologists (A.B. and C.D.) who were blinded to the patient characteristics and outcome. For semi-quantitative analysis of staining, an immunoreactivity scoring system (IRS) was applied. For this purpose, the number of cells stained (0, no cells stained; 1, <10% of cells stained; 2, 11-50% of cells stained; 3, 51-80% of cells stained; 4, >80% of cells stained) as well as staining intensity (0, negative; 1, weak; 2, moderate; 3, strong) was evaluated. Subsequently, the IRS for each case was calculated by multiplication of these two parameters. Those cases, in which disagreement in IRS evaluation between both observers was evident, were discussed using a multi-headed microscope, until agreement was achieved. For statistical analysis the cases with an IRS of 0-6 were grouped in one group ('AMPK-negative') and were compared to cases with an IRS of 7-12 ('AMPK-positive') to divide those cases with clear loss of AMPK expression.

Metabolic profiling. Data on the metabolic profile was available from a previous study for 33 tissue specimens (23), for all 33 tumors data on protein expression of AMPK was available as well. Tissue preparation and metabolic profiling was conducted as described before. In brief, tissue was dissected by a senior pathologist in the operating room and was immediately frozen in liquid nitrogen and stored at -80˚C. Additional H&E sections were done for histopathologic evaluation. Fresh weight (5 mg) of frozen biopsy tissue was prepared under standard operation procedure. Tissue was homogenized in 2 ml Eppendorf tubes for 30 sec at $25s^{-1}$ using 3-mm inner diameter metal balls in a ball mill (Retsch, Haan, Germany). Extraction was carried out using 1 ml of a one-phasic mixture of chloroform/methanol/water (2:5:2, v/v/v) at -20˚C for 5 min (24). After centrifugation, the supernatant was concentrated to complete dryness in a speedvac concentrator. The dried metabolic extract was derivatized in two steps: first, carbonyl functions were protected by methoximation using 20 μl of a 40 mg/ml solution of methoxyamine hydrochloride in pyridine at 28˚C for 90 min. Afterwards, acidic protons (e.g., hydroxyl, amine, sulfhydryl and carboxyl groups) were exchanged against trimethylsilyl group using 180 μl *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Macherey-Nagel, Dueren, Germany) at 37˚C for 30 min to increase the volatility of polar metabolites; 1.5 μl of this solution was injected into an automatic liner exchange system with direct thermodesorption unit (DTD; ATAS GL, Zoetermeer, The Netherlands). For every sample, a fresh liner and microvial was taken to avoid sample carryover and cross-contamination. The sample was introduced at 40˚C using a programmable temperature vaporization OPTIC3 injector (ATAS GL) and heated to 290˚C using a 4˚C/min ramp.

An Agilent 6890 gas chromatography oven (Hewlett-Packard, Atlanta, GA) was coupled to a Pegasus III TOF mass spectrometer from Leco (St. Joseph, MI). A MDN-35 fused silica capillary column of 30-m length, 0.32-mm inner diameter and 0.25-μm film thickness was used for separation. For the liner deactivation procedure, the initial oven temperature was set to 85˚C with an instant ramp of 50˚C/min and a target temperature of 320˚C with a hold time of 3-min duration. For the analysis, the gas chromatography oven was set to 85˚C with duration of 210 sec and a following ramp of 15˚C/min. The target time was 360˚C with duration of 2 min. The transfer line temperature was set to 250˚C. Mass spectra were acquired with a scan range of 83-500 m/z and an acquisition rate of 20 spectra per second. The ionization mode was electron effect at 70 eV. The temperature for the ion

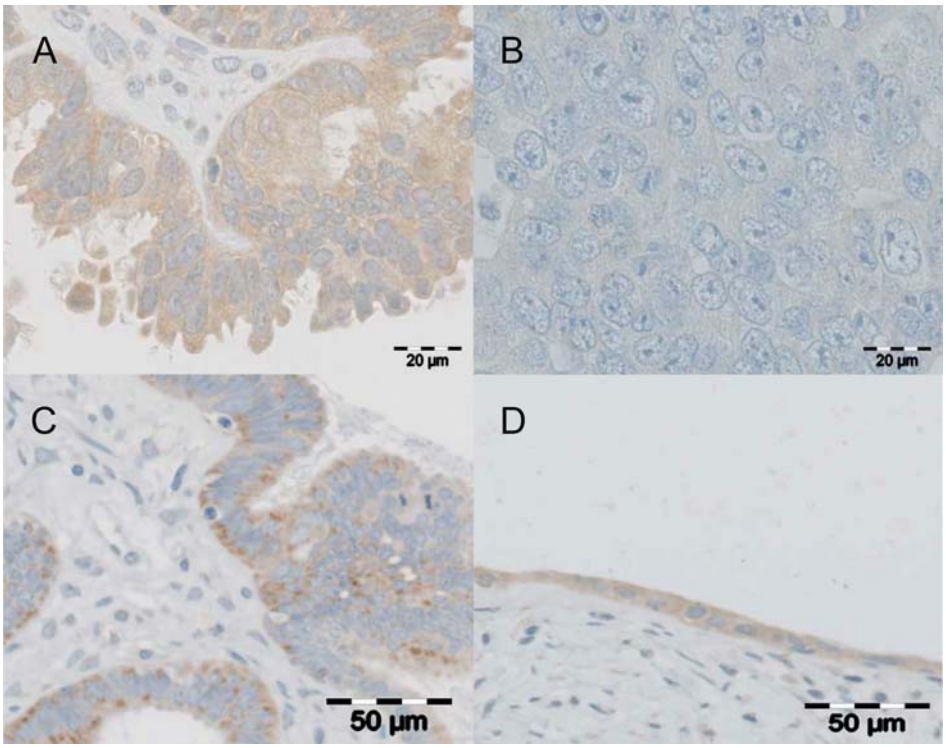

Figure 1. AMPK expression in ovarian tissue as determined by immunohistochemistry. Well differentiated serous ovarian carcinoma with strong homogeneous cytoplasmic staining (A). High grade serous ovarian carcinoma without AMPK expression (B). Cytoplasmic AMPK expression in a serous ovarian borderline tumor (C) and in normal ovarian surface epithelium (D).

source was set to 250˚C. Chromatogram acquisition, data handling, automated peak deconvolution, library search, and retention index calculation were done by the Leco ChromaTOF software (v1.61).

Statistical evaluation. The statistically significance of correlations between AMPK status and clinicopathological data was assessed by Fisher's exact, or χ^2 test for trends, as indicated. Survival curves were estimated by the Kaplan-Meier method and assessed for difference by the log-rank test. Generally, $p<0.05$ was considered as significant. All statistical evaluations were carried out with the SPSS software package 13.0. For statistical evaluation of the correlation between AMPK expression with clinical and pathological characteristics, as well as survival, only those patients with invasive ovarian carcinomas were included.

Further, AMPK expression was correlated with changes in sugar metabolism. To this end, the members of the pathway carbohydrate metabolism were extracted from the KEGG database (http://www.genome.jp/kegg). Metabolic changes were visualized as Profile Clustering, a metabolite grouping method that has been developed before (25). Profile facilitates the functional interpretation of metabolic changes by clustering the metabolites according to their distance in metabolic pathways.

Results

Immunohistochemical AMPK expression in ovarian carcinomas, borderline ovarian tumors and normal ovarian tissue. We performed immunohistochemistry to investigate the AMPK protein expression in normal ovaries and ovarian tumors. In ovarian carcinomas we observed an expression of AMPK predominantly within the cytoplasm. Nearly all positive cases presented a homogeneous staining pattern with >80% of tumor cells being stained (Fig. 1). AMPK expression was present in 39 of the 70 ovarian carcinomas (55.7%), 31 cases showed none or only weak expression (44.3%). Surrounding stromal tissue was negative in the majority of cases; only few cases showed weak stromal staining. To compare the expression patterns we also investigated normal ovarian tissue (n=5) and borderline ovarian tumors (n=14). We found a cytoplasmic AMPK expression in the epithelial cells in 4 cases of borderline tumors (28.6%). Only one normal ovary (20.0%) showed cytoplasmic immunoreactivity for AMPK in the surface epithelium (Fig. 1). No expression of AMPK was detected in ovarian stroma in these entities. There was a significantly higher expression in ovarian carcinomas compared to borderline tumors and normal ovaries ($p=0.038$).

Correlation of AMPK expression with various clinical and pathological characteristics. To investigate a link between AMPK expression and tumor progression in ovarian carcinoma we correlated our data with various clinicopathological factors. Clinical and pathological characteristics of our patient cohort are summarized in Table I. We observed a significant correlation between decreasing expression of AMPK and higher tumor grade ($p=0.009$, χ^2 test for trends, Fig. 2).

Correlation of AMPK expression with survival. In our study cohort the established tumor characteristics FIGO stage, tumor size (pT), nodal status (pN) and intra-operative residual tumor had significant prognostic influence on progression-free survival in univariate Kaplan-Meier analysis. Intra-

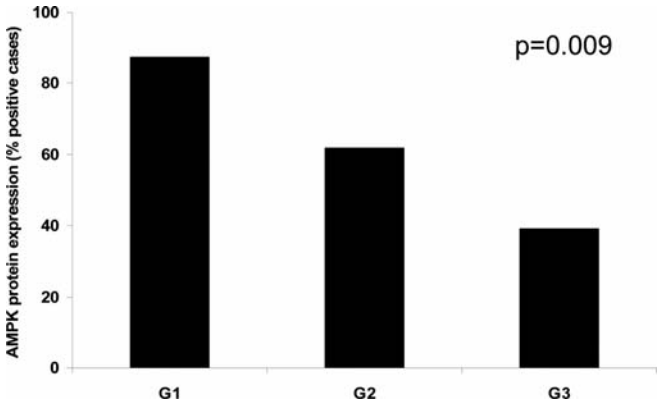

Figure 2. Significantly lower protein expression of AMPK with increased tumor grade (p=0.009, χ^2 test for trends).

operative residual tumor was an important prognostic marker for overall survival. The majority of ovarian carcinoma patients present in advanced tumor stages, and most cases are of serous histological type. As these relatively homogenous subgroups are of clinical relevance, we evaluated the prognostic impact of AMPK expression in serous and high stage tumors by Kaplan-Meier analysis. We observed a significantly shorter overall survival in AMPK-negative serous ovarian carcinomas (p=0.037) as well as in patients with advanced tumor stages (pT 2+3, p=0.016, Fig. 3).

In the complete cohort of ovarian carcinomas, we observed a mean overall survival of 67.6 months in patients with AMPK-positive tumors compared to 50.3 months in AMPK-negative cases. The loss of AMPK expression showed borderline significance to be a negative prognostic marker for overall survival (p=0.081, log-rank test, data not shown). Further, we performed an explorative Cox regression analysis under inclusion of the intra-operative residual tumor and other established prognostic markers as patient age, tumor grade and FIGO stage. Here as well we observed a borderline significance for AMPK overexpression to be an independent predictor of better patient survival (hazard ratio 3.43, confidence interval 0.91-12.93, p=0.069). AMPK expression had no prognostic influence on progression-free survival (data not shown).

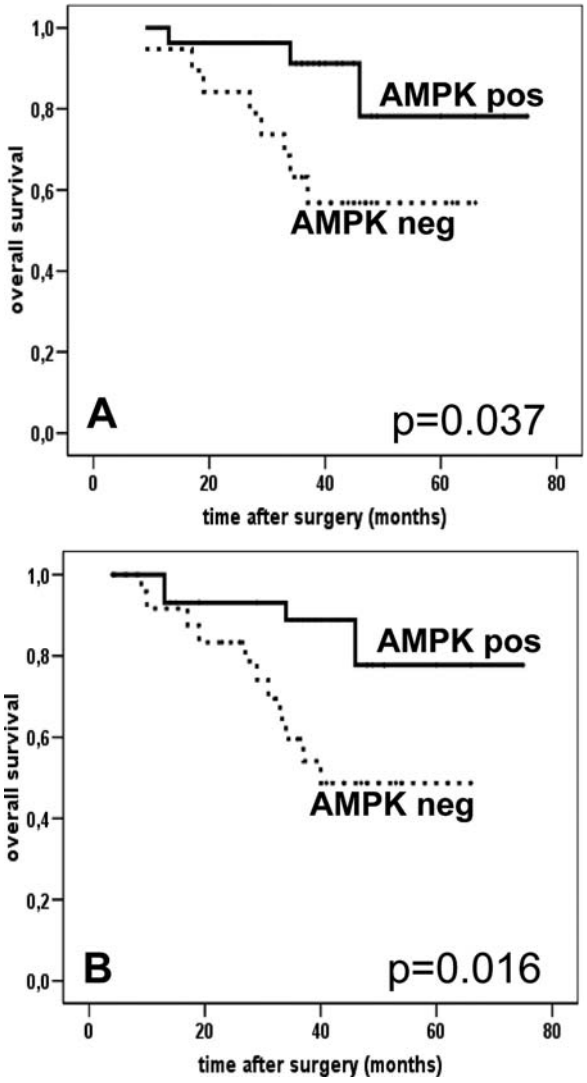

Figure 3. Prognostic influence of AMPK expression on patient overall survival. Loss of AMPK expression and shorter overall survival are significantly correlated in the subgroup of patients with serous ovarian carcinomas [n=46, p=0.037, (A)] as well as in the subgroup of patients with advanced tumor stages [n=54, pT 2+3, p=0.016, (B)].

Metabolic profiling in ovarian carcinomas. We were able to detect 238 different metabolites, 128 of them were identifiable and 85 metabolites were listed in the KEGG database.

Significant changes in the fold change between AMPK-positive and negative carcinomas were observed in five different metabolites: glucose, alanine, threonine, proline and 2-hydroxybutanoate (Fig. 4A). Glucose is used as the primary source of energy in most organisms and either arises from the breakdown of glycogen or is synthesized from pyruvate and glycerol. The non-essential amino acid Alanine functions as a major energy source in muscle, where it is generated during glycoysis and reconverted to glucose after transport to the liver. Threonine is an essential amino acid that can not be synthesized in humans. Proline is a non-essential amino acid synthesized from glutamat and is important in the creation of collagen. 2-Hydroxybutanoate is an organic acid from propanoate metabolism that is generated as a by-product in the synthesis of cystathionine under conditions of oxidative or metabolic stress (26).

In further analysis, we focused on glucose metabolism, since AMPK is known to regulate several enzymes that influence glucose metabolism. Here we investigated a cluster of five metabolites: glucose, fructose, glucose-1-phosphate, sorbitol and sucrose. These metabolites are directly connected by one or two main biochemical reactions according to the KEGG pathway maps (Fig. 4A) (25). For example sucrose is a disaccharide consisting of glucose and fructose, the sugar alcohol sorbitol is obtained by reduction of glucose, and glucose-1-phosphate is generated during the breakdown of glycogen (26).

We compared the metabolite concentration in AMPK-positive and -negative tumors. Of the 33 ovarian carcinomas 17 (51.5%) showed a high protein expression of AMPK and were defined as 'AMPK-positive' tumors according to our scoring system. Sixteen tumors (48.5%) displayed no or only

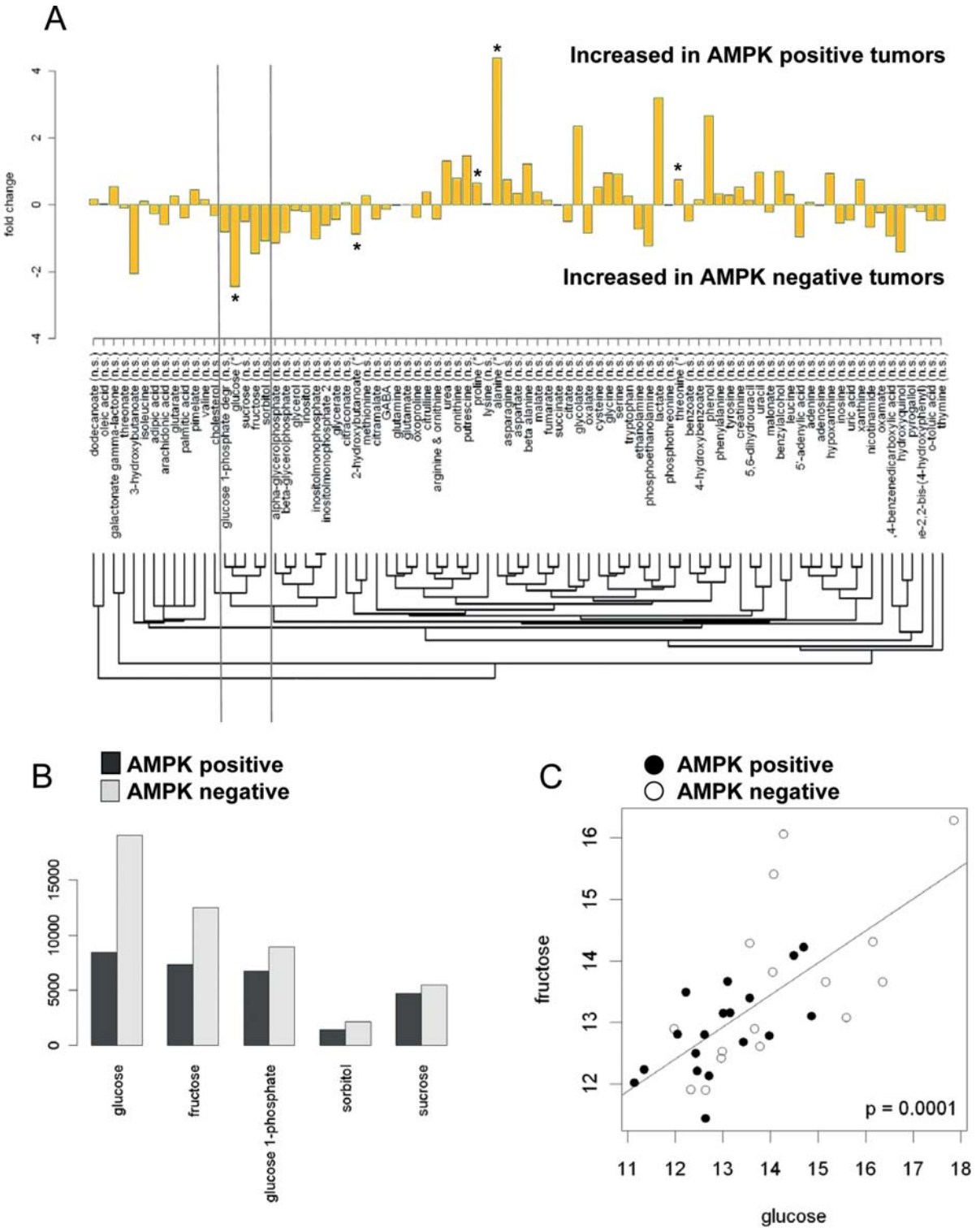

Figure 4. (A) Display of the metabolic differences in ovarian carcinomas. The yellow bars show the fold changes between AMPK positive and negative carcinomas for the single metabolites. Significance is designated by the stars ($p<0.05$). The metabolites are clustered according to the relational pathway information from the KEGG database. Their relation according to biochemical reactions is pictured in the tree diagram in the lower third of the figure. (B) Metabolite concentration of five metabolites from the glucose cluster in (A) in AMPK-positive and negative ovarian carcinomas. Significant up-regulation of glucose in AMPK-negative carcinomas (fold change -2.259, $p=0.022$) as well as an increased concentration of fructose (fold change -2.259, $p=0.060$). Glucose-1-phosphate, sorbitol, and sucrose showed a trend towards overexpression in AMPK-negative carcinomas (fold change 1.324, $p=0.130$ for glucose-1-phosphate; fold change 1.472, $p=0.180$ for sorbitol; fold change 1.158, $p=0.180$ for sucrose). (C) Homogeneity of the tissue was measured using the expression of the two metabolites glucose and fructose, which were significantly correlated comparing all the measured tissue samples (Pearson correlation 0.64, $p=0.00001$).

weak AMPK expression ('AMPK-negative'). We detected a significantly higher concentration of glucose in AMPK-negative carcinomas (fold change 2.259, $p=0.022$, t-test, Fig. 4B). Likewise, the other metabolites fructose, glucose-1-

phosphate, sorbitol and sucrose showed a trend towards overexpression in AMPK-negative carcinomas (fold change 1.70, p=0.061 for fructose; fold change 1.324, p=0.130 for glucose-1-phosphate; fold change 1.472, p=0.180 for sorbitol; fold change 1.158, p=0.180 for sucrose, t-test, Fig. 4B).

The expression of the two metabolites glucose and fructose was significantly correlated comparing all the measured tissue samples (Pearson correlation 0.64, p=0.00001, Fig. 4C). We used this as a quality control to assure homogeneity of the tissue.

Discussion

Recent studies in cell culture models have shown that the growth of tumor cell lines was inhibited by AMPK activation. Xiang *et al* observed a reduction of prostate cancer cell growth after incubation with activators of AMPK (17). These findings were similar in the human gastric cancer cell line GT3-TKB (18), and similarly the activator of AMPK, 5-aminoimidazole-4-carboxamide riboside (AICAR), induces apoptosis in B-cell chronic lymphocytic leukemia cells as well as in liver cells (19,20). Zakikhani *et al* showed that metformin, a drug widely used in the treatment of type-2 diabetes, acts as a growth inhibitor for epithelial cells via AMPK pathway activation with decreased mTOR levels (27). Metformin is an already well established and tested drug that might be adapted to additional indications apart from anti-diabetic treatment. Recently, this has been investigated in a retrospective study where diabetic patients with breast cancer treated with metformin and neoadjuvant chemotherapy had a higher response rate than diabetics not receiving metformin (28).

The expression level and expression pattern of AMPK in human ovarian carcinomas have not been studied, yet. To our knowledge, we are the first to present immunohistochemical findings of AMPK expression in ovarian carcinomas in correlation with clinicopathological data and patient survival. Our results show that AMPK is differently expressed in ovarian carcinomas, borderline tumors and normal ovaries and that loss of AMPK expression in carcinomas is significantly associated with higher tumor grade. Furthermore decreasing AMPK expression is linked to unfavorable prognosis for overall survival in the large and clinically important subgroup of patients with serous carcinomas as well as in patients with advanced tumor stages. These findings are in line with the published results of functional studies, indicating an important role of AMPK as a tumor suppressor or at least as an indispensable part of tumorigenesis. Whether this protective effect of AMPK is mediated via inhibition of the mTor pathway or related to modified ways of energy generation needs to be clarified.

On the other hand, we observed higher levels of AMPK in ovarian carcinomas compared to borderline ovarian tumors and normal ovaries that link AMPK expression to malignant transformation. It is well known that carcinomas show higher glucose uptake because of increased glycolysis compared to adjacent normal tissue, as measured by ^{18}F-deoxyglucose positron emission tomography (FDG-PET) to detect primary cancer sites or distant metastases (29). Gatenby *et al* propose that persistent glycolysis is an adaptation to intermittent hypoxia in pre-malignant lesions. Intra-epithelial proliferations lead to broadened cell layers without vascular supply and thereby cause hypoxic conditions (30). Assigned to our results, this temporary hypoxia could induce up-regulation of AMPK during malignant transformation.

Since AMPK directly and indirectly influences a large amount of downstream targets that regulate glucose and fatty acid metabolism, our hypothesis was that AMPK expression might be linked to measurable metabolic abnormalities in tumor cells. We have analyzed metabolite concentration in ovarian tumor tissue as well as in colorectal carcinomas before. In these studies we were able to identify a significantly different metabolic profile between borderline ovarian tumors and carcinomas (23). Similarly, samples of colorectal carcinomas showed significant differences compared to normal colon mucosa (25). Usually, detection of metabolites is more common from body fluids (e.g., urine, blood or saliva). Issaq *et al* investigated urine from patients with bladder cancer as well as from healthy individuals and were able to detect the carcinoma patients (31). A slightly different method is the use of nuclear magnetic resonance (NMR) spectroscopy instead of mass spectrometry (32). This was performed by Odunsi *et al* to investigate serum profiles from women with ovarian carcinoma, benign ovarian cysts and healthy patients. Here as well it was possible to classify the patients correctly (33). We have now linked metabolomic data to protein expression of the key regulatory protein AMPK, and we are the first to describe the relation between AMPK expression and metabolite concentration. Our results on metabolic profiling in a subset of ovarian carcinomas revealed lower concentrations of glucose, fructose, glucose-1-phosphate, sorbitol and sucrose in AMPK-positive carcinomas. The expression of the two metabolites glucose and fructose was significantly correlated which served as a quality control. These findings are particular interesting based on the fact that AMPK activation physiologically increases catabolic pathways; thereby cellular glucose levels should rise e.g., via activation of different glucose transporters. Our results point towards a dysfunctional regulation of the energy metabolism in ovarian carcinomas with reversed dependence on AMPK. It seems that the increased requirement of energy in malignant tumors cannot be met by up-regulation of AMPK anymore. Alternative ways of energy production are activated, e.g., uncontrolled fermentation according to the Warburg hypothesis. This switch in energy generation from AMPK-dependent to AMPK-independent pathways might be even more advantageous in malignant tumors. To further elucidate the deregulation in energy metabolism functional studies are necessary. The new method of metabolite detection from tumor tissue appears to be very promising and especially helpful for further studies.

In conclusion, our results show, that AMPK is differentially expressed in human ovaries and ovarian tumors and associated with unfavorable prognosis in a subset of ovarian carcinomas. Data from metabolite analysis point towards a dysfunctional regulation of the energy metabolism in ovarian carcinomas. Activation of AMPK, e.g., with the anti-diabetic drug Metformin, might therefore be a future therapeutic approach in ovarian carcinoma patients with advanced tumor stages.

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